AUTOIMMUNITY, THROMBOSIS AND HAEMOSTASIS

Antiphospholipid syndrome

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Introduction

The antiphospholipid syndrome (APS) is a clinicopathologic syndrome in which antibodies binding to phospholipids (PL) are thought to be involved in the development of thrombosis and/or pregnancy complications.1 The notion of this syndrome goes back to observations made more than 50 years ago on the one hand by haematologists who described a circulating anticoagulant in patients with systemic lupus erythematosus (SLE) and on the other hand by rheumatologists who found that patients with SLE frequently had a biologically false-positive serologic test for syphilis. Immunoglobulins were found to be responsible for both laboratory phenomena. Clinicians became intrigued by the occurrence of thrombosis and/or recurrent intrauterine death, explained by thrombosis-related placental insufficiency, despite the presence of a circulating anticoagulant. The aspecific coagulation inhibitor was named lupus anticoagulant (LA) because of its frequent occurrence in patients with SLE and it was suggested that its anticoagulant effect was due to competition of the ‘anticoagulant antibodies’ with the binding of clotting factors to PL surfaces. This and the fact that cardiolipin is a key reagent in the serological test for syphilis, formed the basis for the development of the so-called anticardiolipin antibody (aCL) assay. With the availability of this easy immunoassay, affinity purification of aCL could be monitored leading to the discovery that aCL found in patients with APS do not recognise PLs per se but rather β2-glycoprotein I bound to PLs.2,3

Antigenic targets of antiphospholipid antibodies

Soon after the discovery that β2-glycoprotein I was involved in the binding of aCL to cardiolipin, it was reported that aCL bind to β2-glycoprotein I also in the absence of PL.4 The affinity of the interaction of these antibodies with fluid phase β2-glycoprotein I is, however, low. It was also reported that a subpopulation of aCL possesses LA activity and that certain LAs are directed against prothrombin. Most LAs appear to be directed against β2-glycoprotein I or prothrombin. Autoimmune antiphospholipid antibodies (aPL) have in common that they are directed against proteins with affinity for PL or negatively charged surfaces. The main antigens are β2-glycoprotein I and prothrombin although a number of autoantibodies recognizing other PL-binding proteins like protein C, protein S, annexin V, complement factor H, high- and low- molecular-weight kininogen, prekallikrein, Factor XI, tissue factor pathway inhibitor (TFPI), Factor VII/VIIa, etc have been found in sera of patients with APS.

Anticoagulant mechanism of lupus anticoagulants

The in vitro anticoagulant effect of LAs was originally explained by the assumption that these antibodies compete with clotting factors for anionic PLs acting as catalytic surface for coagulation reactions.5 Since β2-glycoprotein I-dependent LAs are antibodies directed against β2-glycoprotein I, which by itself is at most a very weak anticoagulant, such competition can only be explained by an increased affinity of β2-glycoprotein I for PLs upon antibody binding to this protein. The in vitro anticoagulant effect of β2-glycoprotein I- and prothrombin-dependent LAs is currently explained by the formation of bivalent antigen–antibody complexes of which the affinity for PLs is higher than the antigen alone6–8 (see Figure 1). Evidence for this has been obtained with monoclonal antibodies as well as with affinity purified IgG from patients. The importance of bivalent β2-glycoprotein I complexes was also confirmed via recombinant DNA technology. A recombinant β2-glycoprotein I dimer was constructed and expressed in baby hamster kidney cells.9 The dimer protein had a 35 times higher affinity for PLs than plasma derived β2-glycoprotein I and was able to mimic the anticoagulant effect of bivalent immune complexes.

The fact that not all anti-β2-glycoprotein I antibodies behave as LAs is intriguing and is probably due to the fact that LA-negative antibodies are unable to crosslink two β2-glycoprotein I molecules in such a way that both β2-glycoprotein I molecules of the complex can interact efficiently with the PL surface.

There is also only a subpopulation of prothrombin-dependent aPL showing LA activity. Some of these
markedly enhance binding of prothrombin to negatively charged PL, again likely via the formation of bivalent immune complexes.10

aPL as part of the diagnostic workup of the APS

Definite APS: the Sapporo criteria

APL such as LAs and aCL are mainly determined as part of the diagnostic workup of patients who are suspected to suffer from APS. Definite APS is currently defined by the simultaneous presence of certain clinical and laboratory criteria as agreed upon in an international workshop held in Sapporo in 1998.11 The clinical criteria are one or more objectively confirmed episodes of vascular thrombosis at any site and/or pregnancy morbidity defined as more than one unexplained death of a morphologically normal foetus, more than one premature birth of a morphologically normal neonate because of severe placental insufficiency or more than 3 unexplained consecutive spontaneous abortions before the 10th week of gestation. The laboratory criteria are the presence in the patient's blood of a LA or of medium or high titer β2-glycoprotein I-dependent aCL of the IgG or IgM isotype. β2-glycoprotein I-dependency refers to the fact that persistent autoimmune aCL, in contrast to transient infection related aCL, do not directly bind to negatively charged PLs but to β2-glycoprotein I, bound to PLs.

Other conditions where the detection of aPL may be useful

APL may be found in many clinical conditions that at first glance do not fulfill the criteria of definite APS. In some of these, the detection of aPL seems justified because the diagnosis of an APS may affect the management. These conditions include: unexplained cutaneous circulation disturbances, such as livedo reticularis, blue toe syndrome or ulcers resembling pyoderma gangrenosum, major autoimmune disease, in particular SLE, autoimmune thrombocytopenia or autoimmune hemolytic anemia and non-bacterial thrombotic endocarditis, unless malignancy is an obvious cause.

Primary versus secondary APS

Autoimmune aPL appear either in the context of a generalized immune deregulation as observed in, for example, SLE (so-called secondary APS) or without clear features of an associated immune disorder (so-called primary APS). It should be noted that patients with primary and secondary APS have very similar clinical profiles as they have similar frequencies of arterial and venous thrombosis, pulmonary embolism and pregnancy morbidity. Patients with an APS secondary to SLE more frequently have arthritis, livedo reticularis, thrombocytopenia, leukopenia or hemolytic anemia. It is relatively rare that primary APS (PAPS) evolve to a secondary APS.

Detection of aPL

SSC criteria for Lupus anticoagulant testing

According to the diagnostic criteria set by the Scientific and Standardization Committee (SSC) on Lupus anticoagulants (LAs) and PL-dependent antibodies of the International Society of Thrombosis and Haemostasis, the laboratory diagnosis of an LA should follow a three-step procedure showing the following characteristics: (a) prolongation of a PL dependent clotting test, in particular when the PL-content of the test system is low, (b) lack of correction of the prolonged clotting time by addition of a small amount of normal plasma (thereby excluding a clotting factor deficiency) and (c) correction by the presence of a higher concentration of PL or by use of a reagent that is poorly responsive to the LA effect. Since autoimmune aPL are chronic, the presence of an LA should be confirmed on a second sample taken several weeks later. It should be emphasised that a 6-week interval may not be sufficient to distinguish persistent LAs from those seen in relation with infectious disease since the latter may persist for several months. It is very hard to give precise recommendations on which assays to use for the detection of LAs.12 Selection of assay systems with optimal sensitivity and specificity is almost impossible due to the lack of a ‘golden standard’ (a well-defined LA-positive patient population). Since the introduction of the diagnostic criteria by the SSC and the organization of external quality assessment schemes, several diagnostic companies have introduced reagents and assay kits with improved responsiveness for the LA. Improved responsiveness
means that the degree of prolongation of the clotting time due to the presence of an LA is increased. Although a highly responsive reagent is usually considered to be user-friendlier, it should be stressed that higher responsiveness not necessarily means that the reagent also has a higher sensitivity for the LA.

**Differential diagnosis of LA and specific factor inhibitors**

Confirmation of the PL dependency of the LA (the third SSC criterium) is extremely important to distinguish LAs from specific factor inhibitors because the latter are associated with a significant risk of bleeding. It should, however, be noted that none of the available confirmatory test procedures have an absolute specificity. The use of tests with a diluted PL content to confirm the presence of an LA should be discouraged as they carry the risk for false-positive interpretations in case of specific factor inhibitors. To avoid misdiagnosis of a factor VIII inhibitor or a factor V inhibitor for a LA, we highly advocate the use of a typically LA insensitive or unresponsive aPTT reagent as part of the confirmation procedure. 12

**Immunological assays for aPL:** The anticardiolipin ELISA (semi-) quantifies antibodies that bind to β2-glycoprotein I immobilized on cardiolipin. This assay measures IgG and IgM antibodies separately. The binding activity is expressed either as a binding index or in GPL or MPL units based on standards isolated from patient plasma. 13 A major advantage is that the detection of these antibodies is not affected by anticoagulant treatment. It should be noted, however, that assay does not adequately distinguish 'autoimmune' from 'infectious' antibodies, lacks good standardization and therefore suffers from a poor interlaboratory reproducibility.1 In addition, this assay is of no (or only very limited) help to estimate the risk for (recurrent) thrombosis in a given patient (see further).

β2-glycoprotein I and prothrombin can be immobilized directly on microtiter plates and antibodies to these proteins measured. Retrospective studies suggest that this assay may have a better specificity than the anticardiolipin assay. Direct antiprothrombin antibody assays are also becoming available and some retrospective studies suggest that these antibodies are risk factor for venous thrombosis. However, prospective clinical studies are needed to draw definite conclusions on the risk associated with antibodies measured in direct assays. Moreover, the use of these assays is seriously compromised by lack of standardization and poor interlaboratory reproducibility. Therefore, these direct tests are for the time being better restricted to research rather than to routine diagnostic use.

**aPL and risk for thrombosis**

Although aPL are mainly measured to fulfil the diagnostic criteria for an APS, they are more and more used to estimate the risk for (recurrent) thrombosis or pregnancy morbidity in a given patient. Indeed, there is growing consensus that LAs are stronger risk factors for thrombosis than aCL. It was firstly shown that the presence of an LA correlates better with a history of thrombotic complications than the presence of aCL. The results from a systematic review of the literature formally established that, in patients with previous thrombosis and/or systemic lupus erythematosus, the presence of an LA is a stronger risk factor for future thrombotic complications than the presence of aCL. 14 There is also growing belief that β2-glycoprotein I-dependent aPL may be more strongly associated with thrombosis than prothrombin-dependent aPL. A recent paper describes a method to discriminate β2-glycoprotein I-dependent LAs from prothrombin-dependent ones.15 The method is based on the high-affinity interaction between β2-glycoprotein I and pure cardiolipin. β2-glycoprotein I and LAs dependent on this protein are adsorbed on cardiolipin vesicles when added to patient plasma. Shortening of an LA-sensitive aPTT in the presence of cardiolipin vesicles is an indication for the presence of a β2-glycoprotein I-dependent LA. This assay makes it possible to properly address the question whether the risk for thrombosis is to some extent linked to the target antigen of the LA.

**Pathogenic mechanisms of aPL: surface-mediated thrombogenic characteristics**

In view of the strong association between the presence of (certain) aPL and thrombosis, it is tempting to believe that aPL are involved in the thrombogenesis. Several prothrombotic mechanisms have been proposed which have in common that they interfere with surface-mediated phenomena.

The formation of bivalent immune complex described above is also a surface-dependent phenomenon since the binding between anti-β2-glycoprotein I antibodies and its antigenic target in solution is hampered by the intrinsically low affinity nature of this interaction. The observation that LAs are more closely associated with thrombotic events than aCL also provides an argument in favour of the hypothesis that the prothrombotic activity of aPL may be related to interference with surface-mediated phenomena.

Anionic enriched PL surfaces, needed for the formation of stable bivalent immune complexes, are hardly available within a healthy vessel, since negatively charged PLs are normally sequestered within the inner leaflet of the PL bilayer of the cell membrane. Such surfaces, however, become available during normal hemostatic processes. aPL do probably not cause thrombosis by themselves but rather influence the thrombotic process once negatively charged PL becomes exposed. APL would therefore function as a ‘second hit’. There is indirect clinical support for the concept of a double-hit phenomenon. Firstly, not all patients with aPL develop thrombosis but those who do develop thrombosis have a
high rate of recurrence. Secondly, arterial events are almost always followed by arterial events and venous thrombosis is most likely followed by another venous thrombosis.

There are several ‘anticoagulant’ surface-mediated processes with which aPL are thought to interfere. One major anticoagulant pathway involves protein C and protein S. Protein C, activated by thrombin on endothelial thrombomodulin, and protein S bind to negatively charged PL through their GLA domains; activated protein C in association with protein S cleaves factor Va and factor VIIIa on the PL surface and thereby inactivates the intrinsic tenase and prothrombinase reactions. Occupancy of the surface by immune complexes could impede these interactions and thereby promote further thrombin generation and thrombus growth. Indeed, LAs can induce ‘activated protein C resistance’. A second anticoagulant mechanism involves tissue factor pathway inhibitor. This protein binds to negatively charged PL and to factor Xa on PL. This protein complex then links to the tissue factor–factor VIIa complex, and shuts off further tissue factor-mediated clotting. Again, occupancy of the PL surface by immune complexes may impede this interaction, leading to prolonged thrombin generation.

While these interferences of the immune complexes with anticoagulant pathways have been elegantly demonstrated in vitro, it is difficult to unequivocally extrapolate these observations in vivo, since the immune complexes could impede assembly of the procoagulant complexes to the same extent and the overall result would then be neutral. However, under certain pathological conditions, this may not be the case. Oxidation is believed to play an important role in inflammatory diseases such as atherosclerosis. In addition, it was reported that oxidation of phosphatidyethanolamine containing liposomes potentiates the anticoagulant effect of the protein C pathway without affecting the procoagulant pathway. Total IgG from patients with aPL and thrombosis appears to selectively inhibit the anticoagulant effect of phospholipid oxidation.

The prothrombotic action of aPL may not be limited to inhibition of surface-mediated anticoagulant pathways. Chesterman’s group provided evidence that enhanced antibody-mediated deposition of prothrombin on PL may lead to increased thrombin generation in conditions of flow.

We suggest that the deposition of immune complexes on slightly activated cells may induce further cell activation, leading to generation of microvesicles, that paradoxically provide a much larger PL surface resulting in enhanced thrombin generation (see also Figure 2).

This pathogenetic model was confirmed in an in vivo model of thrombosis in the hamster in whom slight radical-induced endothelial injury of a carotid artery had been provoked after intravenous injection of monoclonal antibodies to human β2-glycoprotein I. One of the tested antibodies had LA activity in hamster plasma, a second bound immobilized hamster β2-glycoprotein I but was without LA activity, and a third had no affinity for hamster β2-glycoprotein I. The antibody with LA activity markedly enhanced arterial thrombosis, the second antibody without LA activity hardly enhanced thrombosis, the third antibody being inactive. F(ab)2 fragments of the LA antibody also enhanced thrombosis, whereas Fab fragments were inactive. These findings confirm that bivalency is required for thrombosis enhancement but also that the Fc component of the antibody is not compulsory for this enhancement. Immunohistological examination revealed that the antibody was mainly localized focally within the platelet thrombus, suggesting thrombus growth spreading from the foci of immune complex deposition on activated platelets. These experiments are in agreement with a double-hit scenario: Following mild endothelial damage, a small platelet thrombus develops (first hit); the slightly activated platelets expose negatively charged PL; this leads to patchy deposition of bivalent β2-glycoprotein I–antibody complexes; these complexes cause further platelet activation and thrombus growth (second hit).

The fact that Fc receptor activation is not compulsory for the prothrombotic action is intriguing and may suggest that another type of receptor-triggered process is involved. Direct evidence for this was obtained from in vitro experiments under flow conditions showing that recombinant β2-glycoprotein I-dimer like β2-glycoprotein I-antibody dimers enhance platelet activation and deposition on collagen surfaces. This platelet
deposition appeared to be dependent on activation of the apolipoprotein E receptor 2.

Antithrombotic therapy in patients with APS

According to the sixth ACCP guidelines for antithrombotic therapy for the prevention and treatment of thrombosis, the mainstay of treatment of arterial or venous thrombosis in patients with established APS is low molecular weight heparin followed by oral anticoagulation at an INR of 2.5 for 12 months or longer (Grade 1C recommendation). There is evidence from retrospective analysis that the treatment should be more intense (INR 3–4). There are now data available from several small prospective studies supporting prolonged treatment with a targeted INR of 2–3.

To prevent pregnancy loss and thrombosis in pregnant patients with aPL and a history of multiple (two or more) early pregnancy losses or one or more late pregnancy losses or preeclampsia, intrauterine growth retardation or placental abruption, treatment with low-dose aspirin plus mini-dose or moderate-dose unfractionated heparin or prophylactic dose low molecular weight heparin (4000–5000 anti-Xa units subcutaneously every 24 h) is recommended (Grade 1B recommendation). This therapy probably should be started as soon as pregnancy is diagnosed.

References


Thrombotic thrombocytopenic purpura

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In this overview, we are first summarizing the history of thrombotic thrombocytopenic purpura (TTP) from its first description in 1924 by Moschcowitz to the discovery in 1998 that most affected patients are severely deficient in von Willebrand factor (vWF)-cleaving protease activity. Next, important advances concerning pathophysiology, diagnosis and treatment of TTP including controversies raised during the years 1998–2004 are presented. Finally, we shortly outline some important open questions on the pathogenesis and treatment of TTP that need to be addressed in future research.

History of TTP from 1924 to 1998

In 1924, Moschcowitz first described TTP in a 16-year-old girl who died within 2 weeks after abrupt onset of petechiae, anemia, microscopic hematuria, fever, paralysis and coma.1 Disseminated microvascular ‘hyaline’ thrombi were found at autopsy and these platelet-rich thrombi in arterioles and capillaries remain the hallmark of pathologic diagnosis of TTP today.2 Reviewing more than 250 published patients and adding 16 new cases, Amorosi and Ultmann,3 in 1966, established the pentad of clinical features that are still considered diagnostic criteria nowadays: microangiopathic hemolysis with schistocytes, thrombocytopenia, neurologic signs and symptoms, renal dysfunction and fever.

Numerous hypotheses on the etiology and pathogenesis of TTP have been proposed (for reviews, see Moake and Chow,4 Ruggenenti and Remuzzi5 and Furlan and Lämmlé6), including, among many others, autoantibodies against endothelial cells and specifically against glycoprotein IV (CD 36), a thrombospondin receptor expressed on endothelial cells, platelets and other cells.7

In 1982, Moake et al.8 described unusually large von Willebrand factor (ULvWF) multimers in the plasma of patients with a chronic relapsing course of TTP. These extremely adhesive ULvWF multimers, found in plasma during remission and disappearing upon relapse of TTP, were supposed to be responsible for platelet clumping in the microvasculature during acute bouts of the disease. Moake et al.8 hypothesized that a lacking ‘depolymerase’ in patients with the relapsing form of TTP resulted in the persistence of ULvWF molecules that were similar in size to those observed in the supernatant of endothelial cell cultures but considerably larger than the largest multimers found in normal plasma.

In 1997, four patients, including two brothers, with chronic relapsing TTP were reported to have a severely deficient plasma activity of vWF-cleaving protease,9 an enzyme isolated from normal plasma in 1996 and shown to cleave the vWF subunit in vitro at Tyr1605-Met1606, the peptide bond in vWF previously found to be cleaved physiologically in vivo. Two retrospective studies on a large number of plasma samples from patients with acute TTP showed that 26 of 30 and 37 of 37 patients, respectively, had a severely deficient vWF-cleaving protease activity,10,11 which in the majority of cases with the nonfamilial form of acute sporadic TTP was caused by circulating IgG autoantibodies inhibiting the vWF-cleaving protease activity. Upon remission of acute acquired TTP most patients showed a normalization of vWF-cleaving protease activity and disappearance of the inhibiting autoantibodies.10,12 In contrast, patients with a familial, obviously hereditary form of TTP remained severely deficient in vWF-cleaving protease activity, even in remission, and their plasma contained no inhibitory autoantibodies.9,10 Patients diagnosed with sporadic or familial hemolytic uremic syndrome (HUS) had a normal or subnormal vWF-cleaving protease activity, in clear distinction to those diagnosed with TTP.10 This almost perfect laboratory discrimination seemed somewhat surprising given the fact that the clinical distinction between TTP and HUS is often very difficult or even arbitrary in view of the largely overlapping clinical and laboratory findings of these two forms of thrombotic microangiopathy (TMA).

Acute TTP was almost universally fatal until the empirical introduction of plasma therapy including plasma infusion and/or plasma exchange with replacement of fresh frozen plasma (FFP) in the 1970s.13 The Canadian Apheresis Study Group reported a prospective randomized study demonstrating that plasma exchange with FFP replacement was superior to FFP infusion alone.14 Using plasmapheresis and FFP replacement up to 80% of patients with acquired TTP may survive the acute episode,14 even though a considerable proportion of survivors will suffer from relapses during the ensuing months or years.13 Patients with acute acquired TTP are often receiving in addition to the established plasma therapy – corticosteroids, vincristine, other immunosuppressive medication and/or splenectomy (for reviews, see Moake and Chow4 Ruggenenti and Remuzzi5 and George13).
These largely empirical treatment strategies seemed to become pathophysiologically reasonable with the detection that many patients with acute acquired TTP had an autoantibody-mediated vWF-cleaving protease deficiency. While plasmapheresis removes the inhibiting autoantibodies, FFP replacement provides the lacking enzyme, and immunosuppressive measures may help to decrease further autoantibody production.

**Current status and controversies in TTP (1998–2004)**

After its partial characterization in 1996, vWF-cleaving protease was purified from normal plasma in 2001, subjected to partial amino-acid sequence analysis and shown to be a member of the so-called ADAMTS (a disintegrin and metalloprotease with thrombospondin type 1 domains) family of proteases, denoted as ADAMTS13. Simultaneously, Levy et al. performed genomewide linkage analysis in patients with hereditary TTP and their family members and detected the responsible gene, ADAMTS13, on chromosome 9q34. They identified several mutations of this gene as being presumably responsible for the severely deficient ADAMTS13 activity and disease in homozygous or compound heterozygous carriers of mutated alleles, whereas family members with a heterozygous ADAMTS13 mutation had approximately 50% of protease activity and were clinically asymptomatic. Various groups of researchers have since reported many new mutations throughout the ADAMTS13 gene consisting of 29 exons encoding 1427 amino acids (Figure 1).

Functionally active recombinant human ADAMTS13 has been expressed in transfected mammalian cells and was shown to restore the vWF-cleaving protease activity when added to plasma from subjects with severe hereditary ADAMTS13 deficiency. Plasma or purified IgG fraction from patients with acquired TTP due to ADAMTS13 inhibiting autoantibodies inhibited the recombinant human ADAMTS13 activity when tested in vitro.

Whereas most researchers in the field now agree that patients with Upshaw–Schulman syndrome, which is synonymous with hereditary TTP, most often, if not always, suffer from severely deficient ADAMTS13 activity (<5% of normal) caused by compound heterozygous or homozygous ADAMTS13 gene defects, the role of acquired, autoantibody-mediated ADAMTS13 deficiency in the pathogenesis of sporadic TTP has been seriously questioned by several authors. Some investigators suggested that deficient ADAMTS13 activity was not restricted to patients with TTP, but was instead found in various other thrombocytopenic states, in disseminated intravascular coagulation, various acute inflammatory conditions, and even in healthy subjects. It should be noted, however, that these researchers either did not use rigorous quantitative assays of ADAMTS13 activity or, if quantitative assays were used, found only slightly or moderately decreased vWF-cleaving protease activity in their non-TTP patients. A formal study on 68 patients with thrombocytopenia caused by severe sepsis or septic shock, heparin-induced thrombocytopenia, or various hematologic diseases different from TTP and HUS revealed that 12 of 68 patients had ADAMTS13 activity values ≤30% of normal, but none was severely deficient (<3–5%), in contrast to many patients with acute TTP. Therefore, it would seem that a severely decreased ADAMTS13 activity is a specific feature of TTP even though a few asymptomatic adult subjects with severe hereditary protease deficiency have been reported.

Another controversy concerning the specificity of severe ADAMTS13 deficiency for TTP was raised by Remuzzi et al. who found lacking vWF-cleaving protease activity not only in TTP but also in some patients they had clinically diagnosed as familial or recurrent HUS. This is probably less surprising given the fact that TTP and HUS cannot always be clearly distinguished clinically.

Even more debated is the sensitivity of severe ADAMTS13 deficiency for the clinical diagnosis of TTP. Whereas Tsai and Lian found 37 of 37 patients clinically diagnosed with acute TTP to completely lack ADAMTS13 activity, other investigators reported 33–87% of acute TTP patients to lack any measurable protease activity. Thus, some workers argue that only severe ADAMTS13 deficiency defines TTP, whereas others find a sizable proportion of patients with acute TTP not being severely ADAMTS13 deficient.

It has been demonstrated that ADAMTS13 cleaves ULvWF multimers on the surface of endothelial cells. Upon their histamine-induced secretion from endothelial cells, these ULvWF multimers seem to remain anchored to the endothelial cell surface in a P-selectin-dependent manner and will be rapidly cleaved when perfused under venous or arterial shear stresses with normal plasma or purified ADAMTS13 but not with TTP plasma. Lopez and Dong provided experimental evidence that ADAMTS13 binds to both the A1
and A3 domains of the vWF subunit and suggested that this binding may be necessary for ADAMTS13-induced cleavage of the Tyr1605-Met1606 bond in the A2 domain of vWF. Hypothetically, structural defects of the ADAMTS13 molecule or antibodies impairing the binding of ADAMTS13 to the endothelial cell and/or to vWF might explain defective processing of endothelial cell-anchored U1vWF molecules in vivo despite normal activity of ADAMTS13 as assessed by the currently used static in vitro assays.

In this regard, it is important to note that Scheifflinger et al.31 reported non-neutralizing (noninhibitory) IgG and IgM autoantibodies in a patient with acquired TTP who showed, however, severe ADAMTS13 deficiency. They postulated an increased clearance of ADAMTS13, possibly by ADAMTS13-autoantibody complex formation.

Recently, an ADAMTS13 epitope mapping of autoantibodies from 25 patients with acquired TTP was performed.32 All patients had severe or borderline severe ADAMTS13 deficiency and circulating autoantibodies inhibiting the ADAMTS13 activity in mixtures of patient and normal plasma. Purified recombinant ADAMTS13 fragments were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and overlaid with the diluted patient plasma samples. All 25 plasma samples contained antibodies directed to the Cys-rich/spacer domain, 16/25 samples reacted with the Cub1+2 domains, 14/25 with the first thrombospondin type 1 (thrombospondin type 1/1) domain, 14/25 with the catalytic-disintegrin-thrombospondin type 1/1 domains, 7/25 with the thrombospondin type 1/2-8 domains, and 5/25 plasma samples recognized the propeptide region (Table 1). This shows that autoantibodies reacting with different antigenic regions of the ADAMTS13 molecule are present in acute TTP. Probably, the antibodies towards the Cys-rich/spacer domain, present in 25/25 investigated patients, are necessary to inhibit ADAMTS13 activity in currently used static ADAMTS13 assays. It is conceivable, however, that antibodies with other specificities may impair the ADAMTS13 interaction with endothelial cell-anchored vWF in vivo, and that such antibodies may lead to clinical manifestations of TTP.

Various assays have been proposed for measuring ADAMTS13 activity in plasma (for review, see Veyradier and Girma33). A multicenter study comparing several of these assays on 30 plasma samples with activity levels from <3 to >100% showed a generally good agreement concerning the identification of severe ADAMTS13 deficiency even though some false-positive and one false-negative result(s) were reported by laboratories using the very delicate collagen binding assay.34 For samples with normal or mildly reduced vWF-cleaving protease activity results among the participating laboratories were less concordant.

Table 1 ADAMTS13 epitope mapping study of autoantibodies in plasma samples of 25 patients with acquired TTP.

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<td>+</td>
</tr>
<tr>
<td>25</td>
<td>&lt;3</td>
<td>5</td>
<td>+</td>
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</tbody>
</table>

All patients had severe (or borderline-severe) ADAMTS13 deficiency and circulating inhibitors of ADAMTS13 activity (inhibitor titre given as Bethesda units (BU)/ml). Summary of the results obtained with immunoblot analyses: + denotes positive reactivity of antibodies towards the respective recombinant ADAMTS13 fragment. Equivalent amounts of reduced ADAMTS13 fragments (see Figure 1) were subjected to SDS-PAGE and electrotransfer to nitrocellulose membranes. Nitrocellulose membranes were blocked and overlaid with patient plasma diluted 1:500, and bound autoantibodies were detected using alkaline phosphatase conjugated goat anti-human Ig. From Klaus et al.,32 with permission of the publisher.
Probably, more physiologic, endothelial cell-dependent assays will improve the diagnosis of thrombotic microangiopathies in the future.39,40 Also, a recombinant peptide of 73 amino acids, spanning the vWF A2 domain sequence from Asp1596 to Arg1668 and containing the minimal specific substrate requirement for ADAMTS13, was expressed and may allow for an easy and fast ADAMTS13 activity assay.41

A clinically most relevant controversy raised during recent years relates to treatment of suspected TTP. Some authors argue that plasma exchange and FFP replacement, shown to be clinically effective14 at a time when knowledge about autoantibody-mediated ADAMTS13 deficiency was not available, should probably be reserved for those patients having severe acquired ADAMTS13 deficiency. Mori et al.,25 in a small study on 18 patients with acute TTP, found that 10/12 with severe acquired ADAMTS13 deficiency survived, whereas 4/6 with ADAMTS13 levels around 25% of normal died despite plasma exchange treatment. In contrast, among the 48 patients with idiopathic TTP from the Oklahoma TTP-HUS registry, the 16 patients with severe ADAMTS13 deficiency and the 32 patients without severely reduced activity did not differ significantly in their apparent response to plasma exchange therapy: 3/16 patients with and 7/32 without severe ADAMTS13 deficiency died from their disease.26 Thus, despite these contradicting findings, in our view plasma therapy clearly remains indicated in all patients diagnosed with acute TTP, at least as long as new treatment modalities, based on the underlying pathophysiology in those without severe acquired ADAMTS13 deficiency, are not yet available.

Acute acquired TTP often affects previously healthy subjects not having any signs or symptoms of coexisting autoimmune or other diseases. Even though some patients with systemic lupus erythematosus developed acute TTP, a severe ADAMTS13 deficiency caused by inhibiting autoantibodies seems rare in those patients. We have observed identical twin sisters having suffered from acquired TTP at ages of 23 and 24 years, respectively.36 This raises the question whether there is a genetic predisposition to develop autoantibodies inactivating ADAMTS13 activity.

Open questions needing further research

As is frequently the case in all areas of biomedical research, the discoveries of the past few years surrounding TTP have answered some questions yet generated even more new ones. Certainly, assays of ADAMTS13 activity need to be improved and simplified in order to become more suitable for the general routine laboratory. Such assays must be able to unambiguously distinguish severely deficient ADAMTS13 activity from moderately decreased levels. Ideally, such assays should measure as closely as possible the in vivo function of ADAMTS13, that is, its ability to rapidly cleave the newly secreted, endothelial cell-anchored ULvWF molecules under shear conditions as exist in the microvasculature. This will allow to appreciate whether some cases of acute TTP without severely decreased ADAMTS13 activity as measured by currently used assays may nevertheless be attributable to impaired ULvWF processing on the endothelial cell surface. Moreover, ADAMTS13 antigen assays are eagerly awaited.

Patients with acquired TTP, mainly when caused by inhibiting autoantibodies leading to severe ADAMTS13 deficiency, frequently relapse after complete recovery.26 The index patient with sporadic TTP in whom autoantibody-mediated severe ADAMTS13 deficiency was discovered12 lost his autoantibody and normalized vWF-cleaving protease activity in parallel with clinical recovery. Clinical relapse was preceded by reappearance of the inhibitor and disappearance of ADAMTS13 activity.12 However, we are currently following a patient who recovered from plasma refractory TTP only after splenectomy that led to disappearance of the inhibitor, normalization of vWF-cleaving protease activity and clinical remission some 4 years ago. Reappearance of severe autoantibody-mediated ADAMTS13 deficiency about 1 year ago did not result in any clinical disease manifestations so far.27 Large prospective studies in survivors of TTP with regular measurement of ADAMTS13 and its inhibitor will reveal whether this approach will allow to detect those patients at high risk of clinical relapse.

A more general question that will need to be addressed relates to the nature of this peculiar autoimmune disease. Are there specific HLA types or other markers of the immune response that predispose to the development of autoantibodies towards ADAMTS13? As mentioned above, it will be necessary to better define the role of plasma exchange and FFP replacement in those patients not suffering from severe ADAMTS13 deficiency. Will immunoadsorption using Protein A-sepharose columns improve and fasten recovery? What is the role of corticosteroids, splenectomy or rituximab in patients with (recurrent) autoimmune TTP? Finally, will the development of recombinant ADAMTS13 for replacement therapy improve treatment of TTP caused by autoantibody-mediated ADAMTS13 deficiency or will it at least facilitate replacement of ADAMTS13 in patients with hereditary TTP who often need regular FFP substitution to prevent relapses.6

The first 80 years of TTP are a fascinating story, both in terms of pathophysiologic discoveries and by the ingenious empirical introduction of plasma therapy that substantially improved the outcome of affected patients. The years to come will further inspire many researchers and hopefully lead to even better management of patients with TTP.

References


2 Hosler GA, Cusumano AM, Hutchins GM. Thrombotic thrombocytopenic purpura and hemolytic uremic syndrome are distinct pathologic entities. A review of


Idiopathic thrombocytopenic purpura (ITP) is a syndrome that may result from multiple mechanisms. Although ITP is commonly assumed to be due to accelerated platelet destruction caused by autoantibodies, recent data suggest that autoantibodies may also inhibit platelet production, consistent with platelet kinetic studies demonstrating decreased platelet production in some patients with ITP. Some patients with a clinical diagnosis of persistent ITP may have clonal hematopoiesis indicating myelodysplasia. Other data suggesting that myelodysplasia can have an autoimmune etiology support a hypothesis that ITP and myelodysplasia may be overlapping syndromes. These observations, together with data that serum thrombopoietin levels are not increased in most patients with ITP, suggest that thrombocytopenia may result from a combination of increased platelet destruction together with decreased or ineffective platelet production.

Diagnosis

The common use of platelet counts in routine evaluations of healthy adults has extended the spectrum of ITP, involving more asymptomatic and older subjects. In most patients, the diagnosis can be confidently established by the history, physical examination, complete blood count, and examination of the peripheral blood smear without additional procedures or laboratory tests. The careful examination of the peripheral blood smear is critical to confirm the presence of thrombocytopenia and exclude the existence of pseudothrombocytopenia caused by platelet clumping. In the rare adult patients who fail to achieve any response to initial treatment, marrow aspiration may reveal an unexpected diagnosis, such as acquired pure amegakaryocytic thrombocytopenia. Tests for antplatelet antibodies are not recommended; the sensitivity, specificity, and reproducibility of multiple types of assays are poor.

The key diagnostic tool is the history. Thrombocytopenia induced by drugs, herbal remedies, or foods must be carefully investigated and confidently excluded. A drug-induced etiology for thrombocytopenia is often not discovered until the ‘ITP’ suddenly recurs (see also Calvin’s story, http://moon.ouhsc.edu/jgeorge). Quinine is the most common drug causing isolated thrombocytopenia, and patients may not tell their doctors about their use of quinine, or other medicines that they regulate themselves, unless they are asked direct, explicit questions. Our website (http://moon.ouhsc.edu/jgeorge) contains a table describing the level of evidence supporting a causal relation of individual drugs to thrombocytopenia in all 1232 published patient case reports of drug-induced thrombocytopenia through July 2002.

Management

A suggestion for sequential management is presented in Table 1.

Initial presentation. The increased frequency of discovery of asymptomatic patients emphasizes the need for conservative management, to be certain that the side effects of treatment are not worse than the symptoms of ITP. In contrast to the common practice of only careful observation for children with acute ITP, adults who present with severe thrombocytopenia, even if no active mucosal bleeding is present, are routinely treated. This is

<table>
<thead>
<tr>
<th>Clinical status</th>
<th>Management</th>
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<tbody>
<tr>
<td><strong>Initial presentation</strong></td>
<td></td>
</tr>
<tr>
<td>Platelet count &gt; 30 000/μl, no or minimal bleeding</td>
<td>Observe</td>
</tr>
<tr>
<td>Platelet count &lt; 30 000/μl</td>
<td>Glucocorticoid</td>
</tr>
<tr>
<td>Platelet count &lt; 10 000/μl, overt bleeding</td>
<td>Glucocorticoid + IVIG or anti-D</td>
</tr>
<tr>
<td><strong>After failure of initial treatment</strong></td>
<td></td>
</tr>
<tr>
<td>Platelet count &gt; 20 000/μl, no or minimal bleeding</td>
<td>Observe</td>
</tr>
<tr>
<td>Platelet count consistently &lt; 20 000/μl or significant bleeding symptoms</td>
<td>Splenectomy</td>
</tr>
<tr>
<td><strong>After failure of splenectomy</strong></td>
<td></td>
</tr>
<tr>
<td>Platelet count &gt; 10 000/μl, no or minimal bleeding</td>
<td>Observe</td>
</tr>
<tr>
<td>Platelet count &lt; 10 000/μl with significant bleeding</td>
<td>Immunosuppressive treatment</td>
</tr>
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</table>
because it is assumed that the clinical course in adults will be chronic and the risk for bleeding is uncertain at the time of initial presentation. The important initial management decision for adults is at what level of thrombocytopenia-specific treatment, rather than reassurance and observation, is indicated. Recent large case series of consecutive patients5,6,13,14 support the practice of observation without specific treatment for patients with mild or moderate thrombocytopenia, often described as a platelet count greater than 30,000/μl. These observations emphasize the important principle that the goal of treatment is the prevention of severe bleeding, not the achievement of a normal platelet count. This principle is critical to avoid unnecessary treatment. The risks of treatment are apparent in a cohort of 134 consecutive patients followed for 9 years; 6 patients died, four (3%) from infection related to treatment and only two (1.5%) from hemorrhage (Table 2).5 Even short courses of glucocorticoids in otherwise immunocompetent patients with ITP can increase the risk for opportunistic fungal infections, such as aspergillosis.15 In a cohort of 245 consecutive patients followed for 5 years, three (1.2%) patients died from bleeding and one (0.4%) patient died from postsplenectomy complications (Table 2).6

For patients who present with platelet counts <30,000/μl or who present with significant bleeding symptoms, standard treatment is to begin with oral prednisone, 1 mg/kg, given once daily7,8 and continued for 2–3 weeks. Most patients will respond, however, many patients will have recurrent thrombocytopenia when the prednisone dose is tapered and discontinued. A recent study reported results with an alternative initial regimen, a 4 day regimen of dexamethasone, 40 mg/day, in 125 consecutive patients who presented with severe (platelet count less than 20,000/μl) or symptomatic thrombocytopenia (platelet count less than 50,000/μl and clinically significant bleeding).14 In this study, 106 (85%) of 125 patients responded to the initial 4 days of treatment and half of the responding patients maintained a safe platelet count, more than 30,000/μl, for the duration of follow-up (median duration, 31 months).14

The appeal of the dexamethasone regimen is the short, defined course that may be more tolerable than prednisone begun with no clear goal for stopping.

Some have suggested that initial treatment with intermittent anti-D treatment may allow patients to maintain a safe platelet count and to avoid splenectomy. However, our randomized clinical trial documented that the rates of splenectomy were the same for patients treated with standard care, prednisone followed by splenectomy, 38%, compared to patients treated with intermittent anti-D, 42%.16

Although most patients may not achieve a complete remission with initial prednisone, most patients appear to achieve a satisfactory result that is defined by a safe platelet count, no or minimal bleeding symptoms, and no requirement for additional treatment. In the recent report of 245 consecutive patients,6 only 12% required splenectomy and most patients eventually achieved a complete remission.

**Splenectomy** Splenectomy is the standard second treatment for patients with severe and symptomatic thrombocytopenia.7,8 Most hematologists recommend splenectomy 4–6 weeks after diagnosis if continuing or recurrent courses of glucocorticoid are required to maintain a safe platelet count.7 Beyond this time, the side effects of prednisone become intolerable and the risk for critical infections becomes too great.5,15 Although some reports suggest that recurrent ITP is frequent following splenectomy, most case series report durable remissions. We have recently analyzed all reported case series of 15 or more patients with splenectomy for ITP and determined that two-thirds of patients achieved a complete response (defined by a normal platelet count without treatment for more than 3 months and continuing for the duration of follow-up) and the reported complete remission rates were not different across case series with median follow-up times up to 13 years. However, the morbidity and mortality from splenectomy are substantial. Portielje et al.5 reported that 26% of patients had early postoperative complications resulting in prolonged hospitalization or readmission. Operative mortality is reported in 0.3–0.9% of patients. Laparoscopic splenectomy may be a safer procedure than open laparotomy. Complications that may occur many years after splenectomy include overwhelming sepsis, heart attack, stroke, and pulmonary hypertension. These data emphasize that splenectomy is appropriate only for patients who have a significant risk for serious bleeding. However, for these patients, no other current treatment is as effective as splenectomy.

**Management of patients who have persistent severe thrombocytopenia following splenectomy** These patients present the most difficult management dilemma. The platelet count may be very low, suggesting serious risk for severe bleeding, but the patient may have minimal or no symptoms. The efficacy of any treatment to cause a

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**Table 2 Causes of death in adults with ITP**

<table>
<thead>
<tr>
<th>Case series</th>
<th>Deaths due to bleeding</th>
<th>Deaths related to treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>134 consecutive patients with platelet counts &lt;100,000/μl followed for 9.4 years</td>
<td>Two (1.5%) (intracerebral hemorrhage)</td>
<td>Four (3.0%) (three, infection related to immunosuppressive treatment; one, pneumococcal sepsis after splenectomy)</td>
</tr>
<tr>
<td>245 consecutive patients with platelet counts &lt;50,000/μl followed for 5 years</td>
<td>Three (1.2%) (location not specified)</td>
<td>One (0.4%) (postsplenectomy complications)</td>
</tr>
</tbody>
</table>

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**Management of patients who have persistent severe thrombocytopenia following splenectomy** These patients present the most difficult management dilemma. The platelet count may be very low, suggesting serious risk for severe bleeding, but the patient may have minimal or no symptoms. The efficacy of any treatment to cause a
sustained remission is low or unknown.17 The treatment options of more intensive immunosuppression carry even greater risk of critical infections, therefore the indications for treatment must be even more restrictive. It may be appropriate to only carefully observe patients who have negligible bleeding symptoms, even if their platelet counts are less than 10,000/μl. Many patients lead active lives with negligible symptoms in spite of very low platelet counts for many decades (see Crystal’s story, http://moon.ouhsc.edu/jgeorge). Other patients have negligible bleeding symptoms, even if their platelet counts are less than 10,000/μl.18 Many patients may be to stimulate platelet production with thrombopoietin or its analogs. This therapy is based on the observations that platelet production may not be maximal, that endogenous serum thrombopoietin levels are not elevated and that stimulation of platelet production by exogenous thrombopoietin can achieve safe platelet counts. Preliminary reports have documented the effectiveness of treatment with thrombopoietin analogs in small numbers of patients.19,20

References


THROMBOPHILIA/THROMBOSIS

Relations between hemostatic variables, insulin resistance and inflammation

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Introduction

Insulin resistance, also called the metabolic syndrome (MS) is a constellation of lipid and nonlipid factors that places subjects at risk for cardiovascular events. MS is large world wide-spread. Its prevalence in USA is around 23% and increases from 7% for subjects aged 20–29 years to 43% for subjects aged 60–69 years.\(^1\) In Framingham, the metabolic syndrome predicted 25% of all new-onset of cardiovascular disease (CVD).\(^2\) It has recently been defined by the Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in adults (ATP-III) which suggests that the diagnosis should be made when three or more of the following characteristics are present: high fasting glucose, high blood pressure, low HDL cholesterol, high triglycerides, and abdominal obesity.\(^3\) ATP-III identified 6 components of the metabolic syndrome that relate to CVD: abdominal obesity, atherogenic dyslipidemia, raised blood pressure, insulin resistance \textit{per se}, proinflammatory state and prothrombotic state. In this chapter, we will focus on the interconnection between these two last factors and the MS.

Metabolic syndrome and inflammation

Several studies add support to the concept that a proinflammatory state is a component of the MS.\(^4\) All the parameters belonging to MS are associated with elevated levels of CRP that has proven to be a strong independent predictor of both incident diabetes\(^5\) and incident CVD.\(^6\) Consistent with these observational studies, insulin sensitizing pharmacological agents such as rosiglitazone reduced CRP levels by 25% in diabetic subjects.\(^7\) In a cohort of 14 719 initially healthy women, Ridker \textit{et al}\(^8\) have demonstrated that at all levels of severity of the metabolic syndrome, CRP added important and independent pronostic in terms of future cardiovascular risk. The reasons for a link between inflammation and metabolic syndrome are not fully understood. One explanation may be that adipose tissue in obese persons with the metabolic syndrome releases increased amounts of cytokines into the circulation; this in turn accounts for a greater production of CRP by the liver.\(^9\) Another possibility is that insulin resistance \textit{per se} is responsible for a higher production of cytokines.\(^10\) Regardless of mechanism, the finding that patients with metabolic syndrome exhibit characteristics of a proinflammatory states provides a new and exciting connection between inflammation and metabolic process. This connection promises to yield new insights into pathways whereby the MS leads to atherosclerosis and acute coronary syndrome.

Metabolic syndrome and prothrombotic state

A prothrombotic state is also present in the MS. Disturbances of the hemostatic system may favor the development of vascular damage, by playing a role in the vessel wall remodeling and the final occlusion event, by promoting thrombosis at the site of a suddenly ruptured atherosclerotic plaque. Among prothrombotic factors, three have been particularly associated with variables belonging to MS: two markers of hypercoagulability: von Willebrand factor (VWF) and fibrinogen and one of decreased fibrinolytic potential : plasminogen activator-inhibitor-1 (PAI-1). These 3 markers predict the risk of CVD and are associated with inflammatory parameters, underscroing the proximal link between inflammation, metabolic disorders, coagulation and CVD.

Von Willebrand factor

VWF is synthesized and secreted by endothelial cells and, to a lesser extent by megakaryocytes. VWF mediates platelet adhesion and plays a role in thrombus formation.\(^11\) The plasma levels of VWF have been shown to increase with endothelial damage, suggesting that it could be reliable marker of endothelial dysfunction.\(^12\) Furthermore an alteration in the production of VWF by the endothelium could directly contribute to atherothrombotic disease. In individuals healthy at baseline, a recent meta-analysis yielded a combined odds ratio of 1.5 for the risk of CVD.\(^13\) However, among the studies included in this analysis, the predictive ability of VWF strongly depends on the variables controlled for. The precise mechanism by which VWF is associated with cardiovascular risk is
unclear. VWF, an acute phase reactant could be only a marker of the inflammatory process. Accordingly, the ECAT study showed in subjects with angina pectoris, that the independent relative risk of cardiovascular mortality associated with VWF disappeared after adjustment for CRP. In the PRIME study conducted in 10,000 healthy men, high VWF levels remained a predictor of myocardial infarction or coronary death even after adjustment for inflammatory parameters suggesting different pathways through which VWF and inflammation determine CVD risk. Several evidences are in favor of an association between high VWF concentrations in plasma and MS. In population studies, concentrations of VWF have been found to correlate with those of insulin, a surrogate for insulin resistance. Furthermore in patients with type II diabetes, Yudkin et al found a relationship between endothelial dysfunction, as evidenced by raised concentrations of VWF and a measure of insulin resistance. This endothelial dysfunction could play a pivotal role in the relation between MS, inflammation and CVD. Compared with nonobese women, obese women had increased basal concentrations of cytokines such as IL-6 and TNF-2 and endothelial dysfunction (measured by evaluation of vascular responses to L-arginine). In obese women, sustained weight loss was associated with reduction of IL-6 and TNF-2 and with improvement of endothelium dysfunction.

Fibrinogen

Early epidemiological evidence has associated high levels of plasma fibrinogen with CVD incidence. Fibrinogen may contribute to CVD via a number of mechanisms, including increased fibrin formation, plasma viscosity and platelet aggregation. On the other hand, fibrinogen is an acute phase reactant, and the association of elevated fibrinogen and CRP levels with risk of arterial thrombotic disease suggests that inflammation that accompanies atherosclerosis may contribute to increased fibrinogen levels. This hypothesis was reinforced by the PRIME study, where the predictive ability of fibrinogen on the risk of CVD disappeared when CRP, IL-6 and fibrinogen were included in the same model. The increased concentration of fibrinogen is quite consistently described in individuals with MS. It is now considered that the fibrinogen level is determined by overall adiposity rather than insulin resistance. It is related to obesity per se and weakly related to abdominal obesity and insulin levels. IL-6 is thought to be at the basis of this abnormality. IL-6 is produced by adipose tissue and will directly stimulate the hepatic synthesis of fibrinogen. IL-6 could therefore represent a link between obesity and fibrinogen levels in plasma.

Plasminogen activator-inhibitor-1

The fibrinolytic system consists of the circulating proenzyme plasminogen which is converted to plasmin by the plasminogen activators, tissue-type plasminogen activator (t-PA) and urokinase. Given its key role in the regulation of fibrinolysis by specific inhibition of t-PA and urokinase, PAI-1 is considered as a good candidate for CVD. High PAI-1 levels is almost consistently associated with the risk of CVD in univariate analysis. However, its predictive ability disappeared after adjustment for parameters belonging to MS. In a recent study conducted in 1276 adults randomly chosen, for the same amount of atherosclerosis measured by a B-mode carotid ultrasound examination, people with MS had a greater prevalence of CVD. This difference in CVD prevalence among the groups was attenuated after adjustment for PAI-1 levels. These observational studies, suggest that fibrinolytic dysfunction mediates the increased risk of CVD in individuals with MS. It is now well established that hypofibrinolysis due to elevated plasma PAI-1 levels is a core feature of the MS. PAI-1 levels are elevated in obese insulin-resistant patients as well as in non-insulin dependent diabetic patients and are in the normal range in type I diabetic patients. Plasma PAI-1 levels have been shown for a long time to be associated with the parameters of the MS. They correlate with BMI, visceral fat, which characterizes android obesity, blood pressure, plasma levels of insulin or proinsulin, triglycerides, free fatty acids, and are inversely related with HDL cholesterol, whereas no correlation is observed to measures of glycemic control. The modulation of insulin resistance by hypocaloric diet with losing weight, physical training, change in dietary composition, the use of oral antidiabetic drugs such as metformin, induce a decrease in PAI-1 levels which correlate with the decrease in weight and in plasma metabolic parameters. It was shown recently that the use of thiazolidinediones such as troglitazone is associated with a decrease in plasma PAI-1 levels in persons with type II diabetes. Although insulin added in vitro to hepatocytes induces an increase PAI-1 synthesis, and hyperinsulinemia is associated with elevated plasma PAI-1 levels, an acute administration of insulin does not modify plasma PAI-1 concentration or proinsulin, triglycerides, free fatty acids and glucose increase the PAI-1 production by cells in culture.

One uncertainty about PAI-1 synthesis during insulin resistance is its cell origin. Unequivocally many cells/tissues could synthesize PAI-1. But since PAI-1 gene expression is inducible rather than constitutive, one of the foremost question is to recognize the inducer that is concerned in insulin resistance. One approach was to propose that metabolic disturbances observed during this syndrome, directly affect PAI-1 synthesis. Indeed, most cell culture experiments confirm these hypotheses. It has been shown that insulin, glucocorticoids, VLDL, free fatty acid and glucose increase the PAI-1 production by cells in culture. Among the other potential inducers of PAI-1 synthesis the proinflammatory cytokines, TNF-2 appears of particular relevance in the context of insulin resistance. Indeed, it has deleterious effects on both glucose homeostasis and beta-cell function, and can disrupt insulin signaling pathways in both pancreatic beta cells and liver and adipose tissue. In mice, the adipose tissue revealed to contain high amounts of
PAI-1 and the invalidation of both TNF receptors results in a significantly reduced adipose tissue PAI-1 expression and plasma PAI-1 levels.\(^4\)

We have described,\(^4\) as others\(^45,43\) a PAI-1 synthesis by human adipose tissue. We have shown a decrease of PAI-1 expression in adipose tissue by weight loss\(^4\) and a variability of PAI-1 expression depending on fat territory. Visceral fat produces more PAI-1 than subcutaneous or femoral fat.\(^4\) The main PAI-1 producing cells have been identified as stroma cells\(^4\) some of them being of monocyte or smooth muscle cell origin. Thus the adipose tissue could represent a PAI-1 reservoir. However these results do not exclude the contribution of other tissues. Indeed, the liver is also a good candidate since some groups have observed a strong relationship between plasma PAI-1 and liver steatosis levels.\(^46\) The PAI-1 antigen content in human adipose tissue was found highly correlated with those of TNF receptors and TGF\(\beta\), but not to that of TNF2 or IL6 which were both strongly correlated to each other.\(^47,48\) These results from mice and human studies suggest that the TNF pathway could be a common link between elevated PAI-1 and insulin resistance. On the other hand, the levels of TGF\(\beta\), one of the strongest inducers of PAI-1 synthesis in vitro are also associated with those of PAI-1 within the human adipose tissue and could be involved in the regulation of PAI-1 during insulin resistance.

The high expression of PAI-1 during insulin resistance queries on the finality of this phenomenon. Several recent data have suggested that PAI-1 could have a role beyond atherothrombosis in insulin resistance. It was recently demonstrated in a large cohort of healthy non diabetic subjects, that patients who develop an incident diabetes within 5 years presented higher levels of CRP and PAI-1 at baseline than nonconverters.\(^49\) PAI-1 predicted type II diabetes independently of insulin resistance and other known risk factors for diabetes. As PAI-1 is involved in tissue remodeling, it can be suggested that the elevated expression of PAI-1 observed in obesity is involved in adipose tissue development. We and others have shown that modulation of the PAI-1 gene in mice leads to modification of adipose tissue development and metabolic parameters.\(^50-53\) This role of PAI-1 has been reinforced by a recent study showing that PAI-1 inhibits insulin signal in vitro.\(^54\) PAI-1 plasma levels is partly under genetic controls, individuals carrying the 4G allele of the ‘675 4G/5G polymorphism present higher plasma levels.\(^24\) In agreement with the hypothesis of a direct role of PAI-1 in the development of MS is the results obtained by Lopes et al.,\(^55\) showing that this potential functional polymorphism modulates obesity-associated phenotypes in human.

Treatment with an ACE inhibitor has been shown to decrease not only PAI-1 levels\(^56\) and the rate of cardiovascular events\(^57\) but also the incidence of type II diabetes.\(^58\) Therefore, PAI-1 could play a part in the development of cardiovascular diseases, and in type II diabetes. Both these properties indicate that PAI-1 represent an ideal target for therapeutic intervention that aims to decrease the risk of both cardiovascular disease and type II diabetes.

**Conclusion**

A procoagulant state, likely metabolically interconnected with a proinflammatory state has been described in MS and favored the development of CVD. Weight loss represent a safe method for downregulating the inflammatory state, for ameliorating fibrinolytic activity and endothelial dysfunction. Drugs that specifically target procoagulant factors, and particularly PAI-1, could represent a good therapy for both reducing the development of MS and of its most important complication, CVD.

**References**


Thrombosis and cancer

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Venous thromboembolism is a common problem in cancer patients. It complicates both the surgical management of those with cancer and has been associated with varying risk for the development of clinical thromboembolism in patients receiving chemotherapy. Unfractionated and low-molecular-weight heparin have been validated both in terms of their efficacy and safety, in the prevention of venous thromboembolic disease in cancer patients undergoing surgical intervention and for the primary treatment and secondary prevention of recurrent venous thromboembolism. Beyond their uses in the prevention and treatment of thrombosis, low-molecular-weight heparins have recently been shown to prolong survival in patients with solid tumour malignancy.


Introduction

Thrombosis is a common problem in patients with malignant disease. An acute venous thromboembolic episode may be the first manifestation of underlying cancer. Studies suggest that between 10 and 17% of patients with a spontaneous venous thromboembolic episode, in which no underlying cause for the development of thrombosis can be identified, and in particular those with recurrent venous thromboembolism, will go on to have the diagnosis of a new cancer within 2 years.1,2 Despite these observations, the implications of cancer-associated thrombosis as a presenting feature of overt malignancy have yet to be fully elucidated. In particular, trials of sufficient power have not yet been able to demonstrate that screening for underlying malignant disease in patients with spontaneous venous thromboembolism can be associated with improved long term survival in such patients, through the earlier detection of tumours, at a more favourable stage in their natural history.3

Venous thromboembolism complicating surgical and medical management of cancer patients

Beyond spontaneous venous thromboembolism in the cancer patients, thrombosis is known to complicate the course of patients having undergone surgery for cancer.4 Cancer is well recognised to be one of the major risk factors for the development of postoperative deep vein thrombosis. The incidence of thromboembolic complications in the immediate postoperative period in cancer patients without routine thrombo prophylaxis is alarming. Historical series suggest an incidence of between 1 and 5% of fatal pulmonary embolism without the use of some form of prophylaxis against VTE in such patients undergoing major abdominal or pelvic surgery for cancer.5 In patients receiving chemo or radiotherapy for management of their cancer, there are fewer data available from prospective studies that have determined the incidence of venous thromboembolic complications. The best-investigated population of patients are women with breast cancer.6 There appears to be a relationship with stage of disease, and a potential interaction with the type of therapy used. For patients in the adjuvant setting, incidence rates as high as 9.6% have been reported where cytotoxic therapy has been combined with hormonal therapy with tamoxifen. Recent studies suggest that use of hormonal agent may impact importantly on the rate of clinical thrombosis, and that substitution of an aromatase inhibitor for tamoxifen is associated with a lower clinical thrombosis rate.7 Beyond breast cancer, there are only limited data with regard to VTE risk for medical oncology patients. However, there is a suggestion that novel biological therapies directed at inhibition of angiogenesis may be associated with higher rates of thromboembolic complications. Such observations with new and exciting anticancer therapies with regard to thrombosis risk may drive a greater awareness of this problem among oncologists.

Prevention of venous thromboembolism

For surgical cancer patients, both low dose unfractionated heparin and the low-molecular-weight heparins have been validated in terms of efficacy and safety for the prevention of venous thromboembolism. Low-dose unfractionated heparin, administered at a dose of 5000 U commenced 2 h prior to operation and continued three times daily in the postoperative period has been shown to significantly reduce the incidence of fatal pulmonary embolism.8 This benefit extends to patients undergoing operation for cancer. The low-molecular-weight heparins, in general, given once daily to general surgical patients undergoing operation for cancer have
also been validated in terms of efficacy and safety in trials where they have been compared to low-dose unfractionated heparin. Studies during the 1990s have demonstrated that more intense perioperative thromboprophylaxis benefits the cancer surgical patient. In a large study of over 2000 patients, 65% of whom underwent laparotomy for cancer; patients received either 2500 or 5000 U of the low-molecular-weight heparin dalteparin sodium for perioperative thromboprophylaxis. The higher dose of dalteparin was associated with a significant reduction in the rate of postoperative deep vein thrombosis without any significant increase in bleeding complications. Current guidelines on antithrombotic therapy advocate either the use of low-dose or low-molecular-weight heparin for perioperative thromboprophylaxis in such patients. In a large trial randomising over 23,000 patients to either of these two regimens, with a primary end point of autopsy proven fatal pulmonary embolism, cancer patients, despite the use of thromboprophylaxis were shown to have nearly three times the rate of fatal pulmonary embolism when compared to those patients undergoing major operation without cancer. These data suggest that further attention needs to be paid to the problem of postoperative thromboembolic disease in patients with malignancy. A further strategy that has recently been evaluated, involved prolongation of thromboprophylaxis into the postdischarge period for patients undergoing laparotomy for cancer. In recent trials, prolonging thromboprophylaxis beyond the period of hospitalisation for up to 4 weeks after operation. This resulted in a significant reduction in the rate of venographically screened deep vein thrombosis at 4 weeks after surgery when compared to patients who received only in-hospital thrombo prophylaxis with low-molecular-weight heparin.

Trials evaluating low-molecular-weight heparin for prophylaxis of venous thromboembolism in ambulatory cancer patients receiving chemotherapy out of hospital have yet to report. The only agent that has been evaluated for the prevention of thromboembolic complications in patients receiving chemotherapy is low-dose warfarin, dosed to achieve an INR of between 1.3 and 1.9 compared to placebo. In this study low-dose warfarin therapy was associated with an 85% reduction in the rate of venous thromboembolic complications among 311 women with advanced breast cancer receiving cytotoxic chemotherapy. The rates of clinical thromboembolism were 4.4% in the placebo group reduced to 0.7% in those patients receiving low-dose warfarin.

Studies in the early 1990s suggested that the use of central venous catheters in cancer patients was associated with a higher rate of thromboembolic complications, and either low-dose warfarin or low-molecular-weight heparin were effective in the prevention of such complications. Recent studies, where catheters have been used in the contemporary setting, have indicated that rates of thrombosis are lower than previously anticipated. Routine antithrombotic therapy for prophylaxis against thrombotic complications in this setting requires further evaluation.

**Treatment of venous thromboembolism in the cancer patient**

Initial treatment of venous thromboembolism in the cancer patient is with either intravenous unfractionated heparin or subcutaneous low-molecular-weight heparin. Unfractionated heparin therapy requires hospitalisation of the patient and daily monitoring of the activated partial thromboplastin time. The advantages of low-molecular-weight heparin for initial management of acute venous thromboembolism include the ability to dose low-molecular-weight heparin on a weight-adjusted basis, without the need for routine laboratory monitoring and its subcutaneous administration and thus the ability to treat patients on an outpatient basis. Beyond initial treatment, where low-molecular-weight heparins have been demonstrated to be as effective and slightly safer than intravenous unfractionated heparin, patients presenting with acute thromboembolism require antithrombotic therapy for long-term secondary prevention. This is usually provided with the oral anticoagulant vitamin K antagonists such as warfarin. Cancer patients receiving such therapy for prevention of recurrent thromboembolism have three times the risk for the development of a clinical recurrence of their thrombosis and twice the risk for the development of clinically important bleeding complications. This group represent a major challenge in thrombosis practice.

Studies have evaluated the potential benefits of prolonged low-molecular-weight heparin therapy for the prevention of recurrent thromboembolism in patients with acute venous thromboembolism. In general, these studies using high-risk prophylaxis doses of low-molecular-weight heparin suggest that there may be some benefit to this strategy. A large randomised multicentre trial ‘the CLOT in Cancer Trial’ randomised 676 patients with objectively documented acute proximal vein thrombosis or pulmonary embolism and active cancer, either low-molecular-weight heparin dalteparin sodium or oral anticoagulant therapy for the prevention of recurrent venous thromboembolic disease. All patients in this study received 5–7 days of the low-molecular-weight heparin dalteparin sodium in a dose of 200 U/kg and were, thereafter, randomised to continue with low-molecular-weight heparin in the full treatment dose for 1 month and 75–80% of the full treatment dose for a further 5 months or to oral anticoagulant therapy with a target INR of 2.5 for 6 months. This trial demonstrated a 52% reduction in the rate of recurrent VTE in cancer patients randomised to receive dalteparin sodium. There was no significant increase in bleeding complications associated with such therapy when compared to vitamin K antagonists.

**Antithrombotic therapy and cancer survival**

Retrospective meta-analyses of DVT treatment studies have indicated that cancer patients, randomised to trials where intravenous unfractionated heparin was
compared to subcutaneous low-molecular-weight heparin for the initial treatment of deep vein thrombosis, had a survival advantage if they received the low-molecular-weight heparin.23 Such analyses undertaken to demonstrate a survival benefit from DVT treatment trials where such an end point was not part of the original trial design are fraught with difficult dangers. However, such analyses stimulated an interest in an evaluation of the potential benefits of low-molecular-weight heparin therapy in terms of survival in patients with advanced malignant disease. The FAMOUS study (Fragmin Advanced Malignancy Outcome Study) randomised 385 patients with a variety of advanced solid tumours to receive the low-molecular-weight heparin dalteparin sodium in a dose of 5000 U once daily or placebo for up to 1 year. Although this study failed to detect a significant difference in survival associated with low-molecular-weight heparin therapy, it did suggest that in a subgroup of patients there may be important survival advantages.22 Further studies have supported this hypothesis demonstrating both an overall survival advantage and a more pronounced survival benefit in patients with a better prognosis randomised to receive low-molecular-weight heparin in a variety of trial designs. The potential mechanistic explanation for such a survival advantage may include either the prevention of silent, but fatal, thromboembolic episodes, and inhibition of the coagulation proteases that are activated in cancer patients and which in a variety of elegant experimental studies have been shown to alter tumour cell phenotype and enhance both tumour growth,

References

14 Reichart P, Kretzschmar A, Biakhov M, Irwin D. A Phase III double-blind, placebo-controlled study evaluating the


Treatment of venous thromboembolism: duration and new options

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Introduction

Treatment for venous thromboembolism (VTE) typically includes a 5–10 day course of (low molecular weight) heparin followed by a course of at least 3 months of vitamin K antagonists (VKA), with adjustments of doses to achieve an INR of 2.0–3.0.1

The first evidence for the need of heparin was provided by a landmark study published in 1960.2 In this study, postsurgical patients were randomised between intravenous unfractionated heparin (UFH) and placebo. This study was stopped since 25% of placebo-treated patients had died of fatal pulmonary embolism (PE) and 25% had recurrent nonfatal PE; in the heparin group no recurrent PE was observed. In the pivotal study demonstrating the need for vitamin K antagonists, patients were randomised between a fixed dose of UFH (5000 I.U. twice daily) and warfarin (INR 2.0–3.0).3 Recurrent VTE events occurred in 27% of patients in the heparin group versus 4% in the warfarin group. Nowadays, low-molecular-weight heparin (LMWH) is the treatment of choice as initial treatment in deep vein thrombosis (DVT). In patients with PE, LMWH is increasingly used, in spite of rather limited evidence.4

The most important side effect associated with any effective dose of an antithrombotic agent is the inherent risk of bleeding of which the main determinants during treatment are the duration and intensity of therapy. In recent years, a number of randomised studies evaluating these issues in patients treated with VKA have been published. The main disadvantages of the antithrombotic regimen consisting of heparin followed by VKA are the need for parenteral drug administration during the acute phase, and the need for monitoring and frequent dose adjustments of VKA as well their interaction with other drugs. It may well be that newer anticoagulants could overcome some of these drawbacks.

Optimal duration of anticoagulant therapy

Two randomised studies have had a major impact on the issue of optimal duration of VKA. In the first study, a statistically significant difference of the 12 months rate of recurrent VTE was found after a 4-week duration of VKA treatment versus a 3-month period.9 Importantly, the risk of recurrence was found to be substantially lower among patients with a temporary risk factor (eg after surgery) than in patients with more permanent risk factors. A drawback of this study was the use of sub-optimal diagnostic methodology regarding the endpoints. In the second study, the odds ratio for recurrent VTE after 2 years of follow-up was 2.1 for patients treated for a 6-week period with VKA versus patients treated during 6 months.6 This difference was not offset by a higher rate of major bleeding or deaths in the longer duration group. In both treatment groups, there was a continuous risk of 4–5% recurrent VTE per year after stopping VKA. After these two studies, three more randomised trials evaluating the optimal duration have been carried out, one confirming the results of the two earlier studies and the other two studies showing no difference between longer and shorter duration.8,9 Two meta-analyses have shown that a longer duration of VKA treatment of at least 6 months is more effective than a shorter duration; the first showed no significantly increased risk of bleeding, the second did show a difference in major bleeding.10,11 It is unsettled whether a 3-month period of VKA treatment is as effective as a 6-month course. For how long VKA should be continued after 6 months is also still a matter of debate. It is clear that during therapy with VKA, the risk of VTE recurrence is effectively reduced by approximately 90% to a 0.7 episodes per 100 person-years,12,13 In the 6–12 months immediately after discontinuation of therapy the absolute incidence of recurrent VTE is 5–10%. This has been observed after 3, 6 and 12 months of VKA therapy8,14 and suggests that prolonging VKA therapy delays rather than stops recurrence of VTE. Careful cohort studies have shown that during subsequent years the risk of recurrence of VTE stabilises and the annual incidence of recurrence is 1–2%15,16

In conclusion, current recommendation is to give a period of at least 3 months of VKA for a first episode of VTE, with reversible or time-limited risk factors; at least 6 months for idiopathic first VTE event and at least 12 months to patients presenting with recurrent idiopathic VTE or a long-term risk factor (eg cancer, antithrombin deficiency, or antiphospholipid syndrome).1

Patients with an increased risk of recurrence

Among the suggestions of selecting these patients are the extent and severity of the thromboembolic event, the
number of events, the results of thrombophilia screening, the presence of residual thrombosis and the results of global testing of hypercoagulability.

**Number of events**

While the severity and extent of an event has never been the subject of a formal trial and is thus more opinion than evidence-based, only one study has randomised patients presenting with a second event of VTE. In this study, treatment for 4-years caused a trend towards more major bleeding than VKA therapy for 6 months, while the recurrence rate was significantly reduced in the 4-year treatment group.

**Thrombophilia as a risk factor for recurrence**

Since the presence of antithrombin deficiency, homozygous Factor V Leiden mutation (FVL) or antiphospholipid syndrome is associated with a high risk for recurrence, there is general consensus that in patients with antithrombin deficiency and homozygous FVL, VKA treatment should be extended.

It has become clear that the presence of neither a heterozygous FVL nor a heterozygous prothrombin mutation selects a patient group in need for a longer duration of VKA therapy. In a prospective study, the incidence of recurrent VTE among 83 patients with heterozygous FVL over a period of 5 years was 20 versus 21% among 204 patients without such a mutation (RR 0.9 for patients with FVL (0.5–1.6, P = 0.60). Likewise, patients heterozygous for the prothrombin 20210A mutation did not have a higher risk of recurrent VTE than patients without the mutation.

Very recently, a randomised study evaluating the effect on recurrent VTE of vitamin therapy in patients with hyperhomocysteinemia and a first thrombotic event was completed. This study in 701 patients did not show a significant reduction in recurrent VTE in patients with VTE who, after a standard period of 3–6 months of VKA, were randomly treated for a period of 2.5 years with daily supplementation of a high-dose multivitamin cocktail consisting of 5 mg folic acid, 50 mg of pyridoxine and 0.4 mg hydroxycoabalin.

**D-dimer and thrombus load**

A global testing of the haemostasis system such as D-dimer proved to have high predictive value (94%; 95% CI 91–96%) of ruling out recurrent VTE when this blood test was carried out 1 month after stopping VKA treatment. The positive predictive value of an abnormal D-dimer test for recurrent VTE after withdrawal of oral anticoagulant treatment proved low with 16% (95% CI 12–22%), which is not surprising giving the characteristics of D-dimer tests. Alternatively, D-dimer testing during VKA therapy was also highly predictive (95%, 95% CI 86–99%) of ruling out a subsequent recurrence of VTE after stopping treatment.

Alternatively, predicting recurrence could be based on assessment of persistent venous obstruction – indicating residual thrombus in the venous system – by compression ultrasonography. In two cohort studies persistent obstruction was predictive for recurrent VTE. In the first study, performed in 313 patients with prior proximal DVT and conventional 3 months of VKA treatment, of 58 recurrent episodes of VTE, 41 occurred in patients with residual thrombosis, for a hazard ratio for recurrent VTE of 2.4 (95% CI 1.3–4.4, P = 0.004). Prospective randomised studies, in which anticoagulant treatment management is based on the results of both D-dimer testing and measurements of residual thrombosis are needed to further define the role of these two tests.

**Alternative regimens for secondary prophylaxis**

Although there is uncertainty as to how long secondary prophylaxis with VKA should be continued, there is general consensus to extend the duration beyond 6 months in patients with recurrent, ‘ipsilateral, DVT, active cancer, and more severe forms of thrombophilia, including antithrombin deficiency and homozygous FVL. It is apparent that bleeding is the major offset after prolonged anticoagulant treatment. To resolve this therapeutic dilemma, two trials have recently been performed. In the first study, called Prevent, warfarin therapy at a low target INR – 1.5–2.0 – was randomly compared to placebo in patients with unprovoked DVT who had all been treated with VKA, aiming at an INR 2.0–3.0 for a median period of 6.5 months prior to randomisation. The mean follow-up period was 2.1 years. The study was prematurely stopped after inclusion of 508 patients. Among 253 patients on placebo, 37 had recurrent VTE (7.2 per 100 person-years) compared with 14 of 255 patients treated with low-dose warfarin (2.6 per 100 person-years) for a significant risk reduction of 64%. Major hemorrhage was an infrequent event and not different in the two groups. In the other study, a regular intensity regimen of warfarin (target INR 2.0–3.0) was compared with low intensity (INR 1.5–1.9) in 738 patients with unprovoked DVT, all of who had had 3 months of regular regimen warfarin. Recurrent VTE was significantly less frequent in the regular intensity group (0.6 per 100 person-years) than in the low-intensity group (1.9 per 100 person-years, HR 3.3, 95% confidence interval 1.2–9.1). There was no significant difference in major bleeding between both groups. The low intensity group had 1.1 per 100 person-years of major bleeding, versus 0.9 per 100 person-years in the regular intensity group. Both rates of recurrent VTE and major bleeding were relatively low in the latter study when compared to rates observed in other studies, in which 10% recurrent VTE during the first year after complete discontinuation of anticoagulation and 3–4% major bleeding per year was observed. In spite of this potential patient selection
in the latter study, it has been argued that the optimal intensity of VKA treatment in patients with VTE is now settled at a target INR of 2.0–3.0.\textsuperscript{38}

**New anticoagulants**

The mainstay for treatment of patients with VTE is the combination of LMWH and VKA. Although this anticoagulant therapy has been available for more than 60 years, both LMWH by its parenteral use and VKAs with their laborious use involving frequent monitoring and dose alterations, have their drawbacks. In recent years, alternative ways to develop new antithrombotic drugs have been sought, among which selective inhibition of one of the coagulation factors is potentially attractive since it may lead to better antithrombotic properties without concomitant increased risk of bleeding.

**Indirect inhibition**

Fondaparinux is an analogue of the smallest entity of heparin with anticoagulant effect, called pentasaccharide sequence. It is produced by chemical synthesis. This drug, which is given subcutaneously, has a high affinity to antithrombin and has selective and irreversible inhibition of the action of factor Xa, leading to inhibition of thrombin. The elimination plasma half-life is around 17 h, independent of the dose. The molecule is not metabolised and up to 84% of the injected dose eliminated by the kidney.

Two randomised studies, one in patients with DVT and one in PE, have been performed to compare the efficacy and safety of 7.5 mg fondaparinux to that of (low molecular weight) heparin as initial treatment.\textsuperscript{26,30} In both studies, it was observed that fondaparinux was non inferior to the comparator regimen, which consisted of enoxaparin 1 mg/kg twice daily in the DVT study, and intravenous unfractionated heparin in the PE study. For long-term treatment, the long-acting pentasaccharide and factor Xa inhibitor idraparinux is injected subcutaneously once a week. At a dose of 2.5 mg, it gave significantly less bleeding than warfarin ($P = 0.029$) without loss of efficacy in a phase 2 dose ranging study in patients with DVT.\textsuperscript{31} Studies in patients with DVT and PE in comparison with a combination of LMWH and warfarin (INR 2.0–3.0) are being performed, the results of which are expected in 2005.

**Direct inhibition**

While the first synthetic direct thrombin inhibitors, recombinant hirudin and several derivatives, have been introduced into the clinical arena many years ago, their main disadvantages are that they have to be administered by the parenteral route and that they exhibit a narrow therapeutic range. Ximelagatran is an orally available prodrug of the direct reversible thrombin inhibitor melagatran. This drug has a plasma elimination half-life of 3–5 h, has predictable pharmacokinetics and does not need laboratory monitoring. In the double-blind placebo-controlled Thrive III trial in 1223 patients with DVT, all of whom had been treated for a period of 6 months with VKA (target INR 2.0–3.0), ximelagatran in a dose of 24 mg twice daily, was randomly compared to placebo and given for 18 months.\textsuperscript{32} The number of recurrent VTE events was reduced from 71 to 12 for a hazard ratio of 0.16 (95% CI 0.09–0.30, $P < 0.001$). The major bleeding rate was not significantly increased in the active treatment group. It should be pointed, however, that the 1% major bleeding rate in this study was relatively low over the 18-month study period. A possible explanation is the exclusion of patients with a higher bleeding tendency during the prior 6 months of VKA treatment. A phase III trial comparing ximelagatran 36 mg twice daily versus enoxaparin 1.0 mg twice daily combined with warfarin (INR 2.0–3.0) for 6 months in 2489 patients with acute DVT has been completed and published in abstract form.\textsuperscript{33} In this study, ximelagatran was shown to be non-inferior to the comparative regimen with respect to recurrent VTE while major bleeding was similar in both treatment groups. An unexpected observation in both studies was an increase in levels of transaminases $> 3 \times$ ULN during treatment with ximelagatran, which occurred in 6.0 and 9.6% of patients respectively with a peak incidence around three months. These elevated transaminases normalised in the Thrive III study in all but four of the 612 patients on ximelagatran, whether or not continuing the drug.\textsuperscript{32} No patient on ximelagatran in this study got clinical symptoms or died of a hepatobiliary disorder. The clinical relevance of this raise in liver enzymes is yet unknown and a full analysis among all phase III studies with ximelagatran should be awaited to draw definitive conclusions. Finally, for both the pentasaccharides and ximelagatran it should be mentioned that no specific antidote is available, although it has been reported at presentations that major bleeding episodes could be controlled with general supportive treatment (local hemostasis management, transfusions of fresh frozen plasma or plasma factor concentrates).

**Conclusions**

While the initial treatment of patients with VTE has become standard over the last years with LMWH, recent studies including the Prevent and ELATE study have shed important light to the questions of optimal duration and intensity of treatment with VKA. In addition, new options to predict recurrence, including thrombus load and D-dimer measurement point towards possibilities of individualised anticoagulant management. New anticoagulants have the potential to replace VKA on long-term therapy, and randomised studies have been finished recently. These novel antithrombotic drugs hold promise in terms of efficacy, safety and convenience, but important questions with respect to safety and antidote management have to be answered when these drugs are introduced into the clinical arena.
References


30 The Matisse Investigators. Fondaparinux in comparison to (low molecular weight) heparin for the initial treatment of symptomatic deep venous thrombosis or pulmonary embolism. *Blood* 2002; **100**: 82a (abstract).


33 Huisman MV, on behalf of the THRIVE Treatment study Investigators. Efficacy and safety of the oral direct thrombin inhibitor ximelagatran compared with current standard therapy for acute symptomatic deep vein thrombosis, with or without pulmonary embolism: a randomized, double-blind, multinational study. *J Thromb Haemost* 2003; **1** (Suppl 1): abstract number OC003.
EBMT-EHA TRANSPLANTATION

Overview of transplant activity in Europe

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This report summarizes current transplant activity in Europe. It is based on the activity survey of the European Group for Blood and Marrow Transplantation (EBMT). Introduced in 1990, the survey captures the annual numbers of hematopoietic stem cell transplantation (HSCT) by indication, donor type and stem cell source from each individual European transplant team. Supplemented by demographical data and economical factors, team density and transplant rates can be calculated, and the impact of economics on HSCT rates can be assessed. As documented in the present analysis, in the year 2002, a total 20,207 HSCT were performed on new patients in Europe by 586 teams in 39 countries: 6,915 being allogeneic and 13,292 autologous HSCT. The main indications for allogeneic HSCT were leukemias, lymphoproliferative disorders and non-malignant diseases; main indications for autologous HSCT were lymphoproliferative disorders, solid tumors and leukemias. The main source of stem cells were peripheral blood (96%) for autologous, peripheral blood (62%) and bone marrow (38%) for allogeneic HSCT. On the basis of its completeness, the EBMT activity survey presents a rapid description of the status quo, assessment of trends and determination of factors influencing transplant rates. As such, it provides up-to-date information for patients, treating physicians and health-care officials.

Keywords: Hematopoietic stem cell transplantation; transplant activity; economic factors; public health

Introduction

Transplantation of hematopoietic stem cells (HSCT) has seen an unprecedented success story over the last few decades. It has evolved from an emergency measure in difficult situations to establish a planned procedure that is integrated in the therapeutic plan of many radio-, chemo- or immunosensitive malignancies as well as severely acquired or congenital disorders of the hematopoietic system. Hematopoietic stem cells from different donor types (autologous, syngeneic, allogeneic related and allogeneic unrelated donors) and different stem cell sources (bone marrow, peripheral blood and cord blood) are applied depending on the clinical situation and need.1–4 However, HSCT also represents an example of modern high-efficiency medical technology under debate in an era of limited resources. It is associated with high costs and limited to selected groups of patients. Decision-making in these situations represents a challenge for treating physicians, patients and health-care agencies. Patients are confronted with immediate risks and late benefits, physicians are challenged to give advice to well-informed patients with access to the internet and the most recent publications and health-care officials are obliged to provide the needed infrastructure for high-tech, high-cost medicine. HSCT itself is not linked to one specific device or drug. It is rather the complex network of highly trained physicians and nurse specialists, and the length of their commitment to individual patients that renders the procedure time and cost intensive. HSCT is also constantly confronted with rapid changes in procedure. The introduction of new concepts, such as reduced intensity conditioning, might change the short-term outlook for patients or open up the technology to new patient categories. The advent of new drugs, such as imatinib mesylate, opens alternative approaches. Up-to-date information at any level is essential in such a changing field. The activity survey of the European Group for Blood and Marrow Transplantation (EBMT; www.ebmt.org) represents such a tool to provide instant information on the current status quo.5
Methods

**EBMT activity survey**

The EBMT activity survey was initiated in 1990 as part of the EBMT-JACIE (Joint Accreditation Committee International Society for Cell Therapy and EBMT Accreditation Office; www.EBMT.org) and as a rapid tool for quality control and trend assessment.\(^5\) It is still closely linked with the EBMT but includes non-EBMT members as well. Its clear aim is to cover all HSCT activity in Europe, from EBMT members and non-member institutions alike. The activity survey collects annually numbers of HSCT from each participating institution, by indication, donor type and stem-cell source on a single-page questionnaire. For EBMT members, it is mandatory to participate by EBMT constitution, and accreditation for unrelated donor transplants depends on participation. Nonmembers are invited to participate. Lists of transplant teams are compared with national agencies wherever such agencies are in function to assure completeness. In 1993, all active teams were asked to provide their numbers for the years 1983 and 1973 in retrospect.

**Participating teams**

In total, 636 stem-cell transplant teams were contacted in 2002 in 39 European countries. In all, 586 teams

<table>
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<tr>
<th>Table 1 Number of patients treated in Europe in the year 2002 by HSCT according to indication, donor type and stem-cell source</th>
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<td>Teams = 586</td>
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<td>Acute lymphatic leukemia</td>
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(Table 1) returned the survey sheet corresponding to a 92% return rate, and include 465 of 473 EBMT member teams. No major transplant team in Europe is missing from this list. According to informal information, no blood and marrow transplants were performed in the following European countries: Albania, Andorra, Armenia, Azerbaijan, Bosnia-Herzegovina, Georgia, Iceland, Liechtenstein, Malta, Moldavia, Monaco, San Marino and the Vatican.

Definitions

Transplants are defined as an infusion of hematopoietic stem cells following a conditioning regimen with the intention of replacing the existing hematopoiesis by the injected stem cells. First transplants refer to the first transplantation of hematopoietic cells and full information is collected only for the first transplants. Therefore, each patient is counted only once, independent of the number of transplant procedures. This prevents multiple reporting. Additional procedures, such as re- or multiple transplants, were collected in total, not specified by the disease, to obtain an estimate of the absolute number of HSCT procedures. Retransplants refer to a situation where recipients receive a second HSCT for relapse or rejection. Multiple transplants refer to a planned programme of sequential HSCT. Donor lymphocyte infusions are not considered as transplants but general information on new patients treated with DLI was collected.

Transplant rates are defined as the numbers of HSCT per 10 million inhabitants. They are computed for each year, disease indication, donor type and country. Team density is defined as the number of HSCT teams per 10 millions inhabitants. The population data are obtained each year from the US census office (http://www.census.gov). Population data are used to determine total transplant rates, for each donor type and indication. Comparison of transplant rates in different countries leads to the calculation of a coefficient of variation (CV) in transplant rates. A high CV corresponds to a high variation of transplant rates, hence disagreement among transplant physicians; a low CV corresponds to a low variation of transplant rates for the given indication (Table 2), hence agreement on the indication for this specific indication.

Table 2 Coefficients of variations in transplant rates for individual disease indications

<table>
<thead>
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<th>CV</th>
<th>Allogeneic HSCT</th>
<th>Autologous HSCT</th>
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<tr>
<td>&lt;50</td>
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<td>MM</td>
</tr>
<tr>
<td></td>
<td>AML not first CR</td>
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<td></td>
<td>MDS</td>
<td>ST</td>
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</tbody>
</table>

Low CVs correspond to agreement, high CVs to disagreement among transplant physicians in Europe on the given indication.

ALL, Acute lymphoid leukemia; AML, Acute myeloid leukemia; CLL, Chronic lymphocytic leukemia; CML, Chronic myeloid leukemia; ES, Ewing’s sarcoma; HD, Hodgkin’s lymphoma; MDS, Myelodysplastic syndrome; MM, Multiple myeloma; NB, Neuroblastoma; NHL, Non Hodgkin’s lymphoma; ST, all other solid tumors.

Results

Reporting of the status quo

Numbers of HSCT by indication, donor type and stem-cell source are collected and rapidly published annually in major hematology journals. Results of the annual surveys are made available to participating members, including corporate pharmaceutical EBMT members, prior to publication. Every effort is made to have the data published not more than 12 years after the survey. These data, which cover more than 90% of the autologous and more than 95% of the allogeneic HSCT in Europe, are an invaluable tool for transplant teams for self-positioning and patient counselling. Figure 1 illustrates the development of allogeneic and autologous HSCT and also transplant teams from 1973 to 2003.

This figure illustrates the massive changes in absolute numbers of HSCT. These changes are not alike for all indications. Allogeneic HSCT has primarily increased for patients with leukemia. There is also a clear increase of lymphoproliferative disorders in the last 3 years, and nonmalignant indications show a steadily low increase. For autologous HSCT, there remains an ongoing increase in HSCT for lymphoproliferative disorders.
less so in hematological malignancies. In contrast, there has been a massive expansion in autologous HSCT for solid tumors in the early 1990’s with a peak in 1997 and a rapid decline thereafter. This was mainly due to the expectations in breast cancer and the deception based on the negative prospective randomized studies.6,8–10

The numbers for the year 2002 are given in Table 1. A total 20207 HSCT, 6915 allogeneic (30%) and 13292 autologous (70%) HSCT were performed by 586 teams in 39 countries. An additional 1103 allogeneic retransplants (759) or double procedure (344) transplants and an additional 2844 autologous retransplants (509) or double procedure (2335) transplants were added, bringing the total number of HSCT in Europe 2002 to 24154.

The comparison of the participating European countries leads to a quantitative assessment of differences among them. The EBMT activity survey revealed such differences in HSCT activity early on, as illustrated in Figure 2 for the year 2002. These differences relate to all aspects analyzed, for example, indication, donor type, stem-cell source, transplant rates and team density. Repetitive examinations of the annual survey provided insight into several mechanisms. Not surprisingly, transplant rates clearly correlate with national economies such as Gross National Product GNP.7 Transplant rates also correlate with team density. Low team density correlates with low transplant rates. This means that there is a need to have several transplant teams in a given country, in order to disseminate the technology. There is also saturation at a level of about 10 teams per 10 million inhabitants.7 The comparison of transplant rates for individual indications provides an instrument for assessing, with quantitative methods, consensus or disagreement among European specialists and transplant indications. A CV in transplant rates provides a numerical description, and a CV of ≤ 50 strongly suggests consensus, a CV > 100 strongly suggests dissent (Table 2).

Changes over time and mid-term projections

HSCT is a highly complex, cost-intensive but powerful therapeutic strategy. It is also an expanding field with additional rapid changes in technology. In 1990 all HSCT were still bone marrow derived. Within a decade, it changed almost completely to peripheral blood as a stem-cell source in the autologous setting and to about 62% in the allogeneic setting (Figure 3).

The EBMT activity survey has now been developed, with the help of health-care management specialists, into a tool for mid-term projections.11 Preliminary data so far gives a clear answer: HSCT rates for individual indications follow clear mathematical models, and trends are highly predictable in the short term. However, changes can occur. When they did occur, they had been rapid, unpredictable and substantial. They occur 2–4 years before major publications relating to these events. Changes in technology, for example, shift from bone marrow to peripheral blood in autologous HSCT, was completed at the time of publication of the leading article. The anticipation of the physicians and patients are currently being discussed as the main factors influencing such decisions.

Discussion

As it stands, the EBMT activity survey provides a unique tool. It covers a whole continent and captures almost all procedures for a given speciality field. As it concentrates on rapid data capture, it reflects the status quo as it is. It provides a very up-to-date basis for decision-making for physicians, patients and health-care administrators alike. It serves as a quality-control instrument for individual teams, national societies and global structures as well.

In addition, it opens the field to new aspects. Risk assessment for individual patients is well established, and decision-making at the individual and patient level follows accepted rules. Little is shown, in contrast,
about factors influencing team decisions. Clearly, more information and better understanding is warranted. Instruments, such as the EBMT activity survey, could indeed provide us with answers.

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References
Reduced intensity transplantation: where are we now?

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Introduction

As a potential solution to the problems of excessive regimen-related toxicity, the reduced intensity or non-myeloablative allograft was introduced in the mid-1990s. The conditioning regimens were designed principally to immunosuppress the host sufficiently to allow donor engraftment, cure of disease being delivered subsequently by the allogeneic graft-versus-leukaemia (GVL) or graft-versus-malignancy (GVM) effect. Although the regimens have varying degrees of antitumour activity in order to control the malignancy until the incoming lymphoid compartment is sufficiently reconstituted to exert such a GVL effect, the aim of the conditioning therapy is no longer principally to eradicate disease, and thus toxicity is significantly reduced. In this way, the benefits of allogeneic transplantation can potentially be extended to patients who would generally not be considered candidates for conventional myeloablative transplant regimens. Although all the regimens devised are broadly grouped together as ‘reduced intensity’ or ‘mini’ transplants, there is significant disparity in the relative degree of immunosuppression and myelosuppression involved. With minimally myelosuppressive or truly non-myeloablative regimens, patients can be conditioned such that donor neutrophil engraftment occurs in the absence of recipient granulocyte aplasia, and this preservation of circulating granulocyte numbers thus reduces the risks of serious bacterial sepsis, such that the treatment can even be delivered in an outpatient setting. Conversely, the more myelosuppressive or ‘reduced intensity’ regimens cause observable aplasia and carry greater toxicity but have the advantage of greater debulking of residual disease, which may be of importance in those malignancies that are aggressive and propagate rapidly.

To facilitate durable engraftment, the host has to be adequately immunosuppressed in order that the incoming stem cells are not rejected. This is often achieved by using the purine analogue, fludarabine, in combination with either low-dose total-body irradiation (TBI) or alkylating drugs such as cyclophosphamide, melphalan or busulphan. Some regimens incorporate, in addition, anti-T-cell serotherapy with either alemtuzumab (Campath-1H) or antithymocyte globulin (ATG) to reduce the incidence of GVHD, although this must be balanced against the need for T cells to facilitate engraftment, contain viral infection, and mediate subsequent GVL effects.

Regimens

Initial reports from Giralt et al.1 demonstrated that conditioning with fludarabine, idarubicin and cytarabine in 15 patients with acute myeloid leukaemia (AML)/myelodysplastic syndrome (MDS), could result in successful engraftment and complete remission, in some cases of reasonable duration.1 This regimen, however, proved insufficiently immunosuppressive to support consistent engraftment in chronic myeloid leukaemia (CML). The same group went on to demonstrate successful engraftment and encouragingly long-lived disease responses in a cohort of 86 patients with haematological malignancies using a purine analogue and melphalan combination.2

The Seattle group have used low-dose TBI-based conditioning with the majority of transplants being performed in the outpatient setting.3 The conditioning consisted of a single fraction of 2 Gy TBI given on the day of stem-cell return, with immunosuppression thereafter by cyclosporin A (CyA) and mycophenolate mofetil (MMF). Although initial engraftment occurred in all patients, with most avoiding severe cytopenia, 20% of the first 45 rejected following discontinuation of their immunosuppression. The protocol was adjusted to include fludarabine in the conditioning, and rejection was seen thereafter in only 3%.

Each of the above regimens involves reinforcing an unmanipulated T-replete graft. As discussed in the previous section, although there are reasons for preferring this approach, it does confer significant risks of acute and chronic GVHD, with its attendant morbidity and mortality. The alternative involves a degree of T-cell depletion of the incoming stem cells, and, in the NMT setting, this has tended to be done by the use of antilymphocyte antibodies in vivo. These antibodies are given as part of the conditioning and thus fulfill a dual role, immunosuppressing both host, and, as a result of their persistence in vivo, incoming donor T cells. The antibodies most commonly used are ATG or alemtuzumab, the former being a mixed product with wide-ranging specificities including T lymphocytes, and the latter an anti-CD52 monoclonal antibody, which targets a variety of cell-types, including T cells, B cells and monocytes.

Slavin et al. reported the use of ATG, busulphan and fludarabine firstly in 26 patients with either haematological malignancies or genetic disease,4 and subsequently...
in 23 patients, all of whom had lymphoma. This regimen has incorporated subsequent donor lymphocyte infusions (DLI) to compensate for the T-depleting effect of in vivo ATG, and disease-free survival was 81% at median follow-up 240 days for the former group and 43% at 675 days for the latter group. Results have been particularly encouraging for patients with chronic myeloid leukaemia.

Several groups have used regimens containing alemtuzumab. While the most prevalent approach is to combine alemtuzumab with fludarabine and an alkylating agent, there are many other combinations under investigation, and the optimal antibody and chemotherapy schedule remains to be established. Kottaridis et al. reported a series of 44 patients with haematological malignancies conditioned with alemtuzumab, fludarabine and melphalan, and observed sustained engraftment in 42 of the 43 evaluable patients. Only two patients developed grade II GVHD with no grade III–IV, and, at a median follow-up of 9 months, progression-free survival was 75%. One of the most striking features of the alemtuzumab-containing regimens is the reduction in GVHD rates.

**Disease-specific outcomes**

**Multiple myeloma**

The combination of allogeneic transplantation as the only demonstrably curative treatment, evidence for a GVM effect, and a high attrition rate from transplant-related causes when conventional conditioning is used, makes this disease an appropriate candidate for reduced-intensity conditioning regimens.

Peggs et al. have reported a series of 20 patients (HLA-matched related donors 12, unrelated donors eight) conditioned with alemtuzumab, fludarabine and melphalan. As expected in this setting of T-cell depletion, GVHD rates are encouragingly low, with only 3/20 patients developing grade II acute GVHD and none developing grade III or IV. Unfortunately, the disease responses post-transplant presumably also reflect the degree of donor T-cell depletion, with only two patients in CR at 6 months, four in PR and two with minimal response. Donor lymphocytes in escalating doses were given to 14 patients, with seven demonstrating further disease responses — mostly but not exclusively in those who also developed GVHD — but these responses were once again not durable, with a 2-year progression-free survival of only 30%.

This and other early experience in myeloma would suggest that debulking of disease to a minimum prior to the allograft, and certainly to a stable plateau, may be necessary to minimise the chance of early progression post-transplant, and several groups have addressed this question with protocols including high-dose chemotherapy with autologous stem cell rescue prior to the allogeneic procedure. Maloney et al. have reported a series of 54 patients, treated with induction chemotherapy followed by high-dose melphalan and autologous stem cell rescue. At a median of 62 days later, patients were then conditioned with 2 Gy TBI and stem cells from HLA-matched related donors were returned, with CyA and MMF given as additional immunosuppression. Overall survival at a median follow-up of 552 days was 78% with a TRM of 17% and extensive chronic GVHD in 46%. Of the 48 patients not in CR at study entry, 25 (52%) subsequently achieved CR, and of those patients (plus the six who entered the study in CR), only three have relapsed. Outcome, both in terms of TRM and disease response, was better in those transplanted with chemoresponsive disease, and the overall progression-free survival at 2 years was estimated at 55%.

The follow-up in these studies is currently relatively short, but the encouraging early results have led to cytoreduction with an autograft becoming widely used prior to allogeneic transplantation with reduced intensity conditioning in this disease.

**Non-Hodgkin’s Lymphoma**

**Low-grade non-Hodgkin’s lymphoma:** Encouraging results have been reported in a cohort of 20 patients conditioned with cyclophosphamide and fludarabine, nine of whom also received rituximab. Although numbers are relatively small, and 12 of the patients were in complete remission already at the time of transplant, all patients achieved a durable complete remission post-procedure, with no disease relapses at a median follow-up of 21 months. In addition, of the six patients who were tested for minimal residual disease by polymerase chain reaction (PCR) for the bcl-2 gene rearrangement pretransplant, all subsequently achieved molecular remission. This gave a 2-year actuarial disease-free survival of 84%, although with a cumulative incidence of chronic GVHD of 64%.

The use of T-depleting regimens has also been reported. Morris et al. conditioned 46 patients with low-grade non-Hodgkin’s lymphoma (NHL) (31 follicular NHL, 12 CLL) with alemtuzumab, melphalan and fludarabine. Most were in partial remission at the time of the transplant, and 25% had had prior autograft procedures. The TRM at 3 years was 10% with 4-year actuarial overall survival of 69% and progression-free survival of 65%. In total, 11 patients received DLI for residual disease or disease progression, of whom six responded. This regimen thus can be given to relatively heavily pretreated patients with an extremely low incidence of GVHD and a respectable procedure-related mortality. Although the infused graft is T depleted by the alemtuzumab in vivo, the conditioning regimen has cytoreductive capacity that appears to protect from the potential relapse risk conferred by the loss of alloreactive T cells in the initial post-transplant period.

Therefore, it can be seen that this approach shows promise in the management of the low-grade lymphomas. There is some evidence supporting the presence of a GVL effect, the regimens have relatively low procedure-related mortality, and, although disease
High-grade NHL: Morris et al. in the cohort described above, also reported 47 patients with high/intermediate-grade NHL. Most were again in partial remission at the time of transplant, and 70% had had a prior autograft. The TRM at 3 years was 37%, with actuarial overall survival and progression-free survival at 4 years of 35 and 29% respectively. Seven patients received DLI, of whom 3 responded.10

The BEAM-Campath regimen has also been used in high/intermediate-grade NHL, with a less encouraging event-free survival than in low-grade disease (17% at 2 years), and a correspondingly high relapse rate (68% at 2 years). Responses to DLI given for disease progression were poor, and this group now plan to reduce post-transplant immunosuppression in an attempt to combat early relapse.11

In addition, Spitzer et al. reported a series of 20 patients with diffuse large B-cell lymphoma, 17 of whom had chemotherapy-refractory disease, who were conditioned with cyclophosphamide, ATG or anti-CD2 antibody and thymic irradiation. Eight patients had a disease response, and, of these, five were alive and progression-free at 13–52 months follow-up (25%). Although four of these five patients had chronic GVHD, not all of the eight initial responders had acute or chronic GVHD.12

There have been many other reports of the use of different conditioning regimens on small numbers of patients with high-grade disease. In general, the data on high-grade disease are less encouraging than in low-grade disease, with usually higher procedure-related mortality and poorer disease response rates, although progression-free survival with 1–2-year follow-up is certainly being seen in a proportion of these otherwise incurable patients.

The generally inferior disease responses seen may partly reflect the more rapid progress of disease growth when compared to low-grade disease, meaning that the risk of early relapse or progression is relatively higher during the period post-transplant prior to the development of a significant allogeneic effect. Furthermore, donor lymphocyte infusions may be unable to exert a sufficiently robust GVL effect within the timescale needed, if administered in the presence of relapsing disease.

In an attempt to maximise disease reduction prior to allogeneic transplantation, and therefore minimise the risk of progression/relapse prior to the establishment of an allogeneic GVL effect, cytoreduction with high-dose chemotherapy plus autologous stem-cell rescue prior to allogeneic transplantation is also being explored.

Hodgkin's lymphoma

Once again, reduced intensity conditioning has been attempted in this cohort with reasonable early results. Using an alemtuzumab-containing regimen, disease responses appear to occur in approximately 50% of patients post-transplant, with complete responses to DLI documented in a number of relapsing patients.

The strategy described previously using preceding high-dose chemotherapy with autologous stem-cell rescue has also been used in patients with high-risk Hodgkin’s lymphoma. Carella et al. described 17 patients treated with a BEAM autograft, followed by fludarabine/cyclophosphamide conditioning and stem-cell return from HLA-matched sibling donors. Responses were seen in 11 patients (CR in nine), and 11 were alive (six in ongoing CR) at a median follow-up of 566 days. Of note, the best disease responses were seen in those patients who achieved full donor status and in whom GVHD developed, suggesting the presence of an allogeneic graft-versus-lymphoma effect.13

CML/myelofibrosis

As CML is the disease in which response to allogeneic donor lymphocyte infusion was first and most comprehensively demonstrated, it is an obvious candidate for the reduced intensity conditioning approach. Or et al. have described 24 patients transplanted in first chronic phase, using fludarabine, busulphan and ATG. The incidence of significant acute GVHD was 54%, and, at a median follow-up of 42 months, 21 of the 24 patients remain well, and in complete molecular remission, giving an actuarial 5-year disease-free survival of 85%.

Encouraging results have recently been reported in 20 patients with myelofibrosis. All patients engrafted following reduced intensity conditioning with no early transplant-related mortality. In all, 18 patients remain alive at a median of 18 months with resolution of splenomegaly and marrow fibrosis.15

This form of transplantation, therefore, is a viable option in patients with CML/myelofibrosis with ample potential for inducing post-transplant disease responses by the administration of donor lymphocytes.

AML/MDS

Giralt et al. first demonstrated the feasibility of the nonmyeloablative approach in a group of 15 patients with refractory or relapsed AML/MDS, who were conditioned with either fludarabine/ctarabine/idarubicin or cladribine/ctarabine prior to infusion of stem cells from sibling donors. Toxicity was acceptably low, and eight patients achieved remission post-transplant, although responses were short-lived, as expected in this high-risk group, and relapse occurred in all but two by a median follow-up of 100 days.1 The same group reported on the use of fludarabine and melphalan in these patients, and found that the additional cytoreduc-
...conferring by this regimen resulted in improved disease-free survival.2

Following this, Parker et al.16 evaluated a fludarabine, busulphan and alemtuzumab containing regimen in 23 MDS patients with MDS. Early TRM was 9%, with a 2-year actuarial overall survival of 48% and disease-free survival of 39%. Thus, transplantation with reduced intensity conditioning was deemed a feasible approach for those patients considered inappropriate for conventional conditioning because of age or comorbidity, yielding comparable results to those found in patients fit for the fully conditioned regimen. Of note, those individuals who fared best were those with good- or intermediate-risk cytogenetics and a low or intermediate International Prognostic Scoring (IPS) system score.

Kroger et al. published a study of 37 patients with MDS or secondary AML, half of whom had a related donor, who were ineligible for conventionally conditioned transplants, who received fludarabine, busulphan and ATG as reduced intensity conditioning. The overall TRM was 27%, with significantly higher mortality in those with poor risk cytogenetics (75 versus 29%) or with an HLA-matched unrelated donor (45 versus 12%). In total, 32% of patients relapsed, and actuarial disease-free survival at 3 years was 38% with a median follow-up of 20 months. One further observation was that the development of chronic GVHD conferred a relative protection from disease relapse (15 versus 70%).17

Sayer et al.18 reported on 113 patients with acute myeloid leukaemia ineligible for conventional transplants receiving reduced intensity conditioning, consisting of fludarabine and busulphan or TBI. Apart from once again demonstrating the feasibility of such conditioning in these patients, this study identified certain poor prognostic factors. These included low Karnofsky performance scores, and the presence of unrelated donors, but the most striking association was that between the level of residual disease at the time of transplant and subsequent outcome. With a median follow-up of 12 months, the probability of event-free survival was 49% for patients in morphological remission on bone marrow examination, 24% in patients with 5–20% blasts, and 14% where >20% blasts remained.

Lastly, Finke et al. have reported a reduced intensity myeloablative conditioning regimen (fludarabine, BCNU, melphanal) in 30 elderly patients with AML/MDS who received transplants from unrelated donors. In spite of the median marrow blast count being 27% at the time of transplant, the disease-free survival with a median follow-up of 11 months was an impressive 55%.19

Thus, reduced-intensity conditioning shows some promise in those patients with AML/MDS ineligible for transplantation using conventional regimens, although follow-up is generally relatively short. Furthermore, outcome is significantly affected by the presence of poor risk features such as high-risk cytogenetic changes, chemorefractory disease, unrelated donors and low IPS and Karnofsky performance scores. These factors should be considered when selecting patients for such procedures. In addition, there seems, in common with other malignancies, to be a beneficial effect conferred by the presence of chronic GVHD on the risk of disease recurrence.

Conclusion

It can therefore be seen that allogeneic transplantation with reduced intensity conditioning can be successfully performed in individuals with a wide variety of different diseases. Procedure-related toxicity has proven significantly less of a problem than in conventional conditioning, and has provided a means of delivering potentially curative treatment in many situations where this has previously not been possible. Longer follow-up and greater numbers are clearly required to delineate the potential for cure in the various different diseases but encouraging results have been published thus far.

The major challenges remain the identification of the optimal conditioning regimen for particular disease groups, and, crucially, the manipulation of the allogeneic effect so as to promote GVM while avoiding undesirable GVHD. While GVM responses are often seen following the development of GVHD, some disease responses have been observed in the absence of GVHD, and attempts are being made to target for example, overexpressed/abnormal tissue antigens or haemopoietic minor histocompatibility antigens which may be relevant for such antitumour activity. If appropriate T-cell clones can be generated and expanded ex vivo, transplantation with nonmyeloablative conditioning could provide an ideal platform for subsequent adoptive cellular therapy.

References


2 Giralt S, Thall PF, Khouri I, Wang X, Braunschweig I, Ippoliti C. Melphanal and purine analog-containing preparative regimens: reduced-intensity conditioning for patients with hematologic malignancies undergoing allo-


4 Slavin, Nagler A, Naparstek E, Kapelushnik Y, Aker M, Cividalli G. Nonmyeloablative stem cell transplantation...
and cell therapy as an alternative to conventional bone
marrow transplantation with lethal cytoreduction for the
treatment of malignant and nonmalignant hematologic
5 Nagler A, Slavin S, Varadi G, Naparstek E, Samuel S, Or
R. Allogeneic peripheral blood stem cell transplantation
using a fludarabine-based low intensity conditioning regi-
men for malignant lymphoma. Bone Marrow Transplant
6 Kottaridis PD, Milligan DW, Chopra R, Chakraverty RK,
Chakrabarti S, Robinson S. In vivo CAMPATH-1H
prevents graft-versus-host disease following nonmyelo-
2425.
7 Pegg KS, Mackinnon S, Williams CD, D'Sa S,
Thuraisundaram D, Kyriakou C. Reduced-intensity
transplantation with in vivo T-cell depletion and adjuvant
dose-escalating donor lymphocyte infusions for
chemotherapy-sensitive myeloma: limited efficacy for
graft-versus-tumor activity. Biol Blood Marrow Transplant
8 Maloney DG, Molina AJ, Sahebi F, Stocker-Goldstein
KE, Sandmaier BM, Bensinger W. Allografting with non-
myeloablative conditioning following cytoreductive auto-
grafts for the treatment of patients with multiple myeloma.
9 Khouri IF, Saliba RM, Giralt SA, Lee MS, Okoroji GJ,
Hagemeister FB. Nonablative allogeneic hematopoietic
transplantation as adoptive immunotherapy for indolent
lymphoma: low incidence of toxicity, acute graft-versus-
host disease, and treatment-related mortality. Blood 2001;
98: 3595–3599.
10 Morris E, Thompson K, Craddock C, Milligan D, Smith
GM, Parker A. Long term follow-up of an alemtuzumab
(Campath-1H) containing reduced intensity allogeneic
transplant regimen for non-Hodgkin’s lymphoma (NHL).
Blood 2002; 100: 139a.
11 Faulkner RD, Craddock C, Byrne JL, Mahendra P,
Haynes AP, Prentice HG. BEAM-Campath reduced
intensity allogeneic stem cell transplantation for lympho-
proliferative diseases: GVHD, toxicity and survival in 65
12 Spitzer TR. The expanding applications of non-myelo-
blative stem cell transplantation. Pediatr Transplant 2003;
7 (Suppl 3) 95–100.
13 Carella AM, Beltrami G, Carella Jr M, Corsetti MT,
Scalzulli RP, Greco M. Immunosuppressive non-myelo-
blative allografting as salvage therapy in advanced
14 Or R, Shapiro MY, Resnick I, Amar A, Ackerstein A,
Samuel S. Nonmyeloablative allogeneic stem cell trans-
plantation for the treatment of chronic myeloid leukemia in
15 Rondelli D, Barosi G, Bacigalupo A, Prchal JT, Popat U,
Alessandrino EP. Non-myeloablative allogeneic HSCT in
high risk patients with myelofibrosis. Blood 2003; 102:
199a.
16 Parker JF, Shafi T, Pagliuca A, Mijovic A, Devereux S,
Potter M. Allogeneic stem cell transplantation in the
myelodysplastic syndromes: interim results of outcome
following reduced-intensity conditioning compared with
standard preparative regimens. Br J Haematol 2002; 119:
144–154.
17 Kroger N, Bornhauser M, Ehninger G, Schwerdtfeger R,
Biersack H, Sayer HG. Allogeneic stem cell transplantation
after a fludarabine/busulfan-based reduced-intensity con-
ditioning in patients with myelodysplastic syndrome or
secondary acute myeloid leukemia. Am Hematol 2003; 82:
336–342.
18 Sayer HG, Kroger M, Kroger N, Beyer J, Kiehl M, Klein SA,
Schaefer-Eckart K. Reduced intensity conditioning for
allogeneic hematopoietic stem cell transplantation in
patients with acute myeloid leukemia: disease status by
marrow blasts is the strongest prognostic factor. Bone
Marrow Transplant 2003; 31: 1089–1095.
19 Finke J, Egger M, Grullich C, Potthoff K, Spyridonidis A,
Waechs R. Myeloablative reduced conditioning with
fludarabine, BCU, melphalan (FBN) and stem cell
transplantation from unrelated donors in elderly patients
Long-term care after stem-cell transplantation

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Introduction

Large numbers of patients now survive long term following stem-cell transplantation (SCT). The late clinical effects of SCT, particularly secondary malignancies, are therefore of major concern in the 21st century. Nonmalignant late effects are heterogeneous, and although often not life-threatening, they significantly impair quality of life. Readers will find extensive literature summary and references in recently published reviews.¹–⁵ Table 1 describes the risk factors associated with some of the nonmalignant complications of SCT.

Endocrine function

Thyroid dysfunction

Compensated hypothyroidism (elevated thyroid stimulating hormone (TSH) with a free thyroxine (fT4) or thyroxine (T4) in the normal range) is the most common and occurs in 7–15.5% of adult patients within 12 months of transplant. In some of these patients, TSH will normalize spontaneously, so treatment of patients with L-thyroxine is controversial. Treatment should be considered if TSH remains elevated or increases.

The frequency of overt hypothyroidism (elevated TSH and fT4/T4 below the normal range) requiring L-thyroxine is highly variable and depends to a large extent on the type of pretransplant conditioning, that is, nearly 90% in patients who have received 10 Gy single-dose total-body irradiation (TBI), 14–15% of patients following fractionated TBI, and smaller numbers after conditioning with busulfan and cyclophosphamide (BuCy). Clinically significant, hypothyroidism may be hidden if the TSH is normal and fT4/T4 is in the lower third of the normal range.⁶ Treatment with L-thyroxine is indicated in all patients with overt hypothyroidism. Elderly patients should have an ECG prior to commencing treatment to exclude associated ischemic heart disease and/or arrhythmias.

Autoimmune disorders such as Hashimoto-thyroiditis and Grave’s disease are thought to be due to the transfer of abnormal cell clones. Thyroid carcinomas account for only 0.4% of all primary, but for 5–10% of all secondary malignancies.⁷,⁸

Gonadal failure

In males, the testicular germinal epithelium (Sertoli cells), where spermatogenesis occurs, is more vulnerable to radiation and chemotherapy than the testicular Leydig cells component, which is involved testosterone secretion. Therefore, testosterone levels are usually normal even where spermatogenesis is reduced or absent. The serum FSH is typically elevated while LH levels may remain in the normal range. The great majority of adult patients will not therefore require testosterone replacement to ensure sexual activity, libido, erection and ejaculation. Sex-hormone replacement therapy (SHRT) with testosterone derivatives in males is indicated for patients with severe uncompensated hypogonadism. In females, the ovaries are more vulnerable to irradiation and chemotherapy than the testes, and hypergonadotropic hypogonadism is almost the rule. Busulfan is one of the most gonadotoxic agents while cyclophosphamide is usually associated with only minor effects on gonadal function. The majority of adult females will need SHRT for the maintenance of menstrual cycles and bone turnover/mineralization. The dose of estrogen treatment will need to be gradually increased and a combination of cyclical estrogen/progesterone treatment introduced after 1–2 years to initiate menstruation and to reduce the risk of future osteoporosis. SHRT can be interrupted once every 2–3 years, for a period of 6 months, to evaluate possible spontaneous recovery of ovarian activity, which occurs in a minority of women.

Special considerations in puberty: Hypogonadism occurs in up to 70% of pediatric patients after SCT. Male patients are more likely to enter puberty and progress than females. During spontaneous puberty, measurement of testosterone is recommended if the pubertal growth spurt is blunted. A high percentage of female patients will need SHRT. The probability of ovarian recovery after fractionated TBI is higher in younger patients but after busulphan hypogonadism prevails.⁹ The timing and progression of puberty have a major influence on growth and final height. This must be considered when initiating replacement therapy. Precocious puberty occurs in few cases and necessitates

**Fertility following SCT**

Overall, the incidence of pregnancy post transplant is low (<2%) except for patients transplanted for SAA. However, many patients may not wish to become parents following the diagnosis of a potentially life-threatening illness, so accurate measurements of the chance of restoration of fertility are problematic.

**Fertility following SCT for nonmalignant disease:** return of gonadal function following cyclophosphamide conditioning for SAA is reported in 50% of adult female survivors and approximately one-quarter subsequently conceived. In adult male survivors, 60% have return of sperm production and 25% may subsequently father children. Gonadal recovery is usual in women under 25 years at the time of transplant but is much less likely in older women. In thalassemia, gonadal failure is common as a result of both transfusional hemosiderosis and conditioning with BuCy and pregnancies are very rare.

**Fertility following SCT for malignant disease:** The majority of patients given TBI conditioning experience gonadal failure. Recovery of gonadal function occurs in 10% of women and the incidence of pregnancy is less than 3%. In men, recovery of gonadal function has been reported in less than 20% patients and use of increasing doses of TBI may be associated with considerably lower recovery. Parenting a child following the administration of TBI is a rare event in men. BuCy is also associated with a high incidence of gonadal failure in women and there have been no pregnancies reported using BuCy for patients with leukemia. In men, this conditioning appears to be associated with return of gonadal function in approximately 15% cases. Few patients have subsequently fathered children naturally with only 20 patients being identified by the EBMT LEWP. Few studies have evaluated gonadal function following autologous SCT and most of these have involved small numbers of patients. The use of BEAM conditioning for autografts may be associated with a high incidence of gonadal recovery in women, but in men azoospermia is almost always the rule.

**Pretransplant counseling and treatment options**

The pretransplant counseling process should include data on the chance of gonadal failure and should assess the relevance of this to the patient. There should be discussion of assisted conception techniques and in women management of a premature menopause. In women with no residual ovarian function following SCT implantation of embryos cryopreserved prior to SCT is currently the only option for parenting her own genetic child. This, however, requires that prior to SCT the management of the underlying disease can tolerate a minimum 4–6 weeks delay in treatment for controlled stimulation of the ovary and subsequent egg collection. Furthermore, the patient should have a committed partner to provide sperm. In situations where treatment cannot be delayed or there is no committed partner, consideration can be given to freezing ovarian tissue and/or oocytes prior to SCT. Centers in some countries will provide this service for young women, but the patients need to be aware that currently these are a research rather than clinical tools and to date there have been no successful pregnancies in human subjects using these methodologies. Semen cryopreserved prior to transplant may be used post-SCT for artificial insemination, in vitro fertilization and embryo transfer or for in vitro injection into the cytoplasm of the oocyte.

Post-transplant management should routinely include symptomatic and biochemical monitoring of gonadal function. Distressing vasomotor symptoms may commence acutely post-SCT, but this may be prevented by initiating SHRT. While the patient should be prepared for infertility a possible need for contraception soon after SCT must also be emphasized, particularly in

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**Table 1** Non malignant late effects, risk factors and therapeutic interventions

<table>
<thead>
<tr>
<th>Late complication</th>
<th>Risk factor</th>
<th>Intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Eye</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cataract</td>
<td>TBI, steroids</td>
<td>Surgical removal</td>
</tr>
<tr>
<td>Sicca syndrome</td>
<td>CGVHD, TBI</td>
<td>Systemic treatment of GVHD, topical lubricants</td>
</tr>
<tr>
<td><strong>Pulmonary</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic obstructive diseases</td>
<td>CGVHD, TBI, hypogammaglobulinemia</td>
<td>Systemic treatment of GVHD, topical lubricants, bronchodilators</td>
</tr>
<tr>
<td><strong>Bone</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avascular necrosis</td>
<td>Steroids, TBI</td>
<td>Analgesia, surgical treatment</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>Steroids, gonadal failure, duration of CsA</td>
<td>Hormone replacement, calcium and vitamin D, bisphosphonates</td>
</tr>
<tr>
<td><strong>Endocrine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypothyroidism</td>
<td>TBI, Busulphan</td>
<td>l-Thyroxine</td>
</tr>
<tr>
<td>Gonadal failure</td>
<td>TBI, chemotherapy, prior treatment</td>
<td>Early SHRT in women, SHRT based on biological need in men</td>
</tr>
</tbody>
</table>
women who resume menstruation or in patients who do not wish to become parents. Patients have conceived within 6 months of SCT and an unexpected pregnancy may result in requests for termination.

Growth

In growth failure after SCT, disentangling the adverse contributions of the numerous factors has proven difficult. In general, chemotherapy with BU/CY alone does not seem to cause growth impairment but there have been reports of growth hormone deficiency (GHD) after such a combination. In contrast, patients with neuroblastoma generally do not reach their target height. Chemotherapy has an additive negative effect when combined with irradiation. \(^1,^{11}\) Cranial (CI), craniospinal (CSI) and total body (TBI) irradiation can result in growth failure. Single-dose TBI (7–8 Gy) resulted in a loss in final height of \(-2.0\) SDS for boys and \(-1.0\) SDS for girls. This loss occurred during puberty. Fractionated TBI (12 Gy) did not cause substantial loss of height with final height above \(-2.0\) SDS. However, these patients were a median of 10.8 years old at the time of transplant, whereas in children younger than 6 years, the final height was markedly reduced at \(-3.5\) SDS. CI prior to SCT and TBI aggravates the negative effect of TBI. \(^1\) There is disproportion with a reduced growth of the spine, which is most severe in CSI. \(^1,^{11}\) GHD is reported at an incidence of 20–70% following TBI and high-dose CY. Fractionation of TBI (12 Gy) does not spare the GH axis. In estimating the risk for an individual patient, prior irradiation to the GH-axis must be considered. The diagnosis of GHD may be difficult to establish and repetitive testing necessary, as the GH response to stimuli may be discordant. Data on spontaneous GH secretion are scarce. IGF-I and IGFBP-3 as markers for GHD have been shown to be unreliable. GH replacement may be started 2 years after SCT on an individual basis. Data on bone mass after SCT are not consistent. Bone mass was reduced in a pediatric cohort treated for ALL (first remission) with CT and CI. Recovery as well as persisting loss of bone mass were reported. \(^1\)

Pulmonary disease

Chronic obstructive pulmonary disease can be detected in up to 20% of long-term survivors after SCT. It has been mainly associated with chronic GVHD, but other potential risk factors including TBI, hypogammaglobulinemia, GVHD prophylaxis with methotrexate, and infections have been described. Mortality is high among these patients, particularly in those with an earlier onset and rapid decline of FEV1. Symptoms consist of nonproductive cough, wheezing and dyspnea; chest radiography is normal in most cases. High-resolution CT scanning may reveal nonspecific abnormalities. Symptomatic relief can be obtained in some patients with bronchodilators, but in most cases obstructive abnormalities are not improved by this treatment. Patients with low IgG and IgA levels should receive immunoglobulins to prevent infections, which may further damage the airways. Immunosuppressive therapy may be of benefit but typically improvements occur in less than 50% of cases. Asymptomatic patients with abnormal PFTs should be closely monitored for the development of respiratory symptoms as early recognition of airflow obstruction allows the initiation of treatment at a potentially reversible stage.

Recently, late-onset pulmonary disease without an infectious agent has been classified as late-onset pulmonary syndrome (LOPS) and includes bronchiolitis obliterans (BO), bronchiolitis obliterans with organizing pneumonia (BOOP), diffuse alveolar damage (DAD) and interstitial pneumonia (IP). \(^12\) Obliterative bronchiolitis (OB) is the best characterized obstructive syndrome and has been reported in 2–14% of allogeneic SCT recipients and has a mortality rate of 50%. OB is strongly associated with cGVHD and low levels of immunoglobulins. Chest radiographs and CT scanning may reveal hyperinflation with or without infiltrates and vascular attenuation; however, radiological findings do not correlate with lung function changes. Bronchoscopy with transbronchial biopsy can help exclude infection and may reveal obliteration of bronchioles with granulation tissue, mononuclear cell infiltration or fibrosis. It is not clear to what extent combined immunosuppressive treatment can be effective in the treatment of this disease, which typically does not respond to treatment with steroids.

Ocular complications

Posterior subcapsular cataracts have long been recognized in recipients of SCT as one of most frequent late complications of TBI. After single-dose TBI, almost all patients develop cataracts within 3–4 years and most if not all need surgical repair. \(^13\) Although the probability of developing cataracts after fractionated TBI lies around 30% at 3 years the incidence may reach over 80% 6–10 years post-SCT. \(^14,^{15}\) Use of TBI, dose fractionation, and the use of steroid treatment for longer than 3 months are associated with a significant risk of cataract development. In prospective studies of patients who receive cyclophosphamide and TBI (Cy/TBI) have been shown to have a higher incidence of cataract formation than those treated with BuCy. The only treatment for cataract is to surgically remove the opacified lens to restore transparency of the visual axis. Today, cataract surgery is a low-risk procedure and improves visual acuity in 95% of eyes that have no other pathology.

The keratoconjunctivitis sicca syndrome is usually part of a more general syndrome with xerostomia, vaginitis and dryness of the skin. All these manifestations are closely related to cGVHD that may lead, in its most extensive forms, to a Sjögren-like syndrome. Ocular manifestations include reduced tear flow, keratoconjunctivitis sicca, sterile conjunctivitis, corneal epithelial

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defects, and corneal ulceration. The actuarial probability of the development of dry eyes was 21% at 15 years. Risk factors for late-onset keratoconjunctivitis include chronic GvHD, female sex, age >20 years, single-dose TBI, and the use of methotrexate for GvHD prophylaxis. Treatment is based on the management of chronic GvHD with repeated use of topical lubricants.

Bones and joints

Avascular necrosis of bone (AVN)

The incidence of AVN varies from 4 to over 10%. Early diagnosis can rarely be made using standard radiography alone and magnetic resonance imaging is the investigation of choice. The hip is the affected site in over 80% of cases with bilateral involvement occurring in more than 60% cases. Symptomatic relief of pain and orthopedic measures to decrease the pressure on the affected joints are of value, but most adult patients with advanced damage will require surgery. Studies evaluating risk factors for AVN have clearly identified steroids (both total dose and duration) as the strongest risk factor. Thus, unnecessary long-term low-dose steroids for nonactive chronic GvHD should be avoided. The second major risk factor for AVN is TBI, the highest risks being associated the delivery of single doses of 10 Gy or higher or >12 Gy in fractionated doses.

Osteoporosis

The degree of reduction in bone mass can be quantified on dual photon densitometry. Cumulative dose and number of days of glucocorticoid therapy and the number of days of cyclosporine or tacrolimus therapy showed significant associations with loss of bone mass. Nontraumatic fractures may occur in 10% of patients. Using WHO criteria, nearly 50% of the patients have low bone density, a third have osteopenia and approximately 10% have osteoporosis 12–18 months post transplant. Preventative measures for osteoporosis must include sex-hormone replacement for patients with gonadal failure; the efficacy of new treatments for osteoporosis in long-term survivors of SCT requires evaluation.

Secondary malignancies

Secondary malignancies after transplant have already been extensively reviewed, in particular the critical problems of myelodysplastic syndrome (MDS) and acute leukemia following autologous transplant, and the rare occurrence of leukemia in donor cells after allogeneic SCT (not reviewed; see additional reading and Ades et al.4). The main risk factors are summarized in Table 2.

Post-transplant lymphoproliferative disorders and lymphomas

Most cases of lymphoproliferative disorders (PTLD) after hematopoietic stem cell transplantation have been observed in allogeneic recipients. B-cell PTLD are clinically and morphologically heterogeneous; usually they are associated with T-cell dysfunction and the presence of EBV. The mean interval from transplantation to the development of B-cell PTLD is 5–6 months. The cumulative incidence of PTLD was 1.0 – 0.3% at 10 years, with the incidence being highest 1–5 months.

The most frequent presenting findings of PTLD are fever, with or without nodal and extrahematopoietic organ involvement. Today, the use of quantitative PCR for EBV DNA has dramatically changed the diagnostic criteria. Using EBV viral load, patients are now frequently diagnosed while they present only isolated fever with low tumor burden and a monoclonal gammaglobulin. B-cell PTLD are almost always of donor origin.

Most recently, the efficacy of anti-CD20 monoclonal antibody (rituximab) in the treatment of PTLD after SCT has been reported. Currently, many groups consider pre-emptive treatment based on increased EBV load in high-risk patients. Whether this approach is safe and efficacious is not yet proven and this will require longitudinal multicenter studies.

Solid tumors

The median time from SCT to the development of solid tumors is 5–6 years. The cumulative incidence of invasive solid cancers is of the order of 8% at 20 years. In a collaborative study, Curtis et al. analyzed the results of 19220 patients transplanted between 1964 and 1992. There were 80 solid tumors giving an observed/expected ratio of 2.7. In patients surviving at least 10 years after transplantation, the risk was increased eightfold. The risk was increased significantly for melanoma, cancers of the buccal cavity, liver, CNS, thyroid, bone, and connective tissue. The risk was highest for the youngest patients and declined with age. Most striking was the link of squamous cell carcinoma with chronic GVHD and male gender. The underlying diagnosis was

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**Table 2 Risk factors of secondary malignancies according to tumor type**

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Secondary malignancies</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBI</td>
<td>Melanoma, thyroid, CNS tumor</td>
</tr>
<tr>
<td>Limited field irradiation</td>
<td>SCC, head and neck</td>
</tr>
<tr>
<td>T-cell depletion</td>
<td>Melanoma, PTLD</td>
</tr>
<tr>
<td>Chronic GVHD</td>
<td>SCC, head and neck, skin</td>
</tr>
<tr>
<td>HLA-mismatch</td>
<td>PTLD</td>
</tr>
<tr>
<td>ATG, OKT3</td>
<td>PTLD</td>
</tr>
<tr>
<td>Acute GvHD</td>
<td>PTLD</td>
</tr>
<tr>
<td>Oncogenic viruses</td>
<td>PTLD, SCC head and neck</td>
</tr>
</tbody>
</table>

TBI, total body irradiation; CNS, central nervous system; SCC, squamous cell carcinoma; GVHD, graft-versus-host disease; ATG, antithymocyte globulin; PTLD, post-transplant proliferative disorder.
important as the risk of solid tumors was higher for patients with leukemia and lower in patients with lymphoma or aplastic anemia. The risk associated with TBI declined if the irradiation was fractionated but increased with the total cumulative dose administered.

References

10. Sanders JE, Hawley J, Levy W, Buckner CD, Deeg HJ, Doney K et al. pregnancies following high-dose cyclophosphamide with or without high-dose busulfan or total-body irradiation and bone marrow transplantation. *Blood* 1996; **87**: 3045–3052.
ACUTE LEUKEMIA

A European network for AML

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Under the European Union Framework 6 Life Sciences, Genomics and Biotechnology for Health Initiative, a European Leukaemia Network has been initiated under the leadership of Dr R Hehlman. A comprehensive portfolio of work packages each with specific aims has been delineated, and groups of interested participants for each have been identified. Work Package (WP) 5 concerns Acute Myeloid Leukaemia. The stated actions for WP5 include:

(i) Establish an information and communication structure
(ii) Web-based communication
(iii) Create a WP management structure with lead participant and steering group
(iv) Create a Europe-wide platform
(v) Hold meetings
(vi) Set up European AML Registry
(vii) Definitions and standardisation of diagnostics and therapeutic procedures
(viii) Spreading excellence by exchange education, training
(ix) List European study protocols
(x) Integrate new trial groups
(xi) Publications

In total, there are 18 work packages, and a high degree of inter-reaction is anticipated across WP Groups. Of particular relevance to AML will be the Informatics Support, and the WP’s for minimal residual disease and DNA microarray.

Aims of WP in AML

Trial portfolio

Some of the most successful collaborative groups already function in Europe who conduct major trials. All have international collaboration within Europe and beyond, and recruit more than 4500 patients per annum. It is not the purpose of the network that collaborative groups reconfigure into a single entity, but rather that there is a greater awareness of and access to large trials. A central aim is to increase the opportunities for patients to enter viable trials. To this end, an early task is to make available structured summaries of existing trials with contact details. This will become available on the Network Website. It is hoped that those physicians who currently do not have access to trials will thereby be provided with opportunities.

Standards

It is not ethical to enter patients into trials that are not conducted to appropriate standards. Of relevance here are issues such as scientific review of trial interventions, specific ethical approval, patient information and consent. In AML, it is useful to aim for consensus in relation to response criteria – such as the recently revised NCI criteria.1

All trials should have an independent trial steering committee and a separate data and ethics monitoring committee. Analysis should, in general, be carried out on an intent-to-treat basis and public presentation of interim analyses – which can introduce bias to subsequent time periods of the trial – should be avoided. The imminent introduction of the EU Directive on Clinical Trials will create substantial difficulties for investigators in publicly funded trials. The WP will provide a checklist of standards to apply to trials and may thereby develop a trial accreditation mechanism.

Evaluation of novel agents

The opportunity for new drug development on Phase 2 trials has never been greater. Network partners are already active in this area, but rapid evaluation of agents, developed based on new molecular knowledge, in order to bring them to formal efficacy evaluation in randomised Phase 3 trials is required. Given the biological heterogeneity of AML, new statistical approaches may be needed to ‘pick winners’.

An example of novel therapy evaluation are the current randomised trials, being undertaken by network partners, involving the anti-CD33 targeted immunotoxin in Gemtuzumab Ozogamicin. The HOVON-SAKK study is currently evaluating its role in maintenance in
older patients with AML. The EORTC-GIEMEMA will shortly open two studies in older patients. The AML17 trial will evaluate sequential use in induction followed by chemotherapy in first-line treatment in older patients followed by simultaneous administration in consolidation. In patients not considered fit for intensive chemotherapy, GO will be used in combination with hydroxyurea and supportive care (EORTC AML 19 Trial). The MRC are evaluating GO given simultaneously with chemotherapy in younger patients (MRC AML 15). Patients are rerandomised to receive GO or not in consolidation. This trial will therefore evaluate GO in induction and/or in consolidation. Pilot data or preliminary experience from the trials suggest that these approaches are feasible.

Studies are underway within or across collaborative study groups of small molecular agents. FLT-3, being the most common mutation in AML of high value in predicting relapse, is an obvious therapeutic target for which at least four agents are now available. Parallel work to this end will be undertaken in Work Package 12.

Questions beyond randomised trials

The heterogeneity of AML is reflected in response to treatment. This means that small groups of patients may require individualised treatment, which will be very difficult to test by the traditional large randomised trial. One such circumstance is how to deal with patients with acute promyeloctic leukemia who relapse. This is now a rare event. Excellent results have been achieved with arsenic trioxide and gemtuzumab ozogamicin, but the prospects of recruiting sufficient patients to confirm this in a randomised manner against conventional chemotherapy are remote. In spite of large databases, which have confirmed the adverse impact of the presence of an FLT-3 mutation, it remains difficult to establish whether stem-cell transplant improves outcome.

Linkage with other work packages

The evaluation of techniques of minimal residual disease monitoring requires consensus with respect to technique, and observations on sufficient patients with sufficient events to evaluate the predictive value of such tests and the potential for effective clinical intervention. Parallel work to this end will be undertaken in Work Package 12.

Similarly, cDNA microarray is emerging as an approach that can provide expression signatures for major cytogenetic groups, and is likely to delineate new diagnostic prognostic, and causative information (WP13).

Many of these issues cannot be answered by a single trial group and will require larger collaboration. The Europe Leukaemia Network is well positioned to make progress.

References


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AK Burnett
Developing a European network for adult ALL

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Introduction

Acute lymphoblastic leukemia (ALL) is a malignant disease of lymphatic cells in the bone marrow or other organs of the lymphoid system with a number of unique features. Thus, in contrast to other types of cancer, ALL affects the whole age spectrum from infants to adults, with peak incidences in children and in the elderly. It was among the first disseminated malignant diseases that could be cured by chemotherapy alone. Cure rates were improved in the past decades first in children and later in adults from less than 10% to 30–40% and even > 50% in distinct subgroups. Important progress was also achieved in basic research, particularly in molecular genetics. In ALL, the malignant cells are precisely defined by development hierarchy, surface markers, cytogenetics and molecular aberrations, and therefore provide excellent pre-requisites for basic research. Thus, molecular mechanisms of pathogenesis, for example, fusion proteins, could be in part identified and the understanding of signalling pathways provides new targets for causal treatment, such as the BCR-ABL tyrosine kinase inhibitor imatinib. ALL was also within the first malignancies which have been characterized by global gene expression analysis with microarrays.

Recently, methods for individual monitoring of treatment response by evaluation of minimal residual disease (MRD) have been developed and have already entered clinical practice. ALL is an ideal disease for MRD evaluation, since more than 90% of the patients display targets for MRD evaluation. This method offers the opportunity to tailor intensity and duration of therapy in individual patients, thereby avoiding undue toxicity in patients with good response and offering therapy intensification in patients with high risk of relapse. The progress in terms of diagnosis and treatment of ALL has stemmed mainly from systematic clinical research by a number of cooperative study groups. However, due to the heterogeneity and rareness of the disease, further improvement is likely to be achieved only through a more integrated approach that requires the cooperation of several study groups. While in childhood ALL, European and US American groups have already achieved a close cooperation, this remains to be accomplished in adult ALL.

European research activities in ALL

The structure of basic and clinical research in adult ALL differs from other types of cancer and provides excellent pre-requisites for a European research network. There is only one nationwide study group for adult ALL in nearly all European countries with participating hospitals distributed over all regions and established networks with specialized laboratories and research groups. These structures guarantee that a high proportion of adult ALL patients is recognized, diagnosed and treated uniformly within clinical studies. Large patient numbers and thereby representative, nearly population-based results are achieved in individual European countries. Owing to these structures, European study groups on ALL have reached a leading position in clinical research worldwide. This is underlined by the fact that all studies with more than 500 adult ALL patients published in the past decade have been reported from Europe, with the exception of the transatlantic cooperation of the British MRC and the US American ECOG group (Table 1).
The study groups are in close cooperation with basic research, since they provide patient samples and offer the opportunity for clinical testing of new diagnostic and therapeutic approaches. This provides the basis for successful activities in translational research, such as molecular genetics, microarray analysis, etc. and also for the investigation of new drugs. It becomes evident, however, that in these rapidly developing fields, European research in ALL may be falling behind US American activities due to cost-intensive procedures and materials, complicated bureaucratic rules and lower visibility for international companies that enter the market with new drugs.

Why is a European network for adult ALL needed?

Although major improvements in outcome have been achieved in adult ALL through the optimization of chemotherapy, supportive care, risk-stratified regimens and stem-cell transplantation (SCT), future significant progress can only be probably expected from innovative approaches evolving from biotechnology. In these rapidly developing research fields, combined efforts throughout Europe would be very useful. Despite large patient numbers, important issues in diagnosis and treatment of ALL cannot be solved within national studies because subgroups of ALL have to be analyzed separately. However, for joint analyses comparable definitions and procedures, which are not available at present, have to be used. In addition, new targeted therapies lead to a further diversification of therapies in subgroups of ALL with even smaller cohorts to be analyzed. Finally, a European cooperative group could act as a highly visible partner for the pharmaceutical industry, since they provide functioning infrastructures and access to patients for evaluation of new drugs in phase I/II studies.

Foundation of a European network for ALL and participating groups

Following these needs, at the end of 2002 the European study groups for adult ALL met to define topics and aims for future collaboration. In order to increase the chances of obtaining financial support and to achieve a broader cooperation, they decided to participate in the application for a network of excellence named ‘European Leukemia Network (ELN)’ within the sixth Framework Programme of the European Union. The ELN, coordinated by R Hehlmann (Mannheim, Germany), encompasses a large group of applicants from all over Europe which represents all leukemia entities and related research fields (www.eln.org).

Nearly all European study groups for adult ALL made an active contribution to the joint application. Table 2 details the participating groups and their representatives. They involve more than 300 centers and more than 8000 adult ALL patients in their last studies. All major research fields in adult ALL, as illustrated by Figure 1, are represented in the activities of the European ALL study groups. Besides large patient numbers with biological and clinical data on more than 10,000 patients with ALL, they provided high-quality scientific publications and major contributions to basic research and clinical implementation of innovative diagnostic and therapeutic procedures. This includes recent contributions for innovative topics such as molecular genetics,18,19 microarray analysis,20,21 minimal residual disease,22–24 targeted therapies,25 treatment of specific subtypes of ALL, such as mature

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**Table 1** Results of European multicenter studies in adult ALL (1993–2003)

<table>
<thead>
<tr>
<th>Group (Reference)</th>
<th>Year</th>
<th>N</th>
<th>Age (years)a</th>
<th>CR(%)</th>
<th>LFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMALL 011</td>
<td>1993</td>
<td>368</td>
<td>25</td>
<td>74</td>
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<td>41%  at 5yearsb</td>
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Weighted mean                               8308     82    33%

*aMedian; bSurvival of CR pts.*

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The Hematology Journal
Table 2 European ALL study groups within the European leukemia network

<table>
<thead>
<tr>
<th>Group</th>
<th>Country</th>
<th>Representatives</th>
<th>N centers</th>
<th>N pts/ last study</th>
<th>Central diagnosis</th>
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<tr>
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<td>GRAALL</td>
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<tr>
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<td>41</td>
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<td></td>
</tr>
<tr>
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<td>B Smedmyr</td>
<td>153</td>
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Abbreviations: EORTC = European Organization on Treatment and Research in Cancer, EORTC Leukemia Group; GIMEMA = Gruppo Italiano Malattie EMatologiche Maligne dell'Adulto; GMALL = German Multicenter Study Group for Adult ALL; GRAALL = Cooperative group combining the French GET-LALA (Groupe d’Etude et de Traitement des LALA de l’Adulte), the GOELAMS (Groupe Ouest Est des Leucemies Aiguees et Maladies du Sang) and the SAKK (Schweizer Arbeitsgemeinschaft für klinische Krebsforschung, Leukämie-Arbeitsgruppe); MRC = Medical Research Council, Leukemia Working Party; NILG = Northern Italy Leukemia Group; PLRG = Polish Lymphoma Research Group; PETHEMA = Program for Study and Treatment of Malignant Hemopathies, Spanish Society of Hematology.

Figure 1 Major research fields in adult ALL.

B-ALL,26,27 or Ph + ALL, treatment of adolescents or SCT.31,32

Aims of a European network for adult ALL

In individual countries, a high level of cooperation and standardization is already reached. One of the major aims for the European collaboration is to transfer these achievements to a broader, international level.

Diagnosis of ALL

Elaborated diagnosis is a pre-requisite for clinical research in ALL and for all diagnostic methods standardization would be very helpful, for example, to make published results comparable and meta-analysis possible. At the national level, standardization is mainly achieved by specialized central labs integrated in the study groups. Most of them also perform central storage of patient samples. These laboratories are also involved in European standardization projects. Thus, for immunophenotyping, the European Group (EGIL) defined a standard panel for immunologic classification of ALL subtypes, but not all groups adhere to it. For cytogenetics, besides technical standards, which are relevant for all types of leukemias, for ALL a standardized definition of the most relevant cytogenetic aberrations would be useful in order to perform intergroup analyses on prognostic impact. For molecular genetics, standardization procedures have been reported from the groups involved in the BIOMED concerted action. There is, however, no consensus panel.
of molecular aberrations to be analyzed in ALL. While detection of BCR-ABL and ALL1-AF4 is certainly performed in all study groups, only some groups perform routine analysis of further markers such as PBX-E2A, TEL-AML1, TAL1 or HOX11. The methods and definition for MRD analysis are even less standardized. This not only refers to different methods, but also to the definition of technical details (cut-points, sensitivity), time-points and prognostic relevance. Microarray technology is another rapidly evolving field in diagnosis of ALL. European study groups have made primary contributions, but there are no common standards or combined efforts at the moment. In order to make study concepts and results comparable, the use of standardized diagnostic procedures and uniform definitions for diagnostic subgroups would be very important. In all these fields, experts of the European ALL study groups can cooperate with corresponding projects of the ELN.

Clinical studies in adult ALL

All European study groups probably carry out national studies for de novo ALL in the main age groups of ALL. These studies show, however, a broad variation regarding entry criteria (eg age limits), chemotherapy concepts ranging from standard protocols up to intensified anthracycline inductions, use of high-dose cycles, a wide spectrum of different consolidation cycles, maintenance therapy (standard or intensified), duration of therapy, risk stratifications and indications for SCT.

ALL has a variety of subgroups with large differences in outcome and also response to subgroup adjusted therapy approaches. Not all ALL study groups perform studies in rarer subgroups (eg elderly patients, adolescents, mature B-ALL, biphenotypic ALL), in later disease stages (eg systemic relapse, CNS relapse) or studies with targeted therapies (eg studies with monoclonal antibodies or with imatinib in Ph/BCR-ABL positive ALL). Some of these subgroups are very rare and it is difficult for individual study groups to define rational treatment approaches and achieve relevant results. Another problem is that phase I/II studies are performed only rarely and in single European centers despite the elaborated structures and high patients number to be reached by European study groups. It would be highly desirable to create first an overview on ongoing studies, for example, in an internet-based registry that would be open to all participating groups. This registry would provide relevant criteria for comparison, for example, overall treatment concepts, diagnostic standards, entry/exclusion criteria, risk stratification, indications for SCT. Based on this information, a discussion on options for harmonization and possible joint studies can be started. Intergroup studies are useful for rare subgroups of ALL in order to achieve relevant patient numbers and results in shorter time. Joint studies would also be of interest for the exploration of innovative approaches in SCT (eg nonmyeloablative transplantation and/or targeted cell therapy) and for phase I/II studies with the pharmaceutical industry for the investigation of new cytostatic drugs, monoclonal antibodies and ‘molecular’ drugs, such as imatinib, FTI, small molecules, etc.

Prognostic models

It is beyond dispute that ALL is characterized by distinct subgroups with largely different prognosis, which are defined by generally accepted prognostic factors. These factors may, however, be used in clinical practice with different definitions. Furthermore, they are used for treatment stratification only by some groups. Prognosis of ALL is getting inferior with increasing age, but the cut-points vary. Upper age limits of studies generally reflect the limit for intensive chemotherapy and particularly SCT and range between 55 and 65 years. Some groups also use the age limit of 35 years, as defined in the first published models33 as a prognostic factor. Age limits may have a considerable effect on overall results and this has to be considered for all comparative analyses.

WBC as a prognostic factor is generally applied with a cut-point of 30 000/l as published initially.33 There are, however, some variations of this cut-point (10–35 000/l) and some groups use a modified cut-point for T-ALL of 100 000/l. Immunophenotype plays an important role for risk stratification and treatment decision. Generally, mature B-ALL (if treated according to specific protocols) and T-ALL are considered as more favorable subgroups. However, some groups still deal with inferior results for T-ALL. Pro-B-ALL and immunologic subtypes of T-ALL (early and mature T-ALL) are not recognized by all groups as poor prognostic subgroups.

By cyogenetics/molecular genetics, the most unfavorable subgroup of adult ALL, Ph/BCR-ABL-positive ALL, is identified. t(4;11)/ALL1-AF4 is also an unfavorable prognostic factor in most groups, although results seem to improve with more intensive therapies including SCT. Some groups use also t(1;19)/E2A-PBX1 as an unfavorable feature. New molecular prognostic factors are emerging such as HOX11/LYL1 in T-ALL or the overexpression of the multidrug-resistance gene (MDR1).

Several, but not all study groups, perform prospective evaluation of MRD. The methods vary widely, as well as number of sequential analysis and the time-points. Some groups have already included MRD in their prognostic models that are at present not readily comparable. Although there is a consensus on major prognostic factors in ALL, they are used in a different way in individual study groups. The aim of a European cooperation could be to create first an overview on prognostic models in use. It would then be important to define a set of potentially relevant prognostic factors and to analyze similar ALL subgroups in national studies. If possible, the final aim could be a consensus model for prognostic factors in ALL.
Indications for stem cell transplantation

Indications for SCT are closely linked to prognostic factors. Undisputed is probably only the fact that patients with Ph/BCR-ABL-positive ALL are candidates for SCT in first CR, as are all patients beyond first CR. All other issues are handled rather differently in the European study groups. This includes age limits for transplantation, indications for unrelated SCT, the use of dose-reduced SCT, conditioning regimens and the role of autologous SCT with additional variables as purging, time-point for stem-cell collection.

Regarding the indications for SCT, two major concepts are under investigation. One is the risk-based model with the use of SCT only in patients with high-risk features. The other is a model based on the availability of a related donor as a ‘biologic’ randomization where all patients with sibling donor receive allogeneic SCT in first CR. Furthermore, several groups have performed randomized comparisons between autologous SCT and chemotherapy with, however, rather different time-points for collection of SC, SCT and compared chemotherapies. As first step, a comparison of indications for SCT and procedures such as the conditioning regimen would be very helpful for the planning of joint studies and meta-analyses.

Preparation of meta-analysis

Classic meta-analysis and evidence-based medicine that is solely focused on randomized studies is probably not very useful in ALL due to the heterogeneity of patients, rareness of the disease and lack of randomized studies. However, the good characterization of patients offers the opportunity to perform meta-analysis of well-defined rare subgroups in which the individual study groups cannot perform analysis with sufficient patient numbers. This applies, for example, for certain cytogenetic/molecular aberrations, specific treatment approaches, for example, autologous or nonmyeloablative SCT or subgroups like adolescents. As a prerequisite of meta-analysis required data sets, procedures (eg data exchange modalities, responsible group, publication) and subgroups of interest have to be defined.

Analysis of gender-specific differences

The recognition of gender-specific issues is an important topic in all programs funded by the EU. ALL shows, as all lymphoid malignancies, a male preponderance. Published data for childhood ALL also show that outcome is influenced by gender with boys having a poorer outcome. For adults this issue remains to be analyzed. Little is also known about late effects of therapy in adult ALL and in particular on gender-related differences. This may include, for instance, fertility, bone density, hormonal disturbances and quality of life. Therefore, an important goal of the ALL network would be to combine available information on gender-specific differences and to consider these issues for specific research projects.

Education and spread of excellence

Clearly, one major aim of a European network on adult ALL is to combine efforts in the field of education. Most important is probably an internet-based information exchange on ongoing studies, information resources on adult ALL, etc, which would be useful for physicians, patients and the public. The exchange of scientific staff and the organization of training programs for researchers and physicians, as well as the organization of meetings (scientific symposia, workshops) during European hematology congresses would be another important goal. Through the open structures of the network, the inclusion of additional participants from European countries’ including Eastern Europe’ should be possible and desirable. At a later time-point, certainly the development of guidelines for diagnosis and even later treatment of ALL would be an important goal.

Operation procedures of the network

The organization and management of the network should be based on equal representation of all involved study groups in the steering committee. All decisions should be made by democratic consensus during regular meetings. Specific topics could be covered in addition by working groups, for example, for diagnostic procedures, prognostic models, etc. For the success of the network, it is of utmost importance to first define procedures and detailed action plans and also to monitor performance parameters regularly. The objectives will be achieved in a stepwise process that has to be associated with a gain of confidence between so far competing study groups. Clearly, the identity and independence of each group should be maintained since this is the basis for the success in their individual countries. On the background of a strong collaborative European group, it will hopefully be possible to obtain additional funding from national and international organizations and to obtain support from the pharmaceutical industries.

First steps

There is certainly an urgent need for cooperation of the European ALL study groups; it should take place independent of funding and a lot of preparative work has already been done. For the grant application, detailed items of a working program with persons in charge and deadlines had to be defined. Therefore, after acquisition of the funding, the work on details of the working program can start immediately. The first step is certainly to gain an overview on ongoing research activities in adult ALL in Europe and to set up structures for exchange of information, such as internet presentation, regular meetings, set-up of working groups, etc.
second step is to define fields of interest for cooperative research activities. These may include meta-analyses, joint studies and other collaborative projects. For the realization of these projects, rules including publication rights, type of exchanged data, etc have to be defined. A further step is to define certain standards, for example, in the field of diagnosis, prognostic factors, clinical studies, etc. Finally, the national activities for education, standard recommendations, etc can be combined and the network could expanded to more European countries.

In the proposed European network for adult ALL, 12 groups with more than 400 participating hospitals, specialized laboratories and research groups will combine their national research networks. In their last studies, these groups included more than 8000 patients. This unique chance will hopefully lead to major progress in basic research, including new complex biotechnologies, rapid translation of findings in clinical practice and will improve standards of public health in ALL all over Europe.

References


Prophylaxis of bacterial infections

Background

Bacterial infections are a major cause of morbidity and mortality in patients with hematological malignancies and chemotherapy-induced neutropenia. Such infections are most frequently caused by microorganisms that colonize the gastrointestinal tract and enter the bloodstream through epithelial surfaces damaged by cytotoxic chemotherapy.

This observation suggested that prophylactic use of antibacterial agents that act against the intestinal flora may be valuable in reducing the rates of infection in neutropenic patients. Preservation of the anaerobic flora of the alimentary tract, while eliminating potentially pathogenic aerobic Gram-negative bacilli, has been considered by some investigators to be especially important. The term ‘selective decontamination’ has been applied to this approach.

Evidence

Several studies have attempted to document the effectiveness of this potentially useful strategy. Various combination of nonabsorbable antibiotics were firstly used but their effectiveness and tolerability were found unsatisfactory and they were abandoned in favor of orally absorbable medications such as trimethoprim-sulphamethoxazole (T/S) and fluoroquinolones.

Several studies demonstrated that the infection rates for T/S-treated patients were significantly lower than were those for placebo-treated patients. T/S, however, did not affect patient mortality and had some additional problems. Among them prolongation of neutropenia, adverse reactions caused by sulfonamide moiety and emergence of drug-resistant bacteria were the most important. These disadvantages prompted the study of oral fluoroquinolones (eg ciprofloxacin, ofloxacin, pefloxacin, norfloxacin). Comparative studies showed these compounds to be more effective than placebo and equally or more efficacious than T/S in preventing febrile episodes of infectious origin in neutropenic patients and reducing Gram-negative bacillary infections. However, the benefits with prophylactic use of fluoroquinolones, were countered by emerged resistance to these drugs in some Gram-negative bacilli and inadequate coverage for Gram-positive bacteria, in particular methicillin-resistant staphylococci and resistant streptococci. The combination of an anti Gram-positive antibiotic (ie penicillin, rifampicin) with the quinolones to overcome this problem gave contrasting results and clinical experience with new fluoroquinolones with improved activity against Gram-positive bacteria (eg levofloxacin, gatifloxacin, moxifloxacin) is still lacking.

Overall, although the majority of the prophylactic trials using quinolones showed a clear trend towards reducing infections caused by Gram-negative bacilli, after more than 20 years of use there is no consensus to recommend antibacterial prophylaxis in neutropenic patients with the only exception of T/S usage in selected patients to prevent Pneumocystis carinii pneumonia. The lack of consensus is based on three considerations: first, the routine use of fluoroquinolones as prophylaxis in neutropenic patients may cause the emergence of antibiotic resistant bacteria; second, prophylaxis has not been shown to reduce mortality rate; third, all the studies on prophylaxis in neutropenic patients were small, and they included an inadequate number of patients for sound statistical analysis to provide a conclusive answer on the real utility of such a prophylaxis.

To overcome these limitations, we conducted a large prospective randomized placebo-controlled double-blind clinical trial to see if prophylaxis with levofloxacin can reduce the number of infections in neutropenic cancer patients and to define which population of neutropenic patients could benefit more from this prophylactic regimen. A total of 760 consecutive afebrile adult patients who had hematologic malignancies or solid tumors and chemotherapy-induced neutropenia expected to last more than 7 days were studied. Patients were randomized to levofloxacin 500mg/daily or placebo. Prophylactic treatment was started concomitantly with chemotherapy and was continued until the resolution of neutropenia.

Efficacy analysis on 675 evaluable patients showed those receiving levofloxacin to have significantly less febrile episodes and microbiologically documented infections. The mean duration of fever and that of antibacterial therapy during the whole period of neutropenia was shorter in patients receiving levofloxacin than
those receiving placebo. The efficacy was similar irrespective of the risk represented by underlying disease and more severe and protracted neutropenia. As expected susceptibility data of the microbiologically documented single agent infections showed a higher rate of fluoroquinolone-resistant strains in patients treated with levofloxacin than in patient treated with placebo.

**Recommendations**

Concern about the problem of emerging drug-resistant bacteria and lack of effect in reducing mortality rates, are the main reasons for not recommending the usage of routine prophylaxis with fluoroquinolones and T/S in neutropenic patient.4-5 This recommendation is in a sense paradoxical as now we have the best evidence (A.I. rating) that neutropenic patients (both at ‘high’ or ‘low’ risk for infection) have reduction in febrile episodes and severe Gram-negative infections if they receive prophylaxis with levofloxacin.

The above-mentioned concerns cannot be fully agreed upon. There is no evidence that the greater percentage of resistant bacteria found among patients receiving prophylaxis with quinolones, represent an ‘induction’ of resistance or instead simply a selection of resistant strain from a normal intestinal flora. Once the quinolone use is interrupted, the epidemiology of resistant bacteria would reverse to normal. The lack of evidence that prophylaxis can cause a reduction in mortality is not surprising as mortality for infection is related to the appropriate use of empiric therapy and to other risk factors rather than prophylaxis by itself. Therefore, reconsideration of the existing recommendations seems wise.4

**Empiric therapy of bacterial infections**

**Background**

Empiric therapy with broad-spectrum antibacterial agents administered with the development of fever or at the first sign or symptom of infection before pathogenic organism can be identified, represent the cornerstone of the successful initial management of febrile neutropenic cancer patients and is now a universally accepted principle. This aggressive approach was found necessary because of the rapid clinical deterioration that can occur when a neutropenic patient develop an infection and because such patients with early bacterial infections at presentation cannot be reliably distinguished from non infected patients as symptoms and signs of inflammation (with exception of fever) may be minimal or even absent. The aim of empiric therapy is therefore to avoid early mortality from severe infections during neutropenia while waiting for infection documentation.5

What constitutes the optimal empiric antibiotic regimen for the initial treatment of febrile neutropenic patient, continues to be debated. That is not surprising as neutropenic patients do not form a homogeneous population and do not share equal risk of infection nor is the infection-related morbidity the same. Therefore, no specific drug or combination of drugs and no specific period of treatment can unequivocally by applied to all febrile neutropenic patients.4

Criteria that dictate the choice of initial empiric therapy are represented by bacterial epidemiology; individual risk factors for infection and clinical documentation of infection at the onset of fever.

**Bacterial epidemiology**

Changes in the general prevalence of various bacterial agents and in the pattern of their sensitivity to antibiotics play a major role in the choice of empiric therapy. In the late 1960s and 1970s the microorganisms that predominated in cancer patients with neutropenia and life-threatening infections were Gram-negative aerobic bacteria, most frequently *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. In more recent years, Gram-positive bacteria, such as coagulase-negative staphylococci, *Staphylococcus aureus* and streptococcal species, were most frequently isolated and now account for approximately 60% of microbiologically documented infections in most centers.

One additional finding particularly worrying in these changes is the increasing prevalence of infections caused by methicillin-resistant staphylococci which require the use of a glycopeptide and consequently increase the risk of selecting bacteria resistant to this important class of drugs.

Three Gram-negative bacteria (*E. coli; Klebsiella sp; P. aeruginosa*) account now for 90% of Gram-negative isolates in febrile neutropenic patients. There is wide consensus that a bacteremic infection caused by Gram-negative (in particular *P. aeruginosa*) should be considered at higher risk of severe complications and death than one caused by Gram-positive bacteria (especially methicillin-resistant staphylococci). Therefore, the therapeutic approach should be prompt and specific.

Finally, one must remember that the epidemiology of prevalent microorganisms and their susceptibility to the antibiotics can vary from one institution to another and that new ‘emergent’ pathogens should be considered. This requires vigilant observation and prompt access to local epidemiology data.

**Individual risk factors for infection**

Neutropenic patients are not all the same. Despite the fact that neutropenia itself is the single most important risk factor for infection, other factors must be considered as they can alter the risk of infection in the single patient.5 They include:

- The degree and the duration of neutropenia. Patients with less than 100 neutrophils/mm³ have the highest risk of infection and the risk increases with its duration. Patients with ≤7–10 days duration of
Since the 1970s, the empiric therapy was commenced when compared with those with >10 days duration that are considered at 'high' risk for infection. 

- The phagocyte function and the status of the patient’s cellular and humoral immune system. Both of them can be impaired by the patient’s underlying disease or its treatment with cytotoxic or immunosuppressive chemotherapy.
- Alterations of physical defence barriers. They can be due to damage of mucosa of the alimentary tract by chemotherapy or to damage of skin due to presence of indwelling intravenous catheters.

Clinical documentation of infection at the onset of fever

Despite the fact that symptoms and signs of infection may by minimal or absent in neutropenic patient, a careful search should be undertaken for a clinically documented focus. The site of infection can in fact suggest a preferential pathogen (eg coagulase-negative staphylococci in vascular access devices, infection or anaerobic bacteria in patients with acute abdominal pain or perianal tenderness) and call for immediate addition of specific antimicrobial coverage. On the other hand, the demonstration of a focus of infection or signs or symptoms suggesting systemic infection (eg rigors, hypotension) indicate a patient at high risk for serious infection complication.

Therefore, when considering empirical therapy, all these factors should be taken into consideration to make the most appropriate choice. Unfortunately, only few studies have produced and validated clinical predictive rules able to identify and differentiate patients with 'high' or 'low' risk of infection by taking in account major risk factors for impending infection.

The approach to the choice of empiric antibacterial therapy has four possible alternatives.

Option 1: Monotherapy

Evidence: Since the 1970s, the empiric therapy was characterized by an antibiotic combination able to ideally cover a wide range of prevalent pathogens, to exert an additive or synergistic effect especially on the more virulent Gram-negative pathogens and to potentially decrease the emergence of resistant organisms. Usually, the effective combinations included an aminoglycoside plus an extended-spectrum penicillin (ticarcillin, piperacillin) or, more rarely, a double beta-lactams combination (eg a broad-spectrum penicillin plus a broad-spectrum cefalosporin, or various two, or three drugs combination. However, no particular combination has been shown to be consistently superior, although some clinical studies have confirmed that synergistic antibiotic effect and high bactericidal activity in serum were critical in the management of most serious Gram-negative infections.8

In the 1990s along with an increased prevalence of Gram-positive bacteria, a number of new antibiotics were made available. These antibacterial drugs included third/fourth-generation cephalosporin, carbapenems, fluoroquinolones and the beta-lactam/beta-lactamase inhibitor combinations. These drugs possessed a broad spectrum of antibacterial activity, including P. aeruginosa and viridans streptococci, had peak serum levels that exceeded the MIC for the most common pathogens recovered from febrile neutropenic patients, had high serum bactericidal levels and lacked of relevant toxicity. They were therefore ideal candidates for use as single-drug empiric therapy. To test this hypothesis, many studies have been carried out which compared monotherapy with standard combination treatment for febrile neutropenia.4,7–10 Although most of these randomized clinical trials do not report any significant difference in efficacy between the two options, their results are difficult to interpret. In fact, the available clinical data on comparison of monotherapy versus combination therapy come from studies which, with few exceptions, have major deficiency in design, including: inappropriate choice of comparator, lack of well-defined uniform outcome criteria and end points; investigator bias due to the lack of study regimen blinding; lack of consideration of sample size and power; analysis of only a small number of clinically or microbiologically documented infections. It is not surprising, therefore, that none of these trials were able to demonstrate in an unequivocable way monotherapy to be at least equivalent to the standardly used synergistic antibiotic combinations containing an aminoglycoside. To summarize the clinical evidence available, overcoming the limitation of lack of statistical power and of uniform outcome definitions in the individual studies, we conducted a meta-analysis of 29 trials including 4795 evaluable febrile episodes.11 The meta-analysis demonstrated that single-drug empiric antibiotic therapy provides an as effective treatment for patients with febrile neutropenia as a combination containing an aminoglycoside does, even when patient are bacteremic and the degree of neutropenia is substantial (Figures 1 and 2).
Option 2: Two drug empirical therapy without a glycopeptide

Evidence: The literature provides a long list of possible combinations that include, in the majority of studies, an aminoglycoside plus a compound with antipseudomonal activity (eg, piperacillin/tazobactam; ceftazidime; cefepime, carbapenems). When directly compared in well-conducted studies, these two drug combinations were shown to be equivalent.4

Recommendations: These combinations will remain a commonly used option, probably due to the fact that potentially synergistic effects against some Gram-negative bacilli and minimal emergence of drug-resistant strains during treatment are seen as potential advantages of this option. Disadvantages are the inferior activity of some component of these combinations against some Gram-positive bacteria (eg, ceftadizime against viridans streptococci) and the nephrotoxicity and ototoxicity associated with aminoglycosides.

The literature provides a long list of possible combinations that include, in the majority of studies, an aminoglycoside plus a compound with antipseudomonal activity (eg, piperacillin/tazobactam; ceftazidime; cefepime, carbapenems). When directly compared in well-conducted studies, these two drug combinations were shown to be equivalent.4

Recommendations: These combinations will remain a commonly used option, probably due to the fact that potentially synergistic effects against some Gram-negative bacilli and minimal emergence of drug-resistant strains during treatment are seen as potential advantages of this option. Disadvantages are the inferior activity of some component of these combinations against some Gram-positive bacteria (eg, ceftadizime against viridans streptococci) and the nephrotoxicity and ototoxicity associated with aminoglycosides.

Quinolone-based combinations with a beta-lactam with anti-
Pseudomonas

Evidence: Infections due to Gram-positive bacteria are now more frequent and some of these pathogens (eg, coagulase-negative staphylococci) are methicillin resistant and therefore are susceptible only to glycopeptides or new compounds such as quinupristin–dalfopristin or linezolid. The question now being asked is whether empiric Gram-positive therapy with a glycopeptide is necessary and appropriate. Infections by Gram-positive vary in their presentation. As already mentioned, some of them (eg infections due to coagulase-negative staphylococci, Corynebacterium KJ) are indolent infections and may be susceptible only to a glycopeptide. Other Gram-positive bacteria (eg, S. aureus, viridans streptococci) may cause instead fulminant infections resulting in serious complications or death if not treated promptly.

To further complicate the answer to the above-mentioned question is the consideration of the necessity to limit the use of glycopeptides to avoid the emergence of vancomycin-resistant microorganism.

Recommends: There is now convincing evidence that a glycopeptide is not in general a necessary part of initial empirical antibiotic therapy. The glycopeptide can be withheld without risk in most cases until the results of cultures indicates the need for this antibiotic.13,14

The most recent IDSA guidelines suggest the inclusion of glycopeptide in initial empiric therapy only for selected circumstances:

- Clinically suspected serious catheter-related infection (bacteremia, cellulitis)
- Hypotension or other evidence of cardiovascular impairment
- Known colonization with penicillin- and cephalosporin-resistant pneumococci or methicillin-resistant S. aureus
- Positive results of blood culture for Gram-positive bacteria while waiting for final identification
- Intensive chemotherapy with high-dose cytarabine
- Use of fluoroquinolones in prophylaxis

Anyway, if the glycopeptide is incorporated into the initial empiric therapy and after 24–28 h later no Gram-positive bacteria necessitating such drug is identified, the glycopeptide should be discontinued.

The glycopeptide must be always used in combination with an agent already tested in empiric therapy of febrile neutropenia. A word of caution may be spent in combining the glycopeptide with an aminoglycoside due to potential increased risk of nephrotoxicity and with ceftadizime as in some centers the risk of emergence of resistance may justify the combination with another compound (eg a carbapenem).

Two other questions may be of interest. Which glycopeptide should be used and which is the therapeutic role of the new anti-Gram-positive agents.

Vancomycin and teicoplanin have been extensively used in neutropenic patients and we have compared them in a large clinical trial that demonstrated similar efficacy and tolerability.

Linezolid, an oxazolidinone, has excellent activity against susceptible or resistant Gram-positive bacteria but may be associated with myelosuppression and therefore should not be used in neutropenic patients.

Quinupristin–dalfopristin has no published data in the empiric treatment of febrile neutropenia.

Option 4: Combination of oral antibiotics

Evidence: The increasing understanding on risk factors for serious infection in the neutropenic patient with fever and the availability of broad spectrum orally administered drugs (eg, fluoroquinolones, amoxicillin–clavulanate, cefixime) have raised the question whether low-risk outpatient management with oral drugs would be feasible. Factors that characterize a low risk for...
severe infection among patients with neutropenia have been identified in controlled studies. These characteristics may serve as guidelines for the selection of patients for outpatient therapy. They include: absolute neutrophil and monocyte count >100 cells/mm³; normal findings on a chest radiograph, nearly normal results of hepatic and renal function tests, duration of neutropenia ≤7 days; resolution of neutropenia expected in <10 days; noninvasive catheter-site infection; evidence of early bone marrow recovery; malignancy in remission; peak temperature <39°C; and no comorbidity complications (e.g., shock, hypoxia, deep organ infection, vomiting or diarrhea). Some of these characteristics were selected to develop a risk-index score that was tested for validation. Similar prediction rule for low-risk pediatric patients has also been validated.

**Recommendations:** Treatment of low-risk selected patients with oral broad-spectrum antibiotics has been shown feasible in a small number of studies. In particular, the combination of amoxicillin–clavulanate plus ciprofloxacin has been shown to be effective as intravenous therapy with ceftriaxone in one study and with amikacin plus ceftriaxone in another.15,16 However, in both studies the enrolled patients were kept in hospital till resolution of infection, because of safety considerations. However, a wide acceptance of this approach to empiric therapy of febrile neutropenic patients may be unrealistic in most cases as it would require a careful and continuous evaluation at home of the patient clinical condition. As a more convincing alternative, early discharge with continued outpatient therapy with oral antibiotics, after a brief in-patient admission during which intravenous antibiotic therapy is initiated, has been proved feasible and cost efficient.17

**Conclusion**

The infectious complications that characterize the neutropenic cancer patient represent a challenge for the clinician. The appropriate clinical and microbiological diagnosis is far from being easy due to the lack of inflammatory response and the large number of pathogens that can be responsible for the infection and that often are not immediately identified. Therefore, the choice of the most appropriate therapy is empirical and need a deep knowledge of the relationship existing between mechanisms of defence of the host and the occurring prevalent pathogens. Knowledge of local epidemiology is a necessary prerequisite for a rational choice of the empirical therapy or prophylaxis. A great help in this choice come from some guidelines.4 However, these recommendations must be used thoughtfully and should never be considered a substitute of a careful, continuous, evaluation of the patient clinical condition.

**References**


Fungal infections: current diagnosis and treatment

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Introduction

Despite considerable progress in the management of infectious complications in hematology patients, fungal infection remains an important cause of morbidity and mortality, mainly after allogeneic stem-cell transplantation (SCT). The major advances in the management of invasive fungal infections (IFI) have come from the understanding of the risk factors for the development of IFI, from the development of new biological markers of IFI, and also from well-designed therapeutic trials. However, much remains to be done to decrease the risk of mortality due to IFI in hematology.

Epidemiology and risk factors for invasive fungal infections

Fungal infection, and especially aspergillosis, is nowadays the first cause of infectious deaths after allogeneic SCT. Despite geographical, and center-to-center variations, Candida and Aspergillus infections are the main leaders of IFI. Despite different mechanisms of acquisition, they share many risk factors that are common to many hematology patients, especially: prolonged neutropenia, corticosteroid administration, and graft-versus-host disease after allogeneic stem-cell transplant. Owing to the development of autologous transplantation with peripheral stem cells, the duration of neutropenia in that setting is usually less than 10–12 days. That practice has erased the risk of aspergillosis in autologous transplant. Although the risk of candidemia is still present, it is far lower than after allogeneic stem-cell transplant. For these reasons, it is usually considered that autologous transplant patients do not need any antifungal prophylaxis, other than nonabsorbable antifungals – especially in case of concomitant antibiotics – whose efficacy remains debatable.

Aspergillus is the first cause of infectious death after allogeneic transplant¹ and remains a major complication in the cursus of leukemia treatment. Because more and more patients with lymphoproliferative diseases are intensively treated with chemotherapy and monoclonal antibodies, it is likely that the Aspergillus incidence will increase in the future in this population that was, until recently, considered as spared of this complication. After allogeneic SCT, reported incidences vary from 0 to 20%, with higher incidences in the older patients, and those receiving unrelated grafts. A first peak of incidence occurs during the neutropenic period, particularly in leukemic patients who had been previously colonized. The second incidence-peak is generally between the second and third months, in patients with severe GvHD. In fact, Aspergillosis may occur at any time after transplant, even years later when corticosteroids have been used for prolonged periods. Recurrence may also occur in roughly one-third of the patients with a previous Aspergillus infection.²

Candida infection is more rare in most centers. However, the mortality of candidemia in hematology patients is very close to that of aspergillosis, around 40–60%. Candida epidemiology has changed over time in onco-hematology.³ Candida albicans nowadays represents no more than 50% of all the candida strains responsible for infections in these patients. More and more C. krusei, C. glabrata or C. torulopsis are reported. A special mention should be made of C. parapsilosis, which is mainly linked to the presence of intravenous central lines, and IV nutrition with intralipids. Central withdrawal is critical for the cure of candidemia, especially for C. parapsilosis.

More and more non-Aspergillus, non-Candida infections are reported in hematology patients.¹ This increasing incidence may be due to multiple causes: more immunocompromised patients, better knowledge of fungi, and overall better consideration of fungi, which were previously considered by the microbiologist are only contaminants in human samples, may be selection pressure by antifungals according to their spectrum (Table 1). Pneumonias due to endemic fungi, such as histoplasmosis or coccidiodomycosis, particularly in North America, must be considered in these patients, as well as in the emerging fungi, including trichosporon, alternaria, fusarium, and the mucorales. Any of these fungi can cause pneumonia in the hematology patients, mainly in the more immunosuppressed of them, the allogeneic SCT recipients with GvHD. It is essential to develop effective collaboration with the microbiologist in order to have precise identification of any fungi possibly related to symptomatic disease in these patients, because this is of overwhelming importance in the therapeutic choice.
Diagnosis of invasive fungal infections

New diagnostic tests being developed to improve diagnosis of invasive fungal infection include detection of *Aspergillus galactomannan* by an ELISA. Early results show good sensitivity during neutropenia and after stem-cell transplant.4,5 This test has been included as a biological marker of probable aspergillosis in the Mycosis Study Group-EORTC definitions.6 Because of a false-positive rate of 5–15%, it is suggested that this test may be most useful in prospective screening of neutropenic patients rather than for diagnosing pneumonia. One of the causes of the false positivity could be the presence of galactomannan in betalactamin antibiotics administered to these patients for documented infection or undocumented febrile neutropenia. Other aspergillus antigens are being evaluated. Nucleic acid detection has also been widely investigated, mainly in stem cell transplant recipients.7 Real-time PCR assays may solve technical problems such as contamination.8 There is no standardization of methods yet, and there have been discrepancies between antigenemia and PCR results,9 so there is currently no consensus on their use in clinical practice.6

Although antigenemia and PCR have improved the early diagnosis of IFI in hematology patients, we should mention that, in case of a clinical focus of IFI, there is no noninvasive test that can replace the diagnostic yield of direct investigation. This is specially important in case of pneumonia, where fibroscopy with bronchoalveolar lavage may not only show fungi at direct examination, identify it by culture, but may also show other pathogens involved in multimicrobial pneumonia.

In case of pneumonia, sputum analysis should be analyzed to look for aspergillus or mucorales. The importance of finding *Aspergillus* in the sputum depends on the degree of immune suppression of the patient and the risk of withholding antifungal therapy until further documentation. In stem-cell transplant patients with pneumonia, a positive sputum culture may be highly suspicious for pulmonary aspergillosis. On the other hand, for *Candida*, in the lack of any data establishing the relationship between a positive sputum culture to *Candida*, and the presence of a *Candida* pneumonia, no conclusion can be drawn from the identification of candida in sputum.

How to manage fungal infections: from prevention to treatment through empirical therapy

**Prophylaxis**

In two randomized trials, fluconazole prophylaxis at the dose of 400 mg/day has been shown to reduce the risk for invasive fungal infections, especially candida infections, in allogeneic stem-cell transplant patients. However, because non-*albicans* candida are more and more frequent in hematology patients, the benefit of fluconazole prophylaxis is likely lower than 10 years ago. Fluconazole may be recommended for allogeneic patients during the neutropenic phase for prophylaxis of *Candida* infections in centres with low rates of non-*albicans* candidemia and of *Aspergillus* infections, but due to its lack of efficacy on *Aspergillus*, better efficacy is expected from newer compounds. Itraconazole, which is active on *Aspergillus*, has been shown to have a better prophylactic effect on the occurrence of IFI after allogeneic stem-cell transplant than fluconazole, but without any impact on survival.10,11 The prophylactic efficacy of itraconazole in acute leukemia patients is controversial despite more than 10 randomized studies.12 Serum levels of itraconazole need to be checked weekly and the use of oral solution is to be preferred to capsules because of its better bioavailability.

Addionnally, itraconazole interferes with a many other drugs, including cyclophosphamide and vinca alkaloids. The concomitant administration of itraconazole and cyclophosphamide should be avoided in the conditioning regimen of stem-cell transplant. Owing to the therapeutic efficacy of newer compounds on fungal pathogens, one may consider that the best prophylactic option for fungal infections in SCT patients has not been found until now. Prospective trials are ongoing with other antifungals.

**Empirical therapy**

The routine practice of empirical therapy with antifungals in case of 3–7-day persistent fever under broad-spectrum antibiotics in neutropenic patients is based on two historical trials who showed some benefit to give amphotericin B deoxycholate to these patients on the
incidence of IFI during the neutropenic episode. Although none of these two trials showed any benefit on survival, it is recommended to maintain this practice, especially for high-risk patients, until a more satisfying strategy be established. Since these historical trials, many comparative studies have compared amphotericin B deoxycholate to new antifungals in this indication, then AmBisome – which was shown to be as efficacious and less toxic than amphotericin B deoxycholate – to new compounds. Until now, no drug has been shown to be superior to AmBisome in the empirical indication of febrile neutropenia. However, recent data on caspofungin suggest that AmBisome is challenged in this indication. Although we can compare drugs, the main problem raised nowadays for the clinician is not so much the choice of the drug rather than the clinical pertinence of the empirical strategy in regards to new biological, sensitive, markers of IFI. Additionnally, the high cost of the new antifungals needs to completely reconsider the empirical strategy with good, prospective, strategy, comparative trials.

Pre-emptive strategy

Since the prognosis of documented IFI is poor, even when treated with new antifungals, and because the empirical strategy is questioned for safety and cost reasons, the concept of a pre-emptive treatment is developed. The rational is: (1) to restrict the use of antifungals to the patients with the higher risk of IFI; (2) to early start treatment on biological markers such as antigenemia or PCR, before the development of overt disease, in order to improve the prognosis. However, until now, pre-emptive strategy is not clearly defined. Mainly, there is no clear demonstration that a more restricted, preemptive strategy does not provide more risks of IFI and of fungal death to the patient, than the classical empirical strategy.

Treatment of documented IFI

**Aspergillus**: Newer antifungal agents (Table 2) have begun to effect a decrease in mortality. Voriconazole has been shown to improve the survival for patients with Aspergillosis when compared to a control group treated with conventional amphotericin B. Echinocandins have been studied in refractory Aspergillosis with encouraging results. Despite these improvements, the mortality of Aspergillus in the very high-risk populations such as allogeneic SCT patients remains superior to 50% in recent series.

Voriconazole, a triazole antifungal agent, is fungicidal against Aspergillus and Candida species, including non-albicans candida, and other rare fungal infections such as *Fusarium* or *Scedosporium* species that occur in leukemic patients. In the first-line treatment of aspergillosis, it has been shown to be more effective and better tolerated than amphotericin B. Despite a substantial number of side effects (ie, visual disturbances, confusion, skin reactions, and liver-function abnormalities), and potential drug interferences, especially with ciclosporine, voriconazole is widely used. Although there is no large prospective study similar to the voriconazole versus amphotericin B study, liposomal amphotericin B has comparative results on first-line treatment of Aspergillosis.

Echinocandins have been studied in refractory *Aspergillosis* with encouraging results but no study of first-line therapy has been published so far. Echinocandins are excellent candidates for combinations with either azoles, or polyenes. However, there are, until now, no prospective data published on first or second line therapy of fungal infections with echinocandin-combinations. These combinations will be extremely expensive, and there are no evidence, until now, that they will provide better results than single drug regimens. Consequently, they should be used in second line for now. Prospective, comparative trials need to be conducted before any conclusion been drawn.

### Table 2  Empirical and therapeutic indications of the main antifungal drugs for hematology patients

<table>
<thead>
<tr>
<th>Empirical therapy</th>
<th>Treatment of candidemia</th>
<th>Treatment of Aspergillus</th>
<th>Treatment of Zygomycetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>First line</td>
<td>AmBisome Amphotericin B deoxycholate for patients with short neutropenia</td>
<td><em>C. albicans</em>: fluconazole</td>
<td>Voriconazole</td>
</tr>
<tr>
<td>Alternative first line</td>
<td>Caspofungin</td>
<td><em>C. krusei, glabrata, norvegensis, tropicalis</em>: polyens or caspofungin&lt;sup&gt;b&lt;/sup&gt;</td>
<td>AmBisome</td>
</tr>
<tr>
<td>Second line</td>
<td>—</td>
<td>Switch to an other class: voriconazole if polyenes were initially given, polyens or caspofungin if azoles were given</td>
<td>Combination (add caspofungin)</td>
</tr>
</tbody>
</table>

<sup>a</sup>In case, the patient has a central line, it is recommended to withdraw it.

<sup>b</sup>Add 5 fluorocytosine in case of meningitis, endocarditis, osteoarthritis or thrombophlebitis.
Candida: In case of suspicion of severe infection due to Candida sp. and before the identification of the Candida, it is cautious to consider the possibility of non-albicans candida, and give either a polyene, or caspofugin. Then, the treatment will be reconsidered 48 h later according to the identification. Fluconazole remains the main drug for treatment of C. albicans infection and should be favored each time the strain – even non-albicans – is susceptible to the drug. A loading dose of 800 mg should be given on the first day, then the conventional dose is 400 mg/day. Candidemia should be treated at least 14 days after the last positive blood culture. The treatment of chronic hepatosplenic candidiasis is not clearly defined, due to the rarity of the disease, but usually needs months of treatment.

Other fungi: Considering the polyenes are the antifungals with the broader spectrum (Table 1), their use should be favored in case of infection due to an undetermined fungi, especially in case of mold infection. One should remind that caspofugin has no activity against Cryptococcus sp, Fusarium, and Zygomyces, and that voriconazole has no activity against Zygomyces. The optimal therapeutic choice for the non-Candida, non-Aspergillus infections must be discussed with the mycologist on an individual basis.

References

Viral infections: current diagnosis and treatment

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The classical types of diseases caused by viruses are pneumonia, meningitis/encephalitis, hepatitis, and gastrointestinal disease. Many viruses can cause each type and the differential diagnosis is, therefore, frequently difficult (Table 1). Since different viruses can cause similar symptoms and since many viral infections can now be treated, it is of paramount importance to make a correct diagnosis.

Risk analysis for viral disease

The risk for severe viral disease in patients with hematological diseases is very variable. Classically, allogeneic SCT recipients have the highest risk, while most other patients with hematological disease have a rather low risk for developing severe disease. The most likely explanation for these different presentations lies in the capacity of the patient to mount an immune response. B-cell function and specific antibodies are the main defense mechanisms reducing the risk for reinfection in already seropositive individuals. Cytotoxic T-cell function is the main mechanism for preventing severe viral disease and also for the control of latent viruses. The degrees of T-cell dysfunction vary in hematology patients. However, the immune defects in SCT patients are frequently complex with defects both in the cytotoxic T-lymphocyte, helper T-lymphocyte, NK-cell, and B-lymphocyte functions. T-cell dysfunction is usually most important early after SCT while B-cell reconstitution may be incomplete for many years. Recent developments with the use of T-cell suppressive agents for therapy of lymphoma (alemtuzumab and fludarabine) have increased the population at risk for disease by viruses controlled by T-cells. Knowledge about the specific immune defect can therefore be helpful both to predict the likelihood for a viral infection causing severe disease symptoms and the likelihood for the disease being caused by a particular virus.

Diagnosis of viral infections

The classical ways of detecting viruses have been by serology and isolation. Serology is usually not useful for diagnosis of active virus infections since many hematology patients are unable to mount antibody responses. However, serology is useful for risk assessment. Classic virus isolation has been either too insensitive or too slow to be of practical use. The use of molecular techniques (including PCR) have improved both sensitivity and speed making specific diagnosis as well as monitoring of viral infections practical and feasible. It is important to choose a technique of appropriate sensitivity for the specimen analyzed and the virus looked for. This can be illustrated by two examples. Viral DNA should normally not be found in the CSF and therefore PCR is an excellent and very sensitive technique for detection of viruses infecting the central nervous system. On the other hand, PCR is frequently an extremely sensitive technique for use on gastrointestinal specimens or bronchoalveolar lavage (BAL).

Cytomegalovirus (CMV)

CMV has been one of the most feared infectious complications to allogeneic SCT. T-cell function is paramount in the control of CMV and recently aggressive anti-T-cell therapy such as alemtuzumab has been introduced also in lymphoma patients and to some extent in acute leukemia patients increasing the risk for CMV disease. In a study from the MD Anderson Cancer Center, the reported incidence of CMV pneumonia was 2.8% in acute leukemia patients with an increased risk during the more recent period.

Several risk factors for CMV disease have been identified. Seropositivity of the patient and/or the donor is the most important risk factor for development of disease. Other risk factors are the use of unrelated or mismatched donors, acute and chronic GVHD, and the viral load. Previously, the peak incidence of CMV disease occurred approximately days 45–60 after SCT, but recently CMV disease has been diagnosed more frequently later after transplantation. This change has been associated to ganciclovir prophylaxis and preemptive therapy and to incomplete T-cell reconstitution particularly associated with unrelated and mismatched grafts.

The symptoms of CMV pneumonia are nonspecific and no clinical or radiological feature distinguishes CMV pneumonia from pneumonia caused by other opportunistic pathogens. The radiograph often shows an interstitial pattern, but other patterns, including...
alveolar changes or diffuse nodular changes, can be seen. The diagnosis should be based on documentation of pneumonia in the appropriate clinical setting combined with identification of CMV from the lower respiratory tract usually by BAL. PCR on BAL has a very high negative predictive value, but the positive predictive value is very low. Therefore, detection of CMV only by PCR from BAL fluid is not accepted as a part of definitions of CMV disease. CMV gastrointestinal disease can occur all the way from the esophagus to the colon. The symptoms vary, depending on the location of the disease. The diagnosis should be based on biopsy material analyzed by appropriate histopathological and virological techniques. CMV DNA detected by PCR is not sufficient for the diagnosis of CMV gastrointestinal disease. Other more rare complications are CMV, encephalitis and retinitis. Symptoms of encephalitis are frequently nonspecific and the diagnosis must therefore rely on detection of CMV in the cerebrospinal fluid. PCR for CMV DNA is both sensitive and highly specific for the diagnosis of CMV CNS disease. Retinitis seems to have become more common during recent years. The diagnosis of retinitis is based on typical retinal lesions.

The mortality in CMV pneumonia after SCT remains high; more than 50% in patients treated during the last decade. There is also no difference in outcome of therapy in early and late CMV pneumonia. The standard therapy for CMV pneumonia in SCT patients is still the combination of ganciclovir and standard intravenous immunoglobulin. It should be noted that the regimen is based only on the results of noncontrolled studies. However, in recently published uncontrolled study no improvement in survival was shown with the combination compared to ganciclovir therapy given alone. New therapy options for patients failing ganciclovir and i.v. immunoglobulin are the combination of ganciclovir and foscarnet or cidofovir. Whether the addition of Ig would improve outcome of CMV pneumonia in other patients than allogeneic SCT patients is unknown. For other CMV manifestations than pneumonia, either ganciclovir or foscarnet can be used for therapy.

### Prevention of CMV infection/disease

CMV-seronegative patients should if possible be transplanted from a CMV-seronegative donor. To reduce the risk of transmission from blood products, blood products from CMV-seronegative donors or leukocyte-depleted blood products should be used. The available strategies for prevention in seropositive patients or seronegative patients receiving transplants from seropositive donors can be divided into prevention of a recurrence/a reactivation of CMV (prophylaxis) or prevention of development of disease when a reactivation has occurred (pre-emptive therapy).

Prophylaxis can be given with low potency antiviral drugs (acyclovir, valacyclovir) or high potency (ganciclovir, valganciclovir, foscarnet). Randomized studies of high-dose acyclovir or valacyclovir prophylaxis have shown efficacy against CMV in SCT recipients. Valacyclovir prophylaxis has also been recommended for patients receiving alemtuzumab therapy. Ganciclovir is effective for prevention of CMV disease. A recent randomized trial could find no difference between the efficacy of valacyclovir and ganciclovir for prevention of CMV disease in SCT patients. Valganciclovir is the prodrug of ganciclovir giving similar blood levels as i.v. ganciclovir and is currently being evaluated in SCT patients.

### Pre-emptive therapy

The pre-emptive strategy is today the most commonly used strategy for prevention of CMV disease after allogeneic SCT. Different diagnostic tests can be used for monitoring and as indicator for starting pre-emptive therapy including pp65 antigenemia, PCR, and most recently quantitative PCR. Either ganciclovir or foscarnet can be used for preemptive therapy.

Resistance to ganciclovir is usually mediated through mutations in the UL97 gene. It has been more commonly reported in AIDS and in solid-organ transplant recipients than in SCT patients. Other therapeutic alternatives are then foscarnet or cidofovir. However, only increasing antigenemia early after initiation of antiviral therapy is usually not a sign of antiviral resistance and does not necessitate change of therapy.

### Adoptive immunotherapy

Despite that major advances have been reached in CMV management, several problems still exist including the increased incidence of late CMV disease. The lack of specific immunity to CMV both regarding cytotoxic T-cell (CTL) response and helper T-cell response to CMV has been associated with a high risk for CMV disease in SCT patients. It has been shown that specific CTL can be cloned in vitro, safely be given to the patient, and their activity be detectable during follow-up. Recently new techniques such as the use of peptide pulsed dendritic cells and the tetramer technology have been
developed that might allow an easier selection of CMV specific T-cells.

**Herpes simplex virus (HSV)**

HSV can cause both local and disseminated infections in hematology patients. In many instances, the initial presentation of mucocutaneous HSV disease does not differ significantly from what is seen in the normal host. However, HSV lesions in immunocompromised patients are more invasive, heal more slowly, might shed virus for a prolonged time, and have the potential to disseminate. Manifestations in immunocompromised patients can also be atypical without classical lesions. Herpetic gingivostomatitis can cause severe mouth pain and may predispose the patient to bacterial superinfections, as well as to HSV esophagitis, and pneumonia. Patients with HSV esophagitis will present with symptoms indistinguishable from those caused by candida or CMV. HSV pneumonia is diagnosed most frequently in transplant recipients, and less frequently in other types of immunocompromised patients. Viremic dissemination of HSV can result in infection of multiple organs particularly the liver causing an aggressive hepatitis. Disseminated visceral HSV is today rare.

The therapy of established HSV disease can be given either orally or intravenously. Intravenous therapy with acyclovir should be given to all patients with disseminated HSV or suspected CNS disease. Acyclovir, valaciclovir, famciclovir, and ganciclovir, all require the viral enzyme thymidine kinase for activation. Mutant and resistant virus strains lacking this enzyme can develop. Although acyclovir has now been in extensive use for almost 20 years, there has been only a moderate increase in the detection rate of resistant strains. More recently, acyclovir-resistant HSV seems, however, to have become more common in particular after unrelated or HLA-mismatched SCT and in patients with GVHD. The recommended drug for acyclovir-resistant HSV is foscarnet, but recent case reports have described mutants resistant to both acyclovir and foscarnet. Currently, the only available antiviral drug available for treatment of double resistant HSV is cidofovir.

**Varicella-zoster virus (VZV)**

A primary VZV infection (varicella; chickenpox) is a very severe complication in severely immunocompromised patients. Owing to the high rate of seropositivity in adults, primary infections occur mainly in children. Before introduction of antiviral therapy, visceral involvement (especially pneumonitis and hepatitis) was reported in almost 50% of immunocompromised children with chickenpox with a risk of mortality up to 20%. The introduction of effective antiviral therapy primarily intravenous acyclovir has greatly improved the outcome of varicella in immunocompromised children bringing down the risk for death to <5%. Reactivated VZV infection ‘herpes zoster’ is common in patients who are suppressed in their T-cell function and the severity seems to depend on the degree of T-cell suppression. Herpes zoster is usually dermatomal but cutaneous dissemination outside the dermatome occurs. Among patients with a varicella-like syndrome, the risk of visceral involvement is 40–50%. In rare cases, visceral involvement of VZV might occur in the absence of skin lesions presenting as severe gastrointestinal pain, rapidly developing hepatitis, or meningoencephalitis.

The recommended therapy for primary varicella or disseminated herpes zoster is intravenous acyclovir 10 mg/kg (or 500 mg/m²) three times daily. For localized cutaneous herpes zoster, oral acyclovir, or famciclovir have been studied. No controlled study has been performed with valacyclovir given for treatment of a herpes zoster in immunocompromised patients but its effectiveness is likely to be similar with acyclovir.

**Epstein–Barr virus (EBV)**

EBV is frequently detected in SCT patients. However, only a few case reports have suggested that it directly causes significant end-organ disease such as meningoencephalitis and hairy leukoplaikia. However, the most important complication due to EBV is lymphoproliferative disease (EBV-LPD). The incidence of EBV-LPD varies and is generally less than 2% following allogeneic SCT, but it may increase up to 20% in patients with several risk factors such as unrelated donor SCT, T-cell depletion, ATG therapy, and other forms of intensified immunosuppression. The spectrum of disease ranges from an indolent self-limited form to a very aggressive disseminated disease. A common presentation is an infectious mononucleosis-like illness. The course is frequently rapidly progressive and is fatal in more than 75% of the patients. Lung involvement and gastrointestinal involvement are common. CNS is involved in fewer than 10% of patients with PTLD.

Early identification of patients at risk can reduce the EBV-LPD-related morbidity and mortality. EBV DNA load monitoring by PCR in peripheral blood seems to be a good predictor for EBV-LPD. The first therapeutic option against EBV-LPD is, if possible, to reduce the immunosuppression. Several antiviral drugs including acyclovir, ganciclovir, foscarnet, and cidofovir have antiviral effects against EBV. However, no study has been performed to study these drugs’ efficacy against EBV in SCT recipients. Since EBV-induced LPD result from an uncontrolled proliferation of EBV-infected B cells, therapy aimed at reduction of B-cells such as monoclonal antibodies directed against the B-cell antigen CD20 (rituximab) are today used as treatment of EBV-LPD. Another option is the use of cloned EBV specific donor T-cells. Both strategies are able to reduce the tumor masses and reduce the EBV viral load.

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**References:**


2. P Ljungman The Hematology Journal
**Human herpesvirus type 6 (HHV-6)**

HHV-6 subtype B is the cause of exanthem subitum in childhood. Since this infection is very common early in life, the rate of seropositivity in adults is very high. HHV-6 has been associated with skin rashes, interstitial pneumonia, encephalitis, hepatitis, and bone marrow suppression after SCT. HHV-6 has a propensity for the CNS and several studies have shown that HHV-6 is an important cause of encephalitis in SCT recipients. The symptoms are frequently uncharacteristic including lethargy, confusion, convulsions, and decreased consciousness but also focal neurological changes has been reported. Magnetic resonance imaging can show abnormalities but it can also be normal. The prognosis is poor unless the encephalitis is treated with antiviral drugs. Both ganciclovir and foscarnet have been reported being effective against HHV-6 meningo-encephalitis after SCT.12,13

**Respiratory viruses**

Respiratory viruses including respiratory syncytial virus (RSV), parainfluenza viruses, and influenza A and B are widespread in the community. The epidemiological situation has been shown to influence the risk for infection in hematology patients. Respiratory viruses can easily be spread nosocomially through immune competent staff, other patients, and family members. The infections can be spread through the air by droplets but are more commonly spread through the hands of staff. Thus, infection control measures are important in the control of respiratory infections.

**RSV:** The first reports on RSV infections in SCT patients showed a mortality of approximately 40%. During recent years, the reported mortality is lower. Whether this is due to early intervention or better awareness of the diagnosis and thereby identification of patients that would not develop severe disease is unknown. Less is known about the importance of RSV in other patients than SCT. In one prospective study of hospitalized adult patients with leukemia, 10% of patients with respiratory symptoms had RSV isolated and the mortality was high in patients who developed pneumonia.14 Martino et al.15 could not see any difference in the risk for lower respiratory tract infection with RSV between allogeneic SCT and nontransplant patients with hematological malignancies.

The available agents for therapy of RSV are ribavirin and either polyclonal or monoclonal antibodies. No controlled study has been reported but early intervention seems to be important. Controlled studies are needed to assess the best therapeutic option for RSV lower respiratory tract infections.

**Parainfluenza viruses:** Parainfluenza viruses can give severe and fatal infections after SCT, although the mortality seems to be lower than for RSV. The subtype most associated with severe infections is type 3. The usefulness of antiviral therapy against parainfluenza is still to be determined. The only antiviral agent available with potential effect against parainfluenza is ribavirin, but the results of uncontrolled treatment studies have varied.16,17

**Influenza viruses:** Influenza is important to consider in hematology patients. The mortality in published studies of SCT patients have been about 15%.18,19 Fatal influenza infections can occur several years after an allogeneic SCT in particular in patients with chronic GVHD. Patients with acute leukemia receiving intensive chemotherapy are also at high risk for influenza pneumonia with significant associated mortality. Patients, who are less intensively treated for malignancies, do not seem to be at such high risk for severe complications.15

The primary mode for prevention of influenza is vaccination and is generally recommended to hematology patients. The main problem is the poor responses to vaccination. Therefore, vaccinated patients might develop severe and even fatal infections. Therefore, it is important to reduce the risk for transmission of influenza to immunocompromised patients for example by vaccination of family members and hospital staff. The possibilities for prevention with antiviral agents include ribavirin, amantadine/rimantadine and the neuramidase inhibitors (zanamivir and oseltamivir). Amantadine/rimantadine has been effective in the elderly but the rate of development of resistance is rapid. There are some anecdotal data regarding the efficacy of the neuramidase inhibitors in SCT and leukemia patients. Additional studies are necessary to define the role of the new neuramidase inhibitors in immunocompromised patients.

**Adenoviruses:** Adenoviruses cause a number of clinical syndromes in immunocompetent individuals. However, more severe manifestations have also been reported. Although 51 distinct adenovirus serotypes have been identified, most human diseases is associated with only one-third of these types. Depending on the serotype, there are considerable differences in the tissue specificity and virulence. In otherwise healthy individuals, adenovirus is typically transmitted from person to person. Other possibilities are reactivation of latent virus in the recipient and and/or transmission from the donor have been suggested as possible sources of infection. The control of adenovirus seems to be mostly T-cell mediated and therefore patients having received T-cell suppressive regimens are at an increased risk for adenovirus disease. Therefore, allogeneic SCT patients and lymphoma patients receiving alemtuzumab therapy are particularly at risk, but occasional cases of disseminated adenovirus disease have been documented also in lymphoma patients treated less intensively. The risk factors for adenovirus disease in allogeneic SCT patients are still to be determined.
recipients are T-cell-depleted grafts and use of mismatched and unrelated donors. Children are at a higher risk than adults. In allogeneic SCT patients, the frequencies of adenovirus infections and disease vary greatly between different studies, but there seems to have been an increase in recent years. The most severe disease manifestations are pneumonia, encephalitis and hepatitis but hemorrhagic cystitis and gastroenteritis are more common. The mortality has varied between the different studies but seems also to have increased during recent years. The available diagnostic techniques are isolation, antigen tests, and PCR. Owing to the high mortality, monitoring by PCR has been investigated in recent years.

There is no definitive or specific treatment for adenoviral infection. It would be logical to try to improve the immune control by a decrease in the immunosuppression similar to what is recommended for EBV. Ribavirin has been used in case reports with varying outcome. Cidofovir have effect in vitro against EBV. Ribavirin has been used in case reports with immunosuppressionsimilartowhatisrecommendedforimprovetheimmunecontrolbyadecreaseintheadenoviral infection. It would be logical to try to recent years.

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Other viral infections

There have been some recent reports of severe and also fatal measles infections. The risk is most likely increased in countries where the vaccination coverage of healthy children is low. Papovavirus infections (JC/BK) have been associated to hemorrhagic cystitis after SCT and progressive multifocal leukoencephalopathy (PML) and BK-virus has been implicated in hemorrhagic cystitis and nephropathy in transplant recipients. No established therapy exists, although ribavirin has been used for measles and cidofovir for papovavirus infections.

References

6. Ljungman P, Delfiers GL, Platbecker U, Matthes-Martin S, Bacigalupo A, Einsele H et al. Cidofovir for cytomegalovirus infection and disease in allogeneic stem cell transplant recipients. The Infectious Diseases Working Party of the EBMT has performed a retrospective study of 29 patients given cidofovir therapy for proven or possible adenovirus disease with a success rate of 69%. Pre-emptive therapy against adenovirus has also been suggested. In the EBMT study, pre-emptive therapy was used in 16 patients with a success rate of 87%.

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References

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Evolution of microbial safety

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Introduction

By a series of increasingly sophisticated (and expensive) interventions, the microbial safety of transfused blood has steadily increased. In the developed world, the microbial risks of transfusion of blood components are now so low that they cannot readily be measured by direct prospective studies of recipients. Such studies would require follow-up of so many patients that they would be untenable. The microbial risks of transfusion are now usually computed from assessments of the incidence of new infections in previously negative ‘repeat’ blood donors and by measuring the ‘window period’ that is, the time at which a test marker becomes positive for a given agent following the point of infection. As tests become more sensitive, the residual risk reduces accordingly. With volunteer selected donors, the incidence of new infections will also be low, so that risk overall is now a tiny fraction of what it was in 1970 before screening for viruses began.

Characteristics of transfusion-transmitted infections (TTIs)

Classically, persistence of a microbial agent has been the characteristic hallmark of a propensity for transmission by blood transfusion. Of course, the agent needs to persist in one or more of the components of blood and it also has to be stable under the conditions of storage (22°C for platelets, 4°C for red cells and frozen, for plasma). The ‘persistence’ may embody one or more of the following phenomena: long incubation period, asymptomatic/subclinical infection, a carrier state or integration of microbial DNA in host white cells as a latent infection.

Acute infections with relatively short incubation periods and no carrier or latent state have typically only been responsible for very few transfusion transmissions, because the period of viraemia is short lived. If the rate of infection in potential donors is high, as in parvovirus B19 infections in epidemic periods, transmission may be more frequent. However, in the case of B19 infection, pathogenicity is low except in patients with aplastic anaemia. More recently, however, in North America an acute infection affecting large numbers of individuals and with significant pathogenicity is spreading from East to West. This is West Nile Fever virus (WNV) and it has provided a new type of challenge to Blood Services in the USA and Canada. They have had to rapidly implement routine nucleic acid detection testing (at epidemic periods) for this acute infection – a hitherto unknown intervention for nonpersistent infections. The main features of TTIs are summarised in Table 1. An extensive overall analysis of TTIs is provided in Chapter 16 of Mollison, Engelfriet and Contreras.

Interventions to reduce microbial risk

The key interventions to reduce microbial risk are:

- donor education and selection to exclude those at risk of contracting TTIs
- development of a panel of nonremunerated volunteer donors
- stepwise introduction of increasingly sensitive serological testing of donations for antibody and/or antigen, for an expanding range of TTIs
- development of nucleic acid amplification tests
- leucodepletion of blood components
- initiatives to reduce bacterial risk
- options for inactivating microbial agents in blood components are now becoming available.

Selection and volunteer donors

Despite counter arguments from commercial collectors of blood from paid donors, both the prevalence and the incidence of all the TTIs has typically been tenfold higher in paid, compared with volunteer unremunerated, blood donors. Indeed a recent re-examination of this issue has confirmed this situation.
The importance of educating donors about the risks from TTIs and requesting that candidate donors who may be at risk of contracting TTIs refrain from donation is an extremely cost-effective basis for reducing the microbial risks from blood. The removal of the majority of the HIV transmission risk in California at the onset of the AIDS epidemic, before specific testing and simply by appropriate donor selection/exclusion, is a clear example of this.
infection. Generally, the yield of ‘extra’ positive donors detected by NAT is very small and because of the high costs of NAT (including royalty fees) the cost–benefit ratios for this technology are far higher than for serological testing. A generalised course of infection illustrating the sequence of detectability for genome, antigen and antibody is represented by Figure 2.

Other interventions to reduce microbial risk

- Leucodepletion of blood components was introduced in the UK to reduce the risk of transmission of vCJD by transfusion. It will also reduce the risk from white cell-associated viruses, such as CMV, HHV8 and HTLV1 and II.
- Interventions to reduce bacterial risk can be summarised as enhancing donor arm cleansing, diverting the first 20 ml of the blood donation (to ‘flush out’ contamination of the venepuncture needle by skin commensals) and bacterial testing of platelet preparations.
- Techniques to inactivate microbial agents in blood components are now becoming available. Such ‘pathogen reduction’ (PR) systems are analogous to the highly successful viral inactivation methods for fractionated plasma products. However, because of the labile nature of platelets and red blood cells, they are more technically demanding. Currently, therefore, they are generally labour intensive, time consuming and very expensive. Until the full inventory of components can be treated (preferably at the ‘whole blood unit’ stage) PR will not be able to assume a more cost-effective ‘base strategy’ for microbial safety rather than being just one more expensive incremental safety intervention. This pertains particularly at a time when blood is already so safe that extra safety yields are likely to be very small and therefore very hard to demonstrate by prospective study. However, one benefit of PR would be that most (if not all) ‘emerging’ microbial threats to blood safety would be manageable proactively rather than retrospectively, that is, only after transmissions have been documented.

Approximate timescale for availability of routine safety interventions

The availability and routine introduction of some of the major microbial safety interventions have evolved as follows:

1958: Flocculation tests to detect syphilis antibody
1970: First-generation (immunodiffusion) assays for HBsAg
1985: Assays for anti-HIV1
1986: Anti-HBc testing in USA as a ‘surrogate’ marker for non-A, non-B hepatitis, in combination with estimation of alanine aminotransferase levels
1986/7: Anti-HTLV-I assays that cross-reacted with HTLV-II introduced in some countries
1991: First-generation anti-HCV assays, following the successful cloning of HCV
1992: Anti-HIV2, in combined assays with anti-HIV1
1996: HIVp24 antigen assay in USA. Subsequently, anti-HIV1 plus 2 with HIV1Ag ‘combined’ assays became available in Europe
1998: Leucodepletion of blood components
1999: Introduction of NAT for HCV RNA. Initially, this was performed on pools of several hundred samples to satisfy requirements for plasma fractionation. Pool sizes then reduced to allow components to be NAT tested with timely release of product
NAT for HIV, HBV and other viruses are now available and used routinely in some countries
HCV antigen testing also became available
2002: Pathogen reduction techniques for various components.

The exact timing and range of tests, techniques and agents tested for, and also other specific interventions implemented, vary considerably from country to country. This applies to developed countries, but even more so in developing countries that lack the resources to implement many of these increasingly expensive interventions. The interacting microbial safety options are summarised diagrammatically in Figure 3.

Residual microbial risks of transfusion

Assessing the residual microbial risk of transfusion is now impracticable by prospective study of recipients because these risks are so small in developed countries. One approach is national ‘haemovigilance’ to collate adverse reactions to transfusion, such as the UK Serious Hazards of Transfusion (SHOT) monitoring scheme, which includes (symptomatic) microbial transmissions. Another approach pioneered by Schreiber et al. computes residual risk per donation on the basis of the incidence of infection, by analysing the rate of seroconversions in established donors, together with the window period of a given agent when tested with a particular assay. When such methods (together with the contribution to risk from testing errors) were applied to English data, the current residual risks per donation were 1 in 900,000, 1 in 8 million and 1 in 30 million for HBV, HIV and HCV, respectively.

Conclusion

The relentless evolution of techniques, technology and interventions to reduce the microbial risks of blood transfusion has (in the developed world at least) made blood transfusion currently one of the safest of medical
Interacting Microbial Safety Options

Figure 3 Interacting microbial safety options.

practices. But ‘absolute zero risk’ is a goal that can only be approached asymptotically. The 10–20% rates of post-transfusion hepatitis in the USA in 1970, the headlines surrounding post-transfusion HIV in the 1980s and HCV in the 1990s are now behind us because of the sophisticated level of interventions applied to the reduction of microbial risk from transfusion. Nevertheless, concerns continue to arise because of ‘emerging’ agents such as WNV in North America and the threat from severe acute respiratory syndrome (SARS) that, fortunately, has so far failed to materialise. The WNV threat in North America was managed (albeit retrospectively), by the rapid introduction of NAT for detection of the virus in blood donations. Pathogen reduction might offer a prospective approach to guard against emerging agents; but it would not prevent infection with prions. The recent possible transmission of vCJD13 to a patient who developed the disease 6 years after receiving red cells from a donor who developed vCJD 3 years after the donation, illustrates the point that we can never be complacent. We must continue to be vigilant and strive to evolve effective, yet practicable and balanced, approaches to the provision of blood free from infection.

References

11 Schreiber GB, Busch MP, Kleiman SH, Korelitz JJ. The risk of transfusion-transmitted viral infections. The


Haemovigilance procedure in transfusion medicine

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In the overall safety concept in blood transfusion it is undisputed nowadays that haemovigilance detains a key role.

Haemovigilance came into life in the beginning of the 1990s; the term was probably created in France and has Greek and Latin roots: ‘haema’ = blood and ‘vigi-lans’ = paying a particular attention to.

The primary aim of haemovigilance is to assure surveillance of the blood transfusion activities, to collect data on (serious) sequelae of blood transfusion and to contribute in informing health policy, in improving transfusion standards, in assisting the formulation of guidelines in the field and in increasing safety and quality of the entire transfusion process.

Definitions

The complexity of the situation starts already with the definition of haemovigilance in the different countries: as simple as traceability to very elaborate definitions.1,2

The following definition is used in several countries around the globe:

Haemovigilance is a set of surveillance procedures covering the whole transfusion chain (from the donation of blood and its components to the follow-up of recipients of transfusions), intended to collect and assess information on unexpected or undesirable effects resulting from the therapeutic use of labile blood products, and to prevent the occurrence or recurrence of such incidents.

In the European Blood Directive 2002/98/EC, the following definitions are given:

• Haemovigilance: a set of organised surveillance procedures relating to serious adverse or unexpected events or reactions in donors or recipients, and the epidemiological follow-up of donors;

• Serious adverse event: any untoward occurrence associated with the collection, testing, processing, storage and distribution of blood and blood components that might lead to death or life-threatening, disabling or incapacitating conditions for patients or which results in, or prolongs, hospitalization or morbidity;

• Serious adverse reaction: an unintended response in donor or in patient associated with the collection or transfusion of blood and blood components that is fatal, life-threatening, disabling or incapacitating or which results in, or prolongs, hospitalisation or morbidity.

For the following occurrences commonly agreed definitions in relation to haemovigilance are necessary, but still pending: events, incidents, effects, side effects — undesirable/unexpected, reactions, accidents, errors, near-misses, etc.

The types of blood components used should be defined according to the Council of Europe: Guide on the preparation, use and quality assurance of blood components; Recommendation No. R(95)15, Part C: Blood components.3

The following aspects should be handled according to the European Haemovigilance Network (EHN, Internet site: www.ehn-org.net) and the Council of Europe: Guide on the preparation, use and quality assurance of blood components; Recommendation No. R(95)15, Chapter 31.3

• grading for severity: 0 = no sign; 1 = immediate signs without vital risk and full resolution; 2 = immediate signs with vital risk; 3 = long-term morbidity; 4 = death of the patient.

• grading for imputability: 0 = no relationship; 1 = possible; 2 = likely; 3 = sure.

• clinical and biological signs:
  ○ immediate reaction (haemolysis; non-haemolytic febrile transfusion reaction/ NHFTR; allergic reactions, like rash, erythema, urticaria, anaphylaxis; transfusion related acute lung injury/ TRALI, etc)
  ○ delayed reaction after transfusion (haemolysis; graft-versus-host disease/ GvHD; post-transfusion purpura/ PTP, etc)
  ○ microbiological/viral transmission
  ○ allo-immunisation (against antigens of RBC, WBC, PLT, etc)
  ○ incorrect blood component transfused/ IBCT
  ○ others.

In the interest of standardization, data should be reported according to the EHN-Standardised Reporting Form (see Appendix A).
The blood transfusion chain

Haemovigilance, as a surveillance system based on ongoing and standardised collection and analysis of data, pursues several objectives:

- monitoring the prevalence and incidence of infectious markers in blood donors
- compiling adverse events/incidents that are suspected or have been confirmed to be associated with blood collection (relating to the donors) or transfusion of labile components (relating to the recipients), including transfusion errors and product-related side effects
- documenting confirmation of the transfusion of blood components to patients
- offering rapid alert/early warning procedures.

The blood transfusion chain hereby covering the entire blood transfusion chain and the respective activities.

General concept and the components of the system

As can be concluded from the definitions and the list above, haemovigilance should cover the entire blood transfusion chain: conceptually it stretches over all the activities, from the donor to the recipient ('from-vein-to-vein').

Some important prerequisites for the success in establishing, running and maintaining a fully functional haemovigilance system may be summarised as follows:

- legal framework in place
- common definitions agreed
- standardised reporting used
- continuous budgeting and financing guaranteed
- central evaluation site built up
- rapid alert/early warning organised
- culture of professionalism established
- Hospital Transfusion Committees working
- mechanisms for corrective and preventive actions introduced
- International/European cooperation anticipated.

It is important to point out that there are interfaces with different other vigilance systems, for example, pharmaco-, materio-, reacto-, and biovigilance. If in theory the limits of the different vigilance systems are defined, in practice it is far from so: there is considerable overlapping and interference.

In this context, it may be useful to come up with an extended version of the classical blood chain. According to this model, there are different (potential) actors in the system: the industry, the blood centres, the hospitals, the competent Authorities, etc.

The key actors should be ready to collaborate in a constructive and coordinated way: if so, haemovigilance can fulfil its overall aim, for example, to increase safety and quality of the blood transfusion process in the best interest of patients with a need for labile blood components.

The role of industry: The manufacturers of materials, disposables, reagents, equipments, etc are serving blood centres and hospitals. Regulatory requirements (community, national) and the post-marketing procedures of the companies have set a strong tool to collect and compile data directly and indirectly related to blood transfusion: in the future the input of industry into haemovigilance needs to increase and speed up.

The role of the blood organizations and the blood centres: The blood centres/blood banks are the users of materials, disposables, reagents, equipments, etc manufactured by industry. At the same time, they are also the producers of labile blood components of all types and the providers of transfusion-related services. In this perspective, they play a key role in haemovigilance, figuring on one hand as users and on the other hand as producers.

The role of the clinical segment (hospitals, physicians, paramedics, transfusion committees, etc): If the focus of haemovigilance is mainly on the patient with a blood transfusion need, then the clinical side of the transfusion chain is of paramount importance in the surveillance. Being at the frontline, physicians and paramedics are at the primary site of blood transfusion: they are in a position to detect and report incidents, events, side effects, reactions, accidents, errors, near-misses, etc. The Hospital Transfusion Committees play a crucial role in drafting guidelines, administering training, ensuring peer review, auditing, supervising the reporting, initiating corrective/preventive actions, etc.

The role of the Authorities (National, Community, etc): The competent Authorities also have an important part in haemovigilance. In some countries, the role of the competent National Authorities (cNA) may have to be developed further in the future: legislating, budgeting, inspecting and accrediting, above all surveilling (directly or by delegation). Each and every haemovigilance system (in whatever form it exists) needs the back-up of the cNA. In some countries the cNA is directly organising and running the system, in others the cNA is offering the platform for operations, and in others its role is limited to ‘accompanying’ an established haemovigilance system. Anyhow, the involvement of cNA needs to become clearer and sometimes more efficient.

Procedure

Haemovigilance is a ‘quality process’ with the aim to improve quality and increase safety of blood transfusion. As each and every process it has an ‘input’ (= transfusion of a patient or intent to do so) and an ‘output’ (= corrective and/or preventive actions and follow-up on them).

As most processes the haemovigilance process has different steps, has interfaces and includes critical elements.

Taking into account that haemovigilance covers and surveils all activities of the blood transfusion chain (to the same degree those related to donors and to recipients), the procedure is very similar for both branches and hereafter is described the general process...
in relation with the recipient of a labile blood component.

In practice and generally speaking, the different steps of this quality process are:

- recognition/assessment of an occurrence (deviating from the ‘normal’)
- reporting (according to criteria set out and using a standard reporting form)
- collection of data (following written instructions)
- compilation (using a predefined matrix)
- evaluation (according to agreed techniques)
- conclusions (fed back to those concerned and published) actions (corrective and/or preventive) and follow-up on them.

In principle, the outline of this process is similar at all levels where haemovigilance applies: wards, hospitals, blood centres, competent authorities, manufacturers, etc and the primary actors in this process are physicians, pharmacists, nurses, medical technicians, etc.

It is of paramount importance to ensure that ascending and descending predefined and established communication channels exist between the different levels.

In order to make this process work at different levels, at different sites, with many different people involved, it is essential that close and constructive cooperation is established between the different actors. For this purpose, it is important to settle the organizational aspects, to define the respective responsibilities and mandates, to increase sensitivity in a ‘no blame environment’, to have clear written procedures, to offer adequate and continuous training, etc.

As a quality process, haemovigilance needs to be deeply and solidly embedded into the quality management systems (QMS) of the different establishments: in the blood centres, in the manufacturing companies, etc but also in the hospitals.

In order to guarantee the final result (safe and efficient blood transfusion of the patient) there should be no exception to this rule, at no stage and for none of the activities of the blood transfusion chain.

Europe

As diverse as the organization of blood transfusion programmes/services in Europe, is the approach when it comes to haemovigilance. In Europe in the context of blood transfusion, reference is generally made either to the European Union (EU) or to the Council of Europe (CoE): this simple approach already shows significant differences.

European Union (EU)

For blood transfusion in the European Community, Directive 89/381/EEC ‘relating to proprietary medicinal products and laying down special provisions for medicinal products derived from human blood or human plasma’ is a milestone in the sense that it establishes legal provisions for plasma derivatives: it rules that they are to be considered as medicinal products and as such come under Community legislation on medicines; this is true also for the vigilance aspects and in that regard they are covered by pharmacovigilance (as required by Directive 75/319/EEC under article 29a). Blood products are excluded ‘expressis verbis’ from these provisions and therefore do not fall under pharmacovigilance.

The European Council in its Resolution of 2 June 1995 on blood safety and self-sufficiency in the Community, invited the European Commission to submit appropriate proposals in the framework of the development of a blood strategy: it was proposed to establish of a haemovigilance system based on existing networks for the collection of epidemiological data in relation to the blood transfusion chain.

A Colloquium was organised in Adare (Ireland) in September 1996 on ‘Blood Safety and Self-Sufficiency: an Agenda for the European Community’. Six areas of action for the European Community were identified: one of them was haemovigilance. It was intended to address the subject at Community level, upon a proposal by the European Commission, which launched end of 1995 a call for tender for a feasibility study for a haemovigilance network within the European Community: the HAEMAN Consortium was constituted in 1997, the MS met in 1998 and a report on the feasibility was published in 1999 by the European Commission’ (at the time DG V/F/4 was in charge of blood issues within Employment, Industrial Relations and Social Affairs–Public Health and Safety at Work: Communicable, rare and emerging diseases): no further action was taken at that time.


In the legal provisions there are two operating articles dealing with haemovigilance:

‘Article 14: Traceability
1. Member States shall take all necessary measures in order to ensure that blood and blood components collected, tested, processed, stored, released and/or distributed on their territory can be traced from donor to recipient and vice versa. To this end, Member States shall ensure that blood establishments
Member States shall ensure that:

1. any serious adverse events (accidents and errors) related to the collection, testing, processing, storage and distribution of blood and blood components which may have an influence on their quality and safety, as well as any serious reactions observed during or after transfusion which may be attributed to the quality and the safety of blood and blood components are notified to the competent authority.

2. blood establishments have in place a procedure accurately, efficiently and verifiably to withdraw from distribution blood or blood components associated with the notification referred to above.

3. Data needed for full traceability in accordance with this Article shall be kept for at least 30 years.

‘Article 15: Notification of serious adverse events and reactions’

1. Member States shall ensure that:

- any serious adverse events (accidents and errors) related to the collection, testing, processing, storage and distribution of blood and blood components thereof enabling full traceability to the donor as well as to the transfusion and the recipient thereof. The system must unmistakably identify each unique donation and type of blood component. This system shall be established in accordance with requirements referred to in Article 29(a). With regard to blood and blood components imported from third countries, Member States shall ensure that the donor identification system to be implemented by blood establishments permits an equivalent level of traceability.

2. Member States shall take all necessary measures in order to ensure that the system used for the labelling of blood and blood components collected, tested, processed, stored, released and/or distributed on their territory complies with the identification system referred to in paragraph 1 and the labelling requirements listed in Annex III.

3. Data needed for full traceability in accordance with this Article shall be kept for at least 30 years.’

The Member States of the European Union (EU) – the national levels

The situation in relation to blood transfusion and haemovigilance is quite heterogeneous and in constant evolution, as already mentioned and shown in Table 1–4.

A similar picture exists when it comes to the data generated by haemovigilance, as shown in Tables 1–4 (situation as in 2001).

All reported incidents/events

In the EU there is a wide range when it comes to the incidence data, as high as 325/100 000 blood components distributed (in France) and as low as 7–8/100 000 blood components distributed (in Denmark and the UK). It should be stressed that in France it is mandatory to report ALL incidents, of ALL severity grades and independently whether the transfusion is the cause of the reaction or not.

Non-haemolytic febrile transfusion reactions (NHFTR): As most of the observed respectively reported incidents/events are in fact NHFTR it is not surprising that France has the highest incidence, despite the fact that universal leucodepletion (u-LD) of all labile blood components is mandatory in France since April 1998 and that the acceptable limit for residual leucocyte-contamination in the blood components is quite below the threshold of the Council of Europe (≤1.0 × 10^5/ unit). As most of the NHFTR show mild signs, Denmark and the UK have very low incidence for NHFTR because their systems only collect cases with more severe symptoms.

Incorrect blood component transfused (IBC-T): Only the systems in Denmark, Ireland and the UK require notification of events/incidents linked to ‘wrong blood to wrong patient’. The data and the
conclusions in the context of IBCT are alarming, as they identify the real weaknesses of the blood transfusion chain and point out where the real risk are to be found in blood transfusion nowadays: they are not so much in the production segment, but much more in the clinical segment where blood components are used.

Particularities: It should be noted that in Germany (and also partially in Austria), there exists a particular situation in relation to the administration of blood products: in Germany, the labile blood components are considered to be medicinal products and come under the German Drug Law. According to the national legal provisions covering medicines, side effects have to be reported according to the rules of pharmacovigilance. Nevertheless, it should be mentioned that the recent German Transfusion Law also established haemovigilance as a separate entity.

Some longitudinal results from existing surveillance systems: The following examples illustrate once again the diversities and differences existing in the different Member States of the European Union.

In France, where the haemovigilance system is centralised and nationwide, with a legal obligation to notify in written form each and every side effect in relation to a blood transfusion (grades 0–4), the number of notifications is important. In 1997, there have been 7604 reports; in 1998, 7725 reports; in 1999, 7089 reports; in 2000, approximately the same number as in 1999 and in 2001, 7452 reports, with an estimated 2.5–2.8 mio. blood components transfused every year.11–16

In the UK where haemovigilance is a national scheme between professionals (SHOT, Serious Hazards of Transfusion), on a voluntary basis, covering transfusion reactions of grades 2–4, the number of notifications is much lower than in France. In 1996/1997 there have

Table 1 Situation in relation to blood transfusion and haemovigilance (situation as in 2001, updated from Faber9)

<table>
<thead>
<tr>
<th>(a) Member State (MS) of the EU</th>
<th>Population (in mio.)</th>
<th>Blood transfusion organisation</th>
<th>Operational responsibility</th>
<th>No. of blood banks</th>
<th>Haemovigilance established</th>
<th>Rapid alert</th>
</tr>
</thead>
<tbody>
<tr>
<td>Austria</td>
<td>7.8</td>
<td>Regional</td>
<td>Red Cross</td>
<td>7</td>
<td>Yes</td>
<td>M*</td>
</tr>
<tr>
<td>Belgium</td>
<td>10.0</td>
<td>Regional</td>
<td>Red Cross</td>
<td>37</td>
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<td>V</td>
</tr>
<tr>
<td>Denmark</td>
<td>5.3</td>
<td>Hospital</td>
<td>Public Health</td>
<td>88</td>
<td>Yes</td>
<td>V</td>
</tr>
<tr>
<td>Finland</td>
<td>5.2</td>
<td>Central</td>
<td>Red Cross</td>
<td>5</td>
<td>Yes</td>
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</tr>
<tr>
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<td>Central</td>
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</tr>
<tr>
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<td>116</td>
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<td>M*</td>
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<tr>
<td>Greece</td>
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<td>Public Health</td>
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<td>Yes</td>
<td>V</td>
</tr>
<tr>
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<td>Regional</td>
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<td>2</td>
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<td>V</td>
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<tr>
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<tr>
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<td>Red Cross</td>
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<td>V</td>
</tr>
<tr>
<td>Netherlands</td>
<td>14.8</td>
<td>Regional</td>
<td>Public Health</td>
<td>4</td>
<td>Yes</td>
<td>V</td>
</tr>
<tr>
<td>Portugal</td>
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<td>Mixed</td>
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<td>48</td>
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<tr>
<td>Spain</td>
<td>38.4</td>
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<td>Public Health</td>
<td>142</td>
<td>No</td>
<td>V</td>
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<tr>
<td>Sweden</td>
<td>8.6</td>
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<tr>
<td>UK</td>
<td>58.8</td>
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<td>Public Health</td>
<td>20</td>
<td>Yes</td>
<td>V</td>
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</table>

<table>
<thead>
<tr>
<th>(b) Member State (MS) of the EU</th>
<th>Mandatory (M)/ voluntary (V)</th>
<th>Institution in charge</th>
<th>Types of reaction to report</th>
<th>Standardised report/form</th>
<th>Epidemiology of donors</th>
<th>Rapid alert</th>
</tr>
</thead>
<tbody>
<tr>
<td>Austria</td>
<td>M</td>
<td>ÖBIG</td>
<td>All</td>
<td>Simple</td>
<td>Red Cross</td>
<td>+</td>
</tr>
<tr>
<td>Belgium</td>
<td>M</td>
<td>?</td>
<td>No</td>
<td>No</td>
<td>Red Cross</td>
<td>+</td>
</tr>
<tr>
<td>Denmark</td>
<td>V</td>
<td>DART</td>
<td>Infection</td>
<td>No</td>
<td>National</td>
<td>+</td>
</tr>
<tr>
<td>Finland</td>
<td>V</td>
<td>Red Cross</td>
<td>No</td>
<td>No</td>
<td>Red Cross</td>
<td>+</td>
</tr>
<tr>
<td>France</td>
<td>M</td>
<td>AFSSAPS</td>
<td>All</td>
<td>Detailed</td>
<td>National</td>
<td>+</td>
</tr>
<tr>
<td>Germany</td>
<td>M</td>
<td>PEI</td>
<td>All</td>
<td>Simple</td>
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<td>+</td>
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<tr>
<td>Greece</td>
<td>V</td>
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<td>No</td>
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<td>National</td>
<td>+</td>
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<tr>
<td>Ireland</td>
<td>V</td>
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<td>Severe</td>
<td>Detailed</td>
<td>National</td>
<td>+</td>
</tr>
<tr>
<td>Italy</td>
<td>V/M</td>
<td>? SIMTI</td>
<td>All</td>
<td>No</td>
<td>National</td>
<td>?</td>
</tr>
<tr>
<td>Luxembourg</td>
<td>V</td>
<td>Red Cross</td>
<td>All</td>
<td>Simple</td>
<td>Red Cross</td>
<td>+</td>
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<td>V</td>
<td>TRIP</td>
<td>All</td>
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<td>National</td>
<td>+</td>
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<td>M?</td>
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<td>National</td>
<td>+</td>
</tr>
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<td>? SSTM</td>
<td>No</td>
<td>No</td>
<td>National</td>
<td>?</td>
</tr>
<tr>
<td>Sweden</td>
<td>V/M</td>
<td>? SATM</td>
<td>All</td>
<td>No</td>
<td>National</td>
<td>?</td>
</tr>
<tr>
<td>UK</td>
<td>V</td>
<td>SHOT</td>
<td>Severe</td>
<td>Detailed</td>
<td>National</td>
<td>+</td>
</tr>
</tbody>
</table>

*Organisation: central = centralised; regional = according to regions; hospital = blood banks are hospital based.
*Haemovigilance established = in place and functioning, not necessarily required by law.
*Blood products are classified as medicinal products (drugs), covered by pharmacovigilance.
*Traceability: central = under the responsibility of a single type of institutions; shared = under the responsibility of more than one type of institutions; Severity of transfusion reactions: for example, grade 0–4 (0 = no sign; 1 = absence of vital threat; 2 = vital threat in the long term; 3 = immediate vital threat; 4 = death of the patient); Epidemiology of donors: means mainly data on infectious markers; Rapid alert: + = exists; ? = no reliable data or no information at all.

**European Haemovigilance Network (EHN)**

Aware of these differences and difficulties, five countries took the initiative back in 1998 to work together in the field of haemovigilance: Belgium, France, Luxembourg, Portugal, The Netherlands. The first meeting was organised in Paris on 10 February 1998: the European Haemovigilance Network (EHN) was born. Later on, Denmark, Greece, Ireland, Finland and the United Kingdom joined the EHN and Switzerland, Norway, Croatia and Canada were adopted as associate members. Several other countries (inside and outside of the EU) have shown their interest in cooperating with the above mentioned.

The objectives of the EHN are as follows:
- facilitating close contacts in the field of haemovigilance between countries in Europe
- allowing rapid and efficient exchange of reliable information and of experience
- maintaining a rapid alert system
- developing joint activities (like, organising European Seminars on Haemovigilance).

**European Haemovigilance Seminars:** The first European Seminar on Haemovigilance was organised by France and took place in Bordeaux, November 1997, followed by the Seminars in Lyon, November 1998, in Montpellier, September 2000, in Athens, December 2001, in Amsterdam, February 2003 and in Zurich, February 2004.

**Internet site of EHN:** www.ehn-orgnet: At the same time, an Internet site was developed and put in place: the website contains a rapid alert module. It
allows efficient and quick exchange of information on different matters in relation to haemovigilance. The website is structured in a public domain and a protected (private) domain, the latter is housing the rapid alert: this section can only be acceded by authorised persons, using a password. The address of the site is: www.ehn-org.net, it is protected by a certificate system.

The Home Page of the website gives access to information on the EHN, contact addresses, the National Menus of the participating countries and the Rapid Alert (protected by password). The general section of each National Menu contains information on the organisation of the blood transfusion system as well as detailed data on donors, donations, blood components, etc. The section on haemovigilance contains specific information and data in relation to the surveillance system in the selected country. The last section of the National Menu presents news, developments and possible problems in the field of blood transfusion.

Rapid alert system (RAS): This is an information channel for very quick diffusion of important information in relation to emerging threats, of whatever kind they may be. It works via fax, e-mail and website (protected domain). The contact person in one member country of the EHN is informed that in his country a problem has emerged, for example through the national haemovigilance system or by other means. This key person analyses the information and decides whether this information is diffused to the contact persons in the other countries, members of EHN. It is the responsibility of the respective contact persons in the other countries to take up the information, evaluate them and decide upon the actions in their country. In the past, the RAS has been used on several occasions, like:

- appearance of clusters of clinical signs after transfusion
- hidden or apparent defects of disposable material used in transfusion (like, leakages of filter housings, holes in collection bags, defects in apheresis material, etc)
- difficulties with reagents (lack of performance in terms of sensitivity or specificity, etc)
- problems with equipment
- other instances.

Exchange of information: Another objective of the EHN is the sharing of data between the member countries concerning

- organisation of the blood transfusion system with emphasis on haemovigilance
- collection, preparation, usage of blood products
- epidemiologies of donors and recipients
- incidents in the context of transfusion.

In order to exchange information and to compare results (for example in relation to haemovigilance), standardisation is important. Therefore it was crucial to find consensus definitions for some of the key parameters.

The difficulties and the problems

There are numerous difficulties when it comes to haemovigilance, at different levels: institutional, regional, national, European/International, etc: none of these problems could not be overcome.23 In general, there is still a deficit in relation to haemovigilance when it comes to common definitions, terminology, standardised reporting, uniform matrix, etc. With a few exceptions, in Europe there are still organisational problems, funding shortages, unclear mandates, undefined responsibilities, low sensitivity, insufficient training, hesitation to move forward by implementation of strong actions, etc.

In several countries across Europe, haemovigilance is really established and working. But not in every European country a national haemovigilance system is in place: in some countries they are required by law, officially established but not functioning; in others they are not mandatory by law, nevertheless established, but generating poor results (underreporting).

At Community level, the intention was expressed to rely on existing national haemovigilance systems and to bundle these activities for community purposes; up until now there is no official structure in place at this top level and the longer this situation will exist the greater the risk that national systems are drifting apart and it will be increasingly difficult to bring them together.

The solutions and the future

With the coming into force of the European Blood Directive, haemovigilance is becoming mandatory and therefore an obligatory element of blood safety and quality. The trend is towards comprehensive national systems, designed in a way that international cooperation and exchange of information is possible. A strong network in European haemovigilance will be vital: common definitions, standards, forms, exchangeability of information, rapid alerts and early warnings are still to come.

Mechanisms of corrective and preventive actions at Community level are to be developed. It would also make good sense to rely on existing European initiatives that have proven to be functioning and to generate results, not for the least because they are working ‘bottom-up’ and have input from experts in the field. The players in the blood transfusion chain will see their respective roles valorised and their input into the system will gain rapidly in importance. The problem of different existing vigilance systems interfering with blood transfusion needs to be resolved: spinning of or bridging and bundling will be crucial issues when it comes to modern, advanced haemovigilance, especially at Community level.
Conclusions

Haemovigilance is indispensable when it comes to safety and quality of blood transfusions. In relation with haemovigilance significant differences exist for the moment in the countries around the world, in terms of definition, organisational schemes, state of development, impact and efficiency, cooperation, etc: each country should have an established system with national coverage.

For the European Community, the European Blood Directive requires haemovigilance in each Member State: it is the intention to rely on existing/building-up national systems. In order to bundle the effort at Community level it would make sense to make an appeal to an existing initiative in this field that has proven that efficient cooperation is possible: the European Haemovigilance Network (EHN) has played a major role to bring this important item forward and to develop it into an efficient tool intended to increase safety and quality in European blood transfusion.

Acknowledgements

We thank all those who provided national data on haemovigilance: Hans Kurz, Austria; Charles Salpeteur, Belgium; Jan Jorgensen, Denmark; Brigitte Keller-Stanislawski, Germany; Constantina Politis, Greece; Jukka Koistinen and Tinja Mäki, Finland; Bernard David, France; Emer Lawlor, Ireland; Giuseppe Aprili, Italy; Cees van der Poel and Paul Strengers, The Netherlands; Jorge Condeco, Portugal; Carmen Martin-Vega and Elena Moro, Spain; Olafur Akerblom, Sweden; Elizabeth Love and Dorothy Stainsby, United Kingdom.

References

Appendix A
A Standardised Reporting Form of the EHN (European Haemovigilance Network)

<table>
<thead>
<tr>
<th>Patient</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>sex: M □ F □; age: ...</td>
<td>file number: ...................</td>
</tr>
<tr>
<td>Date of transfusion: .../.../...</td>
<td></td>
</tr>
<tr>
<td>Delay of reaction after transfusion: ...min / ...hours / ...days / ...years</td>
<td></td>
</tr>
<tr>
<td>(other information are confidential and appear only on the hospital form)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Transfused component</th>
<th></th>
</tr>
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<tbody>
<tr>
<td>Type: □ RBC □ platelets □ plasma □ granulocytes</td>
<td></td>
</tr>
<tr>
<td>□ alllogeneic □ autologous</td>
<td></td>
</tr>
<tr>
<td>Preparation: □ whole blood processing □ apheresis</td>
<td></td>
</tr>
<tr>
<td>characteristics: □ leuocdepleted □ irradiated □ CMV negative</td>
<td></td>
</tr>
<tr>
<td>□ plasma-depleted □ SD-treated □ quarantined</td>
<td></td>
</tr>
<tr>
<td>□ antigen matched □ other (specify): ...................</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Place of distribution</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>□ hospital □ blood bank</td>
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</table>

### Symptoms and clinical / biological signs of reaction

#### Signs before tx after tx
- temperature °C
- blood pressure (mm Hg)
- pulse rate
- haemoglobinuria
- cardiac arrhythmia
- other: ....................

#### Symptoms (1)
- discomfort
- chills
- itching
- urticaria
- redness
- rash
- jaundice
- other: ...........

#### Symptoms (2)
- lower back pain
- chest/abdominal pain
- nausea/vomiting
- dyspnea
- acute renal failure
- shock
- loss consciousness
- other: ...................

### Conclusions or Syndrom (only one for each report):

##### Immunological
- haemolysis ABO
- haemolysis irreg ab
- immunisation:
  - □ RBC □ Granulocyte
  - □ HLA □ IgA
  - □ HPA
- PTP – post-tx purpura
- allergy (mild)
- anaphylaxis
- TRALI – tx related lung injury

##### Infections
- component bacterial contamination
  - germ(s): ......................
- HIV
- HBV
- HCV
- CMV
- other agent: ....................

##### Others
- febrile non-haemolytic tx reaction
- TA-GVHD graft-versus-host disease
- pulmonary oedema (cardiac failure, overload)
- haemosiderosis

### Severity
- 0. no effect
- 1. immediate, no vital
- 2. immediate, vital
- 3. long term morbidity
- 4. death

### Imputability
- 0. excluded
- 1. possible, dubious
- 2. likely, probable
- 3. certain, proven

### Other relevant clinical information:
(e.g. prior condition of the patient)

<table>
<thead>
<tr>
<th>Patient outcome:</th>
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### Transfusion process

<table>
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</thead>
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<tr>
<td>□ op.theatre, □ Intensive Care Unit - ICU, □ medical, □ paediatric,</td>
<td></td>
</tr>
<tr>
<td>□ outpatient clinic, □ other:</td>
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</table>

<table>
<thead>
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<th>Time:</th>
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</tr>
</thead>
<tbody>
<tr>
<td>□ working hours, □ night shift, □ week end</td>
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<table>
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<tr>
<th>Incorrect component transfused</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>yes □, no □</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Associated involvement</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>□ materiovigilance □ pharmacovigilance □ reagent-laboratory failure</td>
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</tbody>
</table>
Safety aspects of blood transfusion and European legislation

Jukka LK Koistinen

1Red Cross Finland, Blood Service, Helsinki, Finland


Background

Blood grouping was the most important safety measure in blood transfusion from the beginning of the 19th century until the discovery of hepatitis B virus and appearance of test methods for it in the beginning of 1970s. Then, prevention of transfusion-transmitted infections became the most important safety aspect and seems to remain such. Its importance has been emphasized by the publicity the transfusion-transmitted infections have received in the press. The mistakes and oversight revealed and resulting legal consequences to blood establishments and regulators seen in several countries added extra fuel to the fire of publicity.

National provisions have regulated the blood grouping and testing for transmissible agents, but common European legislation has been missing. Common recommendations and guidance for practice have been given by the World Health Organization and Council of Europe, but they have been binding to the member countries only if officially ratified by the governments.

Role of the Council of Europe

In December 1958, a European agreement on the exchange of therapeutic substances of human origin was published,¹ which referred specifically to human blood and its derivatives. The purpose of the agreement was to make blood and blood derivatives available to other parties of the agreement in case of urgent need at a cost incurred only by the collection, processing and carriage of the derivatives. The agreement included a protocol² that gave specifications of the minimum requirements for the properties of the therapeutic substances and regulations on labelling, packing and dispatching. There were specific provisions on: (1) whole human blood; (2) human red cell concentrate; (3) dried human plasma; (4) human albumin and human plasma protein fraction; (5) human normal immunoglobulin; (6) human-specific immunoglobulin; (7) dried human fibrinogen; (8) dried or frozen human coagulation factor VIII, and (9) dried human coagulation factor IX. No provisions were included about fresh frozen plasma or platelets, the production methods and quality requirements of which were undoubtedly non-standardized at the time.

The Council of Europe member countries ratified the agreement mostly in the sixties, but some did it first in the middle of 1990s. In 1983, an additional protocol was written about the European Economic Community (EEC) becoming a contracting party to the Agreement,³ and in 1987 the Council of the European Union reached decisions in which it accepted this agreement⁴ together with the one on the exchange of blood-grouping reagents from year 1962.⁵ Thus, these early Council of Europe agreements became legally binding in the EEC member states, and serve as examples of the European Union making use of the work of the Council of Europe experts in transfusion medicine.

A working party that was put together to follow the implementation of the above-mentioned agreement became a committee of the Council of Europe in 1962 (Sub-Committee of Specialists on Blood Problems). Later its name (Santé publique hématologie, SP-HM) and its type of work changed, but it has retained its original aim to harmonize the practice of blood transfusion among the member countries of the Council of Europe. A subcommittee to SP-HM, called SP-R-GS (Santé publique-restant-groupes sanguins) was formed in 1973 to give guidance on a European level on automation in blood group serology. After laboratory automation became commonly established this subcommittee concentrated on quality assurance. It started publishing guidance on quality control in blood transfusion services, which gradually developed into an annually revised book "Guide to the Preparation, Use and Quality Assurance of Blood Components".⁶ This book, although only a guide, has become ‘the bible’ for blood transfusion services in Europe and has been officially accepted as the rule to follow in many countries also outside of Europe.

Legislative process in the European Union

Plasma products have traditionally been regulated by European directives and other legal provisions written for medicinal products. Cellular blood components are considered as medicinal products in some EU member countries, but not in most of them and thus are not covered by their laws and regulations. They have been internationally regulated only by the above-mentioned rather general paragraphs originating from the beginning of 1960s, which have had legal power only from the
The European Commission activities aiming at improving confidence in the safety of the blood transfusion chain and promoting self-sufficiency in the Community as listed in the 1994 Commission Communication

1. Development of scientifically sound policies and agreed procedures in the donor selection process among blood collection establishments within the Community in order to provide the necessary reassurances of the safety of blood products originating from whatever Community source
2. Implementation of efficient validated and reliable screening tests in the Community
3. Development of quality assessment criteria and good manufacturing practices regarding collection, testing, processing, and transfusion of blood and blood products and patient follow-up procedures;
4. Development of a haemovigilance system for the collection of epidemiological data related to the blood transfusion chain
5. Development of educational programmes directed towards health professionals on the optimal use of blood and blood products
6. Support for the dissemination of information on blood and blood products and the collection, processing and transfusion procedures through promotional materials, films, campaigns

The directive gives a legal framework for the design of national regulations, the implementation of which will be closely followed by the European Commission. Deviations from the Directive will be allowed only to the direction of stricter provisions in the national laws and regulations. The oversight of the implementation falls on the national competent authorities which the
directive obliges member states to designate. Although most member states have already had an inspection system for blood banks and an organization for the inspections, many need now officially to designate the competent authority. In many cases, the authority is the National Agency for Medicines that already has experience on licensing and inspections.

The articles about the licensing of blood establishment created some discussion at the drafting stage of the Directive. The idea of these articles was to bring the blood establishments under the control of the national competent authorities. Since this control is reached well also by other means than formal licensing, the final wording indicates that the authorities can not only license the blood establishments but are also allowed to designate, authorize or accredit the blood establishments. It was considered that the compromise in safety that a totally free enterprise in the field could bring, can be prohibited by any of these methods. The main idea was to retain the control in the hands of the national authority, but not limit how it is done. This helps to avoid some of the risks eventually brought by totally free and uncontrolled systems.

National inspections of the blood establishment have been common practice in most European countries for more than 10 years, but only as required by national regulations. Now there must be inspections in the EU Member Countries to ensure that the requirements of the Directive are complied with. Interval between inspections must not exceed 2 years. This may prove to be a special burden in countries that have several independent, often hospital-based blood establishments. One would expect that these are the ones in which the inspections stimulate greatest improvements in the safety of blood by improving national harmonization of quality.

Chain of responsibility in the blood establishment must usually be shown at the beginning of every inspection. The Directive requires that a responsible person for the establishment must be designated and gives the processes that this individual is responsible for as well as defines the minimum conditions of qualification for him/her.

**EU Directive and medical practice**

The Directive deals specifically with the safety of the product, blood and blood components. Strictly taken, any safety measures not dealing with the product are out of the scope of the Directive. The purpose was to leave out anything dealing only with medical practice, patient care proper. The Directive regulates hospital blood banks about their personnel, quality systems, documentation, hemovigilance, storage, transport and distribution conditions as well as data protection and confidentiality, but not on the practice of transfusion of blood or blood components, which is considered as medical practice, over which the directive has no power. It is conceivable that due to the Directive the competent authorities may need to extend their inspections somewhat further to the hospitals than to the pharmacy.

**Role of the European Commission**

Quality management and hemovigilance systems must be in place in member states, but further details of them as well as those of further quality provisions of blood and blood components will be defined by the Directives to be given by the European Commission. The procedure for Commission Directives is sufficiently smooth to allow rapid changes arising from scientific development in the field. A Regulatory Committee of the Commission, which comprises of experts designated by all Member States, is the working arm of the Commission for regular revision of technical requirements that are listed in Article 29 of the Directive (Table 2).

The Council Directive of 2003 indicates that the Commission shall hold regular meetings with the national competent authorities and experts from blood establishments to exchange information on the experience acquired with regard to the implementation of the Directive. The Member States will indeed be followed as to their progress in improving the safety of blood according to what the Directive says.

Besides the regular meetings the Commission will require reporting of the activities undertaken in relation to the provisions of the Directive, including the inspection and control measures by the national authorities. The reporting has already commenced and shall be required every three years. The Commission is under the oversight of the European Parliament, the Council, the Economic and Social Committee and the Committee of the Regions, to which it must report on the implementation of the requirements of the Directive.

In this report, emphasis must be given to inspection and

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**Table 2** Technical requirements and their adaptation to technical and scientific process listed in Article 29 of the Blood Safety Directive, which will be published as directives of the European Commission

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<thead>
<tr>
<th>Number</th>
<th>Description</th>
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<tbody>
<tr>
<td>1</td>
<td>Information provided to donors</td>
</tr>
<tr>
<td>2</td>
<td>Information to be obtained from donors including the identification, health history, and the signature of the donor</td>
</tr>
<tr>
<td>3</td>
<td>Requirements concerning the suitability of blood and plasma donors and the screening of donated blood including Permanent deferral criteria and possible exemption thereof</td>
</tr>
<tr>
<td>4</td>
<td>Storage, transport and distribution requirements</td>
</tr>
<tr>
<td>5</td>
<td>Quality and safety requirements for blood and blood components</td>
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<tr>
<td>6</td>
<td>Requirements applicable to autologous transfusion</td>
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Commission Directive on products, approved by the Commission Regulatory Committee in September 2003 deals with:

<table>
<thead>
<tr>
<th>Number</th>
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<tbody>
<tr>
<td>1</td>
<td>Traceability requirements</td>
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<tr>
<td>2</td>
<td>Community standards and specifications relating to a quality system for blood establishments</td>
</tr>
<tr>
<td>3</td>
<td>Community procedure for notifying serious adverse reactions and events and notification format</td>
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control measures undertaken. The reporting is to start on 1 July 2004 and must be done every three years thereafter.

**Conclusion**

Although all the countries that have participated in the blood safety work of the Council of Europe have more or less followed the guidance given, it is evident that first the binding legal provisions brought by the European Union and its legislative process bring a substantial legal addition to blood safety in the EU Member States. This process in the European Union will reflect to blood safety also in the countries that do not belong to the Community both within and outside of Europe. The blood safety directive of 2003 is the first really pan-European law on blood and blood components. Although the national laws need to follow this first by February 2005, it is already evident that all member countries are forced at least to rethink their national regulations and practices and in many cases also implement visible changes.

The process now started shall improve confidence in safety of blood and blood components across the national boundaries in Europe. Safety of transfusion is improved. Whether it will be possible to demonstrate the improvement in numbers and figures is difficult to predict. It must be kept in mind that, after all, therapy with blood and blood components has already for quite some time been one of the safest interventions in patient care.

**References**

IMMUNOTHERAPY

Immunotherapy with alloreactive natural killer cells in haploidentical haematopoietic transplantation

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HLA haplotype mismatched transplants

Until the early 1990s, transplantation from haploidentical donors, that is, transplants from one HLA haplotype-mismatched (haploidentical) family members, was largely unsuccessful because all patients were at high risk of T-cell-mediated alloreactions in the host-versus-graft (HvG) as well as in the graft-versus-host (GvH) direction. In T-cell-replete grafts, the high frequency of alloreactive donor T cells recognising major histocompatibility (MHC) antigens caused the extremely high incidence of severe, acute GvHD. Although extensive T-cell depletion prevents GvHD, the rejection rates rise steeply because the balance between competing host and donor T cells shifts in favour of the unopposed HvG reaction. These T-cell-mediated responses can be largely controlled by (a) extensive T-cell depletion of the BM graft to prevent GvHD and (b) appropriate immunosuppressive intensity of the conditioning regimen to prevent graft rejection. Engraftment of T-cell-depleted mismatched transplants is only achieved by transplanting a megadose of haematopoietic stem cells, which can easily be obtained by administering G-CSF to the donor to mobilise peripheral blood stem cells.

In 1993, we supplemented bone marrow with G-CSF-mobilised peripheral blood progenitor cells (PBPCs), both depleted of T lymphocytes by soybean agglutination and E-rosetting. A total of 36 adults with advanced stage acute leukaemia were conditioned with a highly immunosuppressive and myeloablative regimen that included total-body irradiation (TBI) in a single fraction at fast dose-rate, Cy, ATG and thiotepa. In total, 80% achieved primary sustained engraftment. Although no post-transplant immunosuppressive therapy was used as prophylaxis, the incidence of GVHD was significantly lower than in T-cell-replete allografts.

In October 1995, fludarabine was substituted for cyclophosphamide in the conditioning regimen to minimise extra haematological toxicity and, with the aim of eliminating GvHD, the number of CD3+ cells in the graft was reduced to a mean of 2 × 10⁶/kg recipient body weight. Analysis of outcomes in the 134 high-risk acute leukaemia patients who have received transplants between October 1995 and August 2003, showed primary sustained engraftment was achieved in >90% and acute (grade 1) GVHD in <10% (F Aversa et al., unpublished). These clinical results indicate the one haplotype mismatched transplant is a viable strategy for cure of high-risk acute leukaemia in patients who do not have a matched donor.

Natural killer cell alloreactivity

In the haploidentical transplant, another type of alloreactivity, mediated by natural killer (NK) cells, is triggered by mismatches between killer cell Ig-like receptors (KIR) on donor NK cells and HLA class I molecules on recipient cells. This is known as donor-versus-recipient NK cell alloreactivity.

NK cells are primed to kill by signals delivered through several, different activating receptors. However, NK killing of autologous cells is prevented as each NK cell coexpresses at least one inhibitory receptor for self-MHC class I molecules. Human NK cells discriminate between allelic forms of MHC molecules via clonally distributed receptors, termed KIRs (from Killer Cell Ig-like Receptor), that are specific for epitopes that are shared by MHC class I alleles, that is, group 1 and group 2 HLA-C alleles, and HLA-Bw4 alleles (Table 1). The lack of expression of self-MHC molecules on mismatched allogeneic targets results in susceptibility to NK cell-mediated lysis (‘missing self’ recognition). Although HLA and KIR genes are inherited independently most individuals possess a full complement of KIR genes for inhibitory receptors for the three major class I ligands (group 1 and group 2 HLA-C alleles, and HLA-Bw4 alleles). In the 162 individuals, we screened for KIR genes, 97% bore KIR2DL1, which is the receptor for HLA-C group 2 alleles; 100% expressed KIR2DL2/3 receptors, which are specific for HLA-C group I alleles and 94% bore KIR3DL1, which is the receptor for HLA-Bw4 alleles (A Mancusi et al., unpublished observations). As KIRs are clonally distributed, the NK population in each individual is constituted of a repertoire of NK cells with each bearing a different receptor (or different receptor combinations).
Donor-versus-recipient NK cell alloreactivity. Individuals who express Group 2 HLA-C alleles and possess NK cells that express KIR specific for Group 2 HLA-C alleles (KIR2DL1 and/or KIR2DL3) are alloreactive against cells from individuals who do not express Group 2 HLA-C alleles (who are homozygous for Group 1 HLA-C alleles) (top). Individuals who express Group 1 HLA-C alleles possess NK cells with KIR specific for Group 1 HLA-C alleles (KIR2DL2 and/or KIR2DL3) and are alloreactive against cells from individuals who do not express Group 1 HLA-C alleles (who are homozygous for Group 2 HLA-C alleles) (bottom).

and therefore with different allospecificities. Some NK cells in the repertoire will mediate alloreactions when the mismatched allogeneic target cells do not express a class I allele group that blocks them (Figure 1).13,14

**NK cell alloreactivity in animal transplant models**

Experimental evidence suggests that NK cells predominantly attack the haematopoietic cells of the host while sparing other tissues that are common targets for T-cell-mediated GvH disease. In a series of experiments,15 human alloreactive NK clones were infused into human AML-engrafted NOD-SCID mice. Mice infused with human AML developed advanced disease in 5–6 weeks. If left untreated, or given nonalloreactive human NK clones, mice died over the following 3 weeks. In contrast, much fewer human alloreactive NK cells cleared leukemia, and mice survived until the time of killing (120 days). We next tested whether alloreactive NK cells ablate the host immune system, thereby allowing engraftment of haploidentical haematopoietic transplants in an MHC-mismatched F1 into parent mouse bone marrow transplant model. In F1 H-2d/b into parent H-2b transplants, donor T cells are tolerant of the recipient MHC, but donor NK cells not expressing H-2b-specific Ly49C/I inhibitory receptor, and bearing instead H-2d-specific Ly49A/G2 receptors, are activated to kill the recipient’s targets. Alloreactive NK cells did not cause GvHD even when infused in high numbers in lethally irradiated recipients. However, in nonlethally irradiated recipients alloreactive NK cells, but not control nonalloreactive NK cells, reduced recipient-type T-cell and granulocyte counts in marrow and spleen to levels observed after lethal irradiation. Mice conditioned with nonlethal (≤7Gy) TBI alone rejected donor marrow grafts. In contrast, all recipients conditioned with nonlethal irradiation and alloreactive NK cells engrafted with durable, donor-type haematopoietic chimera. Notably, as few as 2 × 10^4 alloreactive NK cells resulted in major donor haematopoiesis, nonalloreactive NK cells had no effect. Alloreactive NK cells allowed mismatched BMT even combined with the reduced-intensity conditioning regimens adopted from matched human transplants. Thus, mice given these regimens plus low doses of alloreactive NK cells achieved >80% donor chimerism, unlike controls receiving nonalloreactive NK cells. Even after mild immune suppression, fludarabine alone, followed by the infusion of alloreactive NK cells, recipients achieved a substantial degree of donor chimerism (30%). The infusion of alloreactive NK cells 6 weeks post-transplant was able to convert mixed chimeras to stable full-donor chimera. We next tested whether NK conditioning could reduce the need for extensive T-cell depletion. Lethally irradiated H-2b mice transplanted with H-2d bone marrow containing 1 million T cells died from GVHD in 2–4 weeks. After conditioning with TBI + alloreactive NK cells cohorts of transplanted mice were given escalating doses of H-2d T cells. Even with as many as 2 × 10^7 T cells, 100% of mice survived until the time of killing (120 days) without signs of GVHD. In contrast, administration of nonalloreactive NK cells, even at very high numbers, provided no protection. It could be hypothesized that this protection might be mediated by alloreactive NK cells attacking recipient antigen-presenting cells (APCs), shown to be responsible for initiating GVHD14 and that, consequently, mice with APCs that are resistant to alloreactive NK killing might not be protected from GVHD. We therefore made B6xBALB/c into B6 bone marrow chimera to replace the alloreactive NK cell-sensitive H-2b mouse haematopoietic cells, including APCs, with H-2d/b cells that would be resistant to NK cell killing (H-2d/b into H-2b chimeras). While the H-2d allele protects against alloreactive NK cells, the H-2b molecules can still present antigen to donor H-2d T cells, thus priming GVH reactions. When analysed 4 months post-transplant, >90% bone marrow, spleen, and gut dendritic cells (DC) in these chimeras were of H-2d/b origin. When these chimeras were reconditioned with TBI plus alloreactive NK cells and reconstituted with H-2d/BMT containing 1 million T cells, 100% died from GVHD. Control H-2b→H-2b chimeras given 2 × 10^7 T cells survived with no signs of GVHD. It was also found that alloreactive NK cells accelerated the loss of bone marrow, spleen and gut APCs, as compared to mice conditioned with either TBI or TBI plus nonalloreactive NK cells. Taken together, these data indicate that alloreactive NK cells prevent GVHD via elimination of recipient APCs. Therefore, in the mouse model,
alloreactive NK cells not only fail to cause GvHD, but could also block the T-cell-mediated GvHD, which is initiated by DC. In addition, alloreactive NK cells used in the pretransplant conditioning protected from GvHD to such an extent as to allow a safe infusion of otherwise lethal doses of allogeneic T cells.

**Donor-versus-recipient NK cell alloreactivity in clinical transplantation**

In HLA haplotype-mismatched haematopoietic transplantation with a potential for GvH NK-mediated reactions, the engrafted stem cells give rise to an NK cell wave of donor origin that regenerates the same repertoire as the donor’s, and so includes high-frequencies of donor-versus-recipient alloreactive NK cells. Donor-versus-recipient NK cell alloreactivity dramatically reduced the risk of leukaemia relapse in 57 acute myeloid leukaemia (AML) patients at high risk of relapse, while improving engraftment and protecting against GvHD.

An updated analysis (our unpublished data) of transplantation outcomes of all 85 advanced stage AML patients (80% ≥2nd CR) transplanted from haploidentical donors from 1993 through 2003 demonstrates transplantation from NK alloreactive donors enhances engraftment without increasing the risk of GVHD and most impressively controls AML relapse. Probability of relapse is 79% for the 44 patients transplanted from non-NK alloreactive donors (21 transplanted in CR, 23 in relapse) versus 17% (P < 0.005) for the 36 patients transplanted from NK alloreactive donors (23 transplanted in CR, 13 in relapse). Lack of an NK alloreactive donor is the strongest independent risk factor predicting relapse (transplantation from non-NK alloreactive versus NK alloreactive donor: hazard ratio = 4.24, CI = 1.34–13.45, P = 0.014), when compared with disease status at transplant (relapse versus remission: hazard ratio = 2.76, 2.76, CI = 1.06–7.21, P = 0.038). Probability of event-free survival is 52% for patients with NK alloreactive donors versus 7% for those without (P < 0.005).

Experimental evidence confirms alloreactive NK cells are directly involved in controlling relapse of AML. *In vitro* studies showed 100% myeloid leukaemias were lysed. This was the only nonsusceptible target in all the primary tumours of lymphohaematopoietic lineage we have tested to date (our unpublished results). Moreover, transfer of NK cells into NOD/SCID mice eradicated transplanted human AML provided that the NK cells were alloreactive towards the transplanted AML.

The engraftment rate also improved in NK mismatched transplants. Experimental evidence that NK alloreactivity was directly involved in engraftment was provided by our transplant murine models (see above and Ruggeri et al.15). Even after mild host immune suppression, infusion of donor alloreactive NK cells ablated the host lymphohaematopoietic cells, thus preventing rejection of the MHC-mismatched BMT. These data strongly suggest that alloreactive NK cells that arise spontaneously from ‘NK mismatched’ stem-cell grafts exert powerful GvL effects against myeloid leukaemias and also improve engraftment.

Given such a powerful donor-versus-recipient immune reaction, we wondered why NK cells do not mediate severe GvHD. Experimental evidence (see above and Ruggeri et al.15) suggested NK cells attack predominantly the haematopoietic cells of the host but not other tissues that are common targets for T-cell-mediated GvH disease. Murine alloreactive NK cells, even when infused in large numbers, did not cause GvHD. Moreover, alloreactive NK cells killed host-type DC, thereby preventing presentation of host antigens to graft T cells16 and thus blocking T-cell-mediated GvHD.

In conclusion, unlike T-cell alloreactivity, NK alloreactivity combines all the features that make it uniquely suited for transplantation. As depicted in Figure 2, alloreactive NK cells eradicate leukaemia, favour engraftment by killing host lymphohaematopoietic cells, and reduce GvHD by eliminating host-type DCs. One consequence of these studies is the need to rapidly exploit these results by revising current criteria for haploidentical donor selection. Donor selection for AML now involves a search for the donor who is able to mount donor-versus-recipient NK cell alloreactivity.

**Work-up for NK-alloreactive donor selection**

The search for NK-alloreactive donors may require extension from the immediate family (parents and siblings) to other family members such as aunts, uncles and cousins. An extended search raises the chance of finding an NK-alloreactive donor from the random 30% to >60% (which is close to the maximum, bearing in mind that HLA type of about 30% of the population makes them resistant to alloreactive NK killing (see below).

For NK-alloreactive donor selection, first HLA type the recipient by high-resolution molecular techniques. Recipients who express class I alleles belonging to the three major class I groups (HLA-C group 1, HLA-C
group 2, and HLA-Bw4 alleles) will block all NK cells from every donor. Recipients who express alleles belonging to one or two of these three class I groups have the chance of finding NK alloreactive donors. Table 1 shows HLA-C group 1, HLA-C group 2 alleles and the HLA-B alleles sharing the Bw4 supertypic specificity along with the amino-acid sequences that are the basis of the group classification. Donor HLA typing will identify the relative who expresses the allele in the class I group that is not expressed by the patient. Most of these donors will possess NK cells that are alloreactive against the recipients.

KIR genotyping is done by PCR on donor DNA. As we have observed 3% of the population lack KIR2DL1 and 6% lack KIR2DL1, while 100% have KIR2DL2/3 (see also Parham and McQueen19), KIR genotyping of the donors is necessary to make sure the donor possesses the relevant KIR gene and, thus, to improve accuracy of NK alloreactive donor identification.

In some individuals, allelic variants in the HLA-Bw4 inhibitory NK receptor gene KIR3DL1 may not allow full receptor expression at the cell membrane.20 Others may express the corresponding NK clones in very low frequencies (our unpublished observations). Direct functional assessment of the donor alloreactive NK repertoire through the generation of large numbers of donor NK clones and cytotoxicity assays against recipient target cells may be necessary to identify these donors.

Acknowledgements
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References
20 Pando MJ, Gardiner CM, Gleimer M, McQueen KL, Parham P. The protein made from a common allele of KIR3DL1 (3DL*004) is poorly expressed at cell surfaces due to substitution at position 86 in Ig domain 0 and 182 in Ig domain 1. J Immunol 2003; 171: 6640–6647.
Immunotherapy with alloreactive T-cells?

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The introduction of z-interferon (IFN-\(\alpha\)) in the early 1980s contributed to an increase of overall survival compared to chemotherapy of about 20 months. Furthermore, the complete cytogenetic response in 6–25\% of chronic myelogenous leukaemia (CML) patients treated in chronic phase was ascribed to its immunomodulatory effect (upregulation of human leucocyte antigen (HLA) class I and II, and of adhesion molecules). It appeared therefore that the increase in HLA molecules induced by interferon \(\alpha\) therapy had a part to play in the cytogenetic responses seen in some parties.

Allogeneic haematopoietic stem cell transplantation (HSCT), in addition to the diminution and eradication of some of the tumour burden by high-dose pretransplant chemo and radiotherapy regimens, HSCT provides an allogeneic antitumour effect or graft-versus-leukaemia effect (GvL) and truly constitutes the only current curative treatment for CML. In both situations MHC molecules are involved as key mediators of the effects seen. On the one hand by increasing the display of leukaemia specific peptides to the immune system thus enhancing the generation of an effective anti-leukaemia response. In the HSCT setting donor effector cells are able to recognise HLA presentation of a target peptide that may be over-expressed or not, or the recipients leukaemic cells. This peptide target is perceived as foreign and cells displaying it are eliminated. HLA molecules or alloantigens are therefore important in effective immunotherapeutic responses.

When considering HSCT, the donor of first choice will be an HLA-identical sibling, but because of the low proportion of such donor availability, it has become more common to use an unrelated HLA-matched or partially mismatched donor. The improvement of HLA molecular typing together with the understanding of crucial matches versus permissive mismatches and the expansion of Bone Marrow Registers, has allowed transplant physicians to find suitable donors soon after diagnosis. This has resulted in comparable long-term survival rates similar to patients receiving HLA-related donors.

It is now clear that the donor T cells infused with the graft are active in eradicating the residual tumour burden. This has arisen from a number of clinical observations that (a) the rate of leukaemic relapse for an allogeneic grafts is lower than that for a syngeneic graft; (b) that an increased risk of disease relapse was observed in the absence of GvHD, or in patient receiving T-cell-depleted (TCD) grafts, (c) and finally that donor leukocyte infusions (DLI) are effective for patients who have relapsed, implying that immunocompetent cells transferred as part of the infusion are able to exert a potent allogeneic graft-versus-leukaemia effect (GvL).

In addition to mediating GvL, the immunocompetent cells from the donor are able to mount an immune response against the recipient cells causing graft-versus-host-disease (GvHD). This remains a significant cause of morbidity and mortality for patients post-HSCT. Although GvHD and GvL were long believed to be mediated by the same populations of immune cells, disease clearance in CML patients has been obtained without the manifestation of GvHD leading to the suggestion that it may be possible to isolate the cells responsible for GvL away from the cells giving rise to GvHD. Various strategies aimed at improving the outcome of allogeneic HSCT have been tried, all aimed towards preventing GvHD while retaining and/or stimulating the GvL effect. Certainly, it is clear that T-cell depletion (TCD) of the graft is an effective GvHD prophylaxis, yet at the same time, the depletion of the antileukaemic cells often leads to disease relapse.

\textbf{Segregating antitumour from anti-host reactions}

The observation that donor leukocyte infusion (DLI) is an effective salvage therapy for 70–80\% of CML patients who relapsed after HSCT suggests an important role for donor T cells in the GVL effect. GvHD still remains a possible outcome and many efforts at defining the optimal T-cell dose as well as the time of infusion have been studied.

The antileukaemic response or GVL has been observed in CML patients without the presence of GvHD. This suggests that two different T-cell populations recognising two different sets of antigens are responsible for each response. The definition of a specific tumour antigen would enable the development of a safe immunotherapy approach. This is the focus of work being undertaken by the Immunotherapy Group at the Anthony Nolan Research Institute in conjunction with collaborators in other centres.

\textbf{CML molecular biology and bcr/abl specific targeting}

The isolation of the two effects GVHD and GVL in order to promote the beneficial side GVL while reducing
the GVHD side has proved to be difficult to achieve. The work of Ibisch et al. (1999) relating the lysis of leukaemic blasts by allogeneic DP-specific T cells is interesting in forming a link between class II mismatches and the presence of a GVL-like effect and some of the work from our institute in attempting to raise GVL-specific T cell lines.

The target antigens on leukaemia cells and effector cells responsible for the GVL effect post-BMT are at present not well defined. One possibility, minor histocompatibility antigens are suggested as being the targets for cytotoxic T cells that mediate GVHD and they are also obvious candidates for the GVL response, and class II antigens may also have a role to play here. Cytotoxic T-cell clones reacting with leukaemia cells have been raised from HLA-identical sibling donors. In these studies, tissue restricted distribution of minor antigens could help to explain why some patients respond to lymphocyte transfusion without developing GVHD. In order to try and dissect and isolate the GVL initiating cells from cells mediating GVHD, we examined a number of post-BMT CML patients, and attempted to raise line or clones using frozen pretransplant leukaemic cells as a source of antigen. These cells were used to initiate the cultures and to provide subsequent restimulations for the lines. Having established the most favourable conditions for culture growth and expansion. A variety of different culture parameters were investigated, utilising patients post-transplant or donor pretransplant T cells as responders. In one patient donor combination where the recipient was undergoing an episode of aGVHD, immunosuppression was withdrawn in an attempt to resolve the GVHD the patient went into aGVHD, immunosuppression was withdrawn in an attempt to resolve the GVHD the patient went into remission and we were able to obtain a peripheral blood sample at this specific time point. In this setting, we were able to establish lines that were cytotoxic for the patient’s pre-transplant leukaemic cells but were not reactive with purified T cells from the same patient (Figure 1). All of the lines and clones raised from this patients’ bulk cultures were CD4 positive and their proliferative responses could be blocked by the use of anticlass II antibodies in vitro. The lines and clones raised from these cultures were extremely difficult to maintain in culture for extended periods. It was interesting to find that the cells that displayed leukaemia specificity were CD4-positive cells and not CD8 as expected.

Given that the GVL effect is most prominent in CML patients, we attempted to determine whether we could find the presence of leukaemia specific peptides on the surface of CML cells that might be being presented by Class I antigens and which could act as targets for the GVL response.

CML is a clonal expansion of hematopoietic progenitor cells characterized by the presence of the Philadelphia (Ph) chromosome. Ph chromosome is the result of t(9;22) (q34;q11) translocation involving the Ab1 protooncogene on chromosome 9 and a breakpoint cluster region of the Bcr gene on chromosome 22. Therefore, one prime candidate for this, which our laboratory has focused on, is the BCR/ABL chimeric gene that encodes for a p190 or p210 BCR-ABL fusion protein that could after processing be presented at the cell surface and possibly provide a neoantigen for a tumour-specific T-cell response, although suggested peptides derived from this antigen had not been convincingly demonstrated previously on the surface of CML cells. The BCR-ABL gene rearrangement also results in the upregulation of a number of intracellular proteins downstream from p210 and p190 BCR-ABL such as GRAB-2 or RAS, which could perhaps also elicit specific T-cell responses.

The aberrant bcr/abl chimeric gene translates into an active protein with enhanced tyrosine kinase activity compared with the Abl wild-type protein. This chimeric protein is central to the pathogenesis of CML, proved by (1) its ability to transform primary myeloid cells and induce a CML-like disease in mice and (2) the response induced in CML patients when targeting the bcr/abl protein with the drug Gleevec (STI571). The constitutively active tyrosine kinases have different cellular substrates (including RAS, ERK, PI-3 kinase) leading to the activation of multiple signal transduction pathways that in turn cause profound effects on cell growth and differentiation. STI competitively binds to the adenosine triphosphate (ATP)-binding site of the abl tyrosine kinase thereby preventing signalling by the oncogenic protein. Its active effect of proliferation inhibition and induction of apoptosis was shown in vitro in CML and Ph+ ALL at different stages of disease. It was also demonstrated in vivo where haematological responses were obtained in 69% and a major cytogenetic response in 24 % of CML patients. However, resistance or insensitivity of quiescent Ph+ stem cells from CML patients could explain the relapse observed in STI-treated patients. In fact, acquired resistance was demonstrated by a single amino-acid substitution in the abl kinase domain that affected STI binding. Recently, five distinct point mutations in the bcr/abl
domain were found in seven patients, proved to affect STI 571 binding and therefore conferred resistance to the drug in vitro. Furthermore, STI has been shown to induce the downregulation of ICAM-1 on leukaemic cells, inhibiting the recognition and killing by NK cells. Because of the described resistance as well as its interference with the immune system, the STI drug may not, at least on its own, promise to be a long-term cure for CML patients. This adds a further reason why it is important to define whether specific peptide epitopes are being presented and if so whether they are immunogenic and are able to act as targets for cytotoxic lymphocytes. These targets could then be utilised to devise additional treatment strategies if STI treatment begins to allow a relapse in specific patients.

The two major chimeric bcr/abl protein transcripts found in CML patients are b2a2 and b3a2 as shown in Figure 1. The junctional sequence not only joins a set of amino acids not normally expressed on normal cells but also inserts a new amino acid at the exact fusion point: this constitutes a potential CML-specific tumour antigen. The immunogenicity of such an antigen depends on many factors, including its level of expression, expression, its peptide sequences following intracellular processing and finally the ability of the potential peptides to be presented by class I molecules. As the bcr/abl protein is an endogenous cellular protein these fusion proteins may be processed and presented on the cell surface via the HLA class I molecule pathway and thus be accessible to cytotoxic CD8+ cell.

Reports in the literature had previously described potential HLA-A3-binding peptides derived from the BCR/ABL p210 splice region were a novel amino acid is generated by the fusion event that could be seen as a new antigen by the patients immune system. Further evidence in the literature also described a lower than normal frequency of HLA-A3 and HLA-B8 in patients with CML, possibly suggesting that a subgroup of patients with these alleles were able to process and present peptides form BCR/ABL and initiate an immune response to their leukaemia. We therefore chose to focus on these antigens to investigate and confirm the presence on the surface of CML patients cells of leukaemia-specific peptides from the BCR/ABL region.

Utilising a p210-positive cell line K562 that is negative for class I antigens, we generated single HLA transfec-tants for HLA-A3; our aim being to simplify the peptide spectrum found at the cell surface.

These cells were expanded and peptides eluted from the cell surface, concentrated and subjected to analysis by mass spectrometry. The same exercise was conducted on CML cells from p210 b3a2-positive CML patients and the resultant peptide spectrums showing the presence of the BCR/ABL peptide from the junctional region in both cases is shown in (Figure 3). This clearly demonstrates that the BCR/ABL peptide is processed and presented by HLA class I antigens in CML patients.
Additionally, we can demonstrate the presence of BCR/ABL reactive CD8+ T cells in the circulation of CML patients pregrafting (Figure 4). We are currently investigating the functional capacity of these cells and their interactions with the CD4+ population that we have also shown can be detected in CML patients.

A peptide vaccination study has already been reported in the literature using a mixture of bcr/abl-derived peptides in a Phase 1 Clinical Trial. In this study, specific T-cell proliferation was demonstrated after three vaccinations of high-dose peptide. Nevertheless, no specific cytotoxic responses were obtained and this could be due to a number of reasons; one being that the patients enrolled in this trial were in an accelerated phase, bearing a large tumour burden, or this may be indicative of an in vivo anergy toward bcr/abl peptides. Reducing the tumour burden and allowing the patients’ own immune response to deal with residual tumour cells may be an appropriate strategy.

Having defined the presence on CML cells of specific peptides presented by alloantigens, a vaccination study was set up with our clinical collaborators in Liverpool. Reviewing the previous studies with peptide immunisation, we structured our program to provide in addition to the peptides that we knew to be presented on class I alloantigens, a pan Class II covalently linked peptide in order to induce a memory response and to provide class II help for the class I response. The vaccinations would be given in patients who were on STI so that their tumour burden was significantly reduced, thus allowing the patients’ own leukaemia-specific response to predominate.

This study has now started and we are currently undergoing the second phase of a dose escalation study that is required by the regulatory bodies. However, it is encouraging to find that even at these early time points that patients are showing large DTH responses following immunisation.

In terms of immunotherapy, peptide vaccination does look appealing, but will require further trials in chronic phase CML patients in order to test peptide immunogenicity in this more rigorous setting. It may be that we could boost the vaccination response in these patients with HLA-matched donor dendritic cells, as there is some evidence that DC derived from CML patients, do not seem to function efficiently. With the efficiency and safety of adoptive T-cell therapy to restore CMV immunity, specific bcr/abl-primed T cells, derived from an HLA-matched donor may promise to be an efficient immunotherapeutic approach to treat CML patients. The promising in vitro results obtained with bcr/abl peptides in the context of HLA-A*0301 alleles emphasise the importance of testing the in vitro immunogenicity of other bcr/abl-derived HLA-associated epitopes such as for (HLA-B8, HLA-A2) and defining epitope sequences for other HLA class I/II molecules to target a wider patient population. HLA-class I tetramers represent a powerful tool for the selection of Ag-specific CD8+ T cells, and, together with the development of HLA class II tetramers, will allow the development of adoptive transfer of specific T cells as a future treatment of choice in conjunction with treatments such as STI to initially debulk the leukaemic cells.

Another possible avenue is to use the presentation of specific target peptides by alloantigens themselves in the
form of tetramers that are basically alloantigens bearing the appropriate peptide moiety. These can be used to isolate antigen-specific T cells and then it may be possible to further stimulate and expand these cells with tetramer-like reagents, and subsequently give these expanded antigen-specific T cells back to the patient. In time, these type of strategies may be could reduce the need for treating patients with allo-reactive T cells and thus reduce the risks associated with GVHD.

In conclusion, we feel that the identification of the target antigens and effector cells of the GVL response may allow for the separation of GVHD from GVL and could result in novel treatment strategies that could substantially improve transplant outcome.

References


Immunotherapy of hematological malignancies with dendritic cells

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Introduction

Dendritic cells (DC) are antigen-presenting cells (APC) that play an essential role in the initiation of the immune response.1 DC are characterized by their specific morphology allowing them to simultaneously interact with multiple cells from the immune system and their characteristic expression of HLA class I and class II molecules, costimulatory molecules, adhesion molecules, chemokine receptors and production of cytokines depending on the maturation state of these cells.2 Immature DC express low levels of HLA class II molecules, do not significantly express molecules belonging to the B7 family like CD80 and CD86, and do not produce immune activating cytokines. Upon activation DC upregulate HLA class II molecules, express CD80 and/or CD86 and may upregulate chemokine receptors such as CCR7. These changes in surface molecules and cytokines may cause appropriate trafficking of DCs and allow interaction with other cells of the immune system. Under appropriate ‘danger signals’, the DC may further progress into fully mature DCs expressing high levels of all costimulatory molecules including CD40, CD80, CD86, CD83, HLA class I and class II, producing multiple cytokines capable of activating the immune response. During their development and education in the thymus, the T-cell compartment is educated to discriminate between self and nonself peptides that can be recognized in the context of HLA class I or class II molecules. Thymic education results in the deletion of autoreactive T cells allowing T cells capable of recognizing foreign antigens to enter the circulation. However, not all T cells leaving the thymic compartment are nonautoreactive. Therefore, peripheral mechanisms are required to prevent the occurrence of autoimmune diseases. Immature DC expressing self-peptides are thought to play an important role in the peripheral tolerance against autoantigens. If naive T-cells interact with their specific peptide HLA complexes in the context of nonprofessional APC or immature dendritic (like) cells, these T cells may render anergic to the antigens. If, however, the DC expressing the relevant antigens have been activated by (pro)inflammatory cytokines and/or various products from microorganisms like CPGs of LPS, a full immune response may occur. Crosstalk between the various cells from the immune system may further enhance the activation of DC for instance by activation of CD40 on their cell membrane. These interactions may result in fully equipped professional APCs expressing high levels of costimulatory molecules like CD80, CD86, CD83 and adhesion molecules like CD54 and CD58, and producing activating cytokines like IL-12 resulting in an immune response leading to eradication of antigen-expressing cells. The immune response will be specifically activated against antigens that are picked up by DC during their development from immature into mature DCs. However, if antigens are endocytosed in the absence of a ‘danger signal’, semi-mature DC may trigger regulatory T cells like IL-10 producing CD4+ CD25+ cells capable of actively silencing the immune response. This crosstalk between DC and T cells may explain in part the inability of the immune response to eradicate tumors. Specific manipulation of malignant cells into APCs with a mature DC phenotype may trigger the immune system to suppress or eradicate malignancies.

Tolerance to hematological malignancies

There is little evidence of spontaneous autoreactivity in patients resulting in the control of hematological malignancies. Only in lymphoproliferative disorders caused by Epstein–Barr virus (EBV) transformation, autologous suppression of the outgrowth of these transformed cells is obvious. In chronic myeloid leukemia (CML) and hairy cell leukemia (HCL), there may be indirect evidence of autologous immune responses against the malignancy. Both diseases can be controlled by treatment with α-Interferon, and auto-immune T cell responses have been demonstrated. In CML, responses to α-Interferon have been associated with the appearance of anti-proteinase-3 (PR3) T cell responses that were capable of suppressing CML precursor cells and not normal hematopoietic cells.3 It may be plausible that hematological malignancies may express fusion proteins that can be recognized by the immune system as nonself, or may upregulate proteins that are not expressed under normal circumstances in normal tissues. Unique tumor-associated antigens may include bcr/abl peptides in CML, AML/ETO or PML-RARα in acute myeloid leukemia (AML).4,5 Furthermore, idiotypic specific immunoglobulins may act as tumor specific antigens in B-cell lymphoma.6,7 Mutated antigens in lymphoblastic leukemia/lymphoma (B-ALL) are recognized by autologous CD8+ T cells.8 In contrast, tumor regression was observed in association with autologous CD8+ T cell responses, which had been induced by administration of vaccine materials from the tumor associated antigens.9 These findings are consistent with the hypothesis that tumor regression may result from the presentation of tumor antigens by DCs or by other APCs [the tumor autoreactive T cells] that were capable of silencing the immune response leading to eradication of antigen-expressing cells.10 More recently, autologous T cells were shown to recognize tumor antigens and could be used to induce tumor regression in vivo.11 In addition, a role for CD4+ T helper cells was also shown in the generation of immunity against myeloma cells.12 However, autologous T cells and/or T helper cells are not always sufficient to eradicate malignancies. In fact, it has been suggested that autologous T cells may have a regulatory function in the immune response to certain malignancies as a result of the balance between pro- and anti-inflammatory cytokines.
oncogenes like P53 and RAS may be expressed in many tumors. Overexpressed antigens may include proteinase 3 in AML or CML, and WT1 in AML/CML or ALL. Although it may not always be evident that immunogenic peptides derived from these proteins are appropriately processed by the malignant cells and expressed in HLA molecules on the cell membrane, it can be speculated that even in the case of appropriate presentation of these antigens, anergy or suppression of the immune response may occur. Under steady-state conditions, most hematological malignancies will not have a phenotype mimicking DCs necessary for the induction of a primary T-cell response. In contrast, in most diseases the expression of costimulatory and adhesion molecules is absent or low on the malignant cells, and therefore they may serve at best as immature DCs or semimature DC. It therefore seems likely, that if T cells with a specificity for the appropriate antigens will interact with the malignancy, anergy will occur, or regulatory T cells will be activated leading to silencing of the immune response. If tumor-specific proteins are released by the tumor, and picked up by immature dendritic cells, the absence of a concurrent danger signal may lead to suppression of the immune response rather than induction of an anti tumor T cell activity. If active silencing of the immune response against the autologous tumor is present, vaccination with tumor-specific peptides or even with tumor specific peptides loaded onto dendritic cells may not be sufficient to overcome the immune suppression. In several mouse studies, it has been demonstrated that vaccination with tumor-associated antigens prior to inoculation of the tumor may lead to protection against the malignancy, but once the tumor is established, appropriate immune responses leading to eradication of the malignancy can hardly be established. To overcome not only anergy, but also active suppression of the immune response against the tumor, it may be necessary to ablate the immune response prior to vaccination with tumor associated antigens, and provide after reconstitution of the immune system appropriate presentation of tumor-associated antigens in the presence of fully matured DC. Alternatively, it may be necessary to bypass the silencing in vivo effect of regulatory T cells by generating an immune response in vitro using mature APCs as stimulator cells and infuse the ongoing immune response into patients with (minimal) residual disease.

Transformation of hematological malignancies into APC

A logical approach to improve the immunostimulatory capacity of hematological malignancies to allow the induction of an appropriate immune response appears to be transformation of the malignant cells into DC-like cells. Based on the mechanisms by which normal DC can be generated and matured, attempts have been made to differentiate hematological malignancies into DC-like cells, and activate these cells to mimic the phenotype of fully matured DC. Since it had been demonstrated that normal DC can easily be generated from CD34+ progenitor cells, and since many features of CML progenitor cells mimic normal progenitor cells, CML CD34+ cells have been cultured in vitro to obtain DC-like cells. Several authors have reported the successful generation of DC-like cells expressing the malignant genotype, and demonstrated the improved stimulatory capacity of these cells. In addition, AML blasts from the majority of patients could be manipulated in vitro to express costimulatory molecules and improve their stimulatory capacity. However, in many cases the phenotype of fully matured DC could not be obtained and therefore still in only a limited number of cases sufficient maturation of these leukemic DC can be obtained to induce an appropriate T cell response. Similarly, malignant cells from patients with acute lymphoblastic leukemia (ALL) or chronic lymphocytic leukemia (CLL) can be induced to differentiate into DC-like malignant cells by combinations of cytokines including IL-4, activation of toll-like receptors by CPG, and triggering of CD40 expressed on the neoplastic B-cells resulting in upregulation of all costimulatory and adhesion molecules and production of IL-12.

Since successful transformation of the malignant cells into malignant DC mimicking fully matured normal DC was not possible in all cases, alternative approaches have been explored. By feeding normal DC during their maturation with apoptotic malignant cells or by fusion of malignant cells with maturing DC, normal mature DC may be forced to express malignancy associated antigens. Several of these approaches have led to the induction of immune responses in vitro resulting in cytolytic activity of T cells directed against the malignancy. However, the specificity of these immune responses is generally unknown, and characterization have been hampered by the lack of clonal isolation of malignancy-specific T cells. Thus, although it has been demonstrated that hematological malignancies can be induced to differentiate into malignant DC-like cells, the ability of these cells to induce an autologous T cell response capable of controlling the disease is still under investigation.

Normal and malignant DC to control disease after allogeneic stem cell transplantation (SCT)

The major advantage of allogeneic SCT over autologous SCT is the immune mediated graft-versus-leukemia (GvL) effect. After HLA-matched allogeneic SCT, this GvL effect has been demonstrated to be mediated by donor-derived T cells capable of recognizing alloantigens or tumor-associated antigens in the context of HLA molecules expressed on the malignant cells. In particular the successful application of donor lymphocyte infusion (DLI) as a treatment for relapsed leukemia or lymphoma after transplantation has illustrated the potential of T cell immunity to control hematological malignancies. Several lessons can be learned for future application of immunotherapy of hematological malig-
nancies from the characterization of graft-versus-host disease (GvHD) after transplantation. Elegant mouse studies have illustrated that the presence of (recipient derived) DC after allogeneic SCT is essential for the development of T cell-mediated GvHD.16 In the absence of these DC, no GvHD occurs even over major antigenic barriers between donor and recipients. These studies confirm that immune responses may be silenced in the absence of appropriate APC. In humans, a strong association has been found between the occurrence of GvHD after allogeneic SCT and the ability of T cells to control the malignant disease. These observations indicate that as soon as an immune response is initiated it is capable of recognizing the malignancy, resulting in clinically significant suppression of the malignancy. Alternatively, it may be hypothesized that if no professional APC from recipient origin are present in the patient, and the hematological malignancy itself does not appropriately express the relevant costimulatory and adhesion molecules, T-cell anergy, or silencing of an immune response may occur. Although this hypothesis has not been proven in the context of allogeneic transplantation, several observations may support this. First, DLI appears to be most successful in the treatment of relapse CML after transplantation both in the presence and absence of clinically significant GvHD.17 This may be caused by the ability of CML precursor cells to spontaneously differentiate into professional APC in the patient, allowing to develop a T-cell response specific for these malignant cells. Since these T cells are derived from the donor in which they have likely not been silenced by encountering the specific antigens prior to transfusion, the immune response may be of sufficient amplitude to control or eradicate the disease. However, in cases where the infused T cells may first interact with large numbers of nonprofessional malignant APC as in overt leukemia or lymphoma, T cells may become anergic or suppressor T cells may develop before interaction of T cells with matured normal or malignant DC will occur.

Several approaches may lead to a more successful treatment of AML, ALL or CLL by cellular immunotherapeutic approaches. First, since it has been demonstrated that these malignancies can be induced to differentiate and mature into relatively mature malignant DC capable of generating an alloimmune response, vaccination of patients in a state of minimum residual disease after allogeneic SCT may allow to evoke an appropriate immune response to control the disease. In vitro experiments have illustrated the ability of these malignant APC to induce leukemia reactive or minor histocompatibility antigen-specific T cell responses that may specifically recognize recipient-derived (malignant) hematopoietic cells. Alternatively, these malignant APC can be used to induce a leukemia reactive T cell response from donor cells in vitro resulting in the production of large numbers of activated T cells that may be capable of controlling the disease after allogeneic SCT.18 Although the feasibility of this approach has been demonstrated, the logistics of the generation of these T cell responses is still complex, and the requirements of in vitro-activated T cells to survive and proliferate in vivo, and specifically home to the right tissues are still largely unknown. Further clinical and preclinical investigations are needed to improve the specificity and efficacy of these in vitro amplified immune responses.

Recently, a number of specific peptides have been characterized that may be appropriate targets for immune responses in the context of allogeneic SCT. The hematopoiesis-specific minor histocompatibility antigens HA-1 and HA-2, and possibly the polymorphic peptides derived from the BCL2A1 proteins may act as malignancy-specific targets in the context of allogeneic SCT.19,20 Primary T cell responses may be generated in vitro from donor cells by producing fully matured DCs from donor origin and loading these APC with the specific peptides to generate a T cell response against these antigens. With these methods, the inability to produce normal or malignant DC from patients-derived hematopoietic cells can be bypassed.

**Summary**

Dendritic cells play an essential role in the induction of appropriate immune responses, and the balance between tolerance and immunity against hematological malignancies. Most hematological malignancies appear to be inappropriate APC to evoke a clinically significant immune response. Many hematological malignancies, however, can be forced to differentiate and mature into DC-like cells that may evoke a clinically significant T cell response. Alternatively, normal DCs loaded with undefined or defined tumor-associated antigens may be used to evoke a specific immune response. The context of allogeneic stem-cell transplantation may be most appropriate to explore the efficacy of T cell mediated immunotherapy of hematological malignancies, since the likelihood of pre-established tolerance against the malignancies is relatively low in this context. The precise requirements for the induction of an appropriate immune response still need to be defined in more detail, before cellular immunotherapy can be successfully applied at large scale in the control of hematological malignancies.

**References**


RBC ERYTHROPOIETIN PATHOPHYSIOLOGY

Erythropoietin pathophysiology and erythropoietin deficiency anemia

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This educational session on erythropoietin pathophysiology involves three presentations:
- erythropoietin deficiency anemia by Mario Cazzola;
- epoetin-induced autoimmune pure red cell aplasia by Nicole Casadevall;
- polycythemia due to disruption of oxygen homeostasis by Josef T Prchal.

The purpose of this session is to review the pathophysiology of erythropoietin production, to examine erythropoietin deficiency anemia and its treatment with recombinant human erythropoietin (including adverse effects of this drug), and to present the new chapter of polycythemia due to disruption of oxygen homeostasis.

In this meeting, the regulation of Erythropoiesis by hypoxia is discussed by Christopher W Pugh in the hematology-in-focus session on ‘Molecular connections between erythropoiesis and iron metabolism’.

Since two presentations concern the use of recombinant human erythropoietin (rHuEpo), it is necessary to clarify the following issues of terminology. rHuEpo or epoetin is a general term used to define erythropoietin molecules that are produced through molecular genetic technology. Epoetin alfa, epoetin beta and darbepoetin alfa are currently employed as therapeutic agents. Epoetin beta (NeoRecormon) and darbepoetin alfa (Aranesp) are available in just one formulation each although different brands are present in different countries. Epoegen and Procrit are identical formulations of epoetin alfa distributed only within the United States, while Eprex is a different formulation of epoetin alfa distributed only outside the United States. In this presentation, the term rHuEpo will be employed for any erythropoietin molecule that is produced through molecular genetic technology.

Pathophysiology of erythropoietin production and its implications for the clinical use of recombinant human erythropoietin (rHuEpo)

Erythropoietin is essentially made by a single organ, the kidney, outside the bone marrow and participates in a classic negative feedback control system. Erythropoietin-producing cells in the kidney are peritubular fibroblast-like interstitial cells, and hypoxia is the fundamental physiologic stimulus that causes a rapid increase in renal production of the hormone (up to 1000-fold) through an exponential increase in the number of these cells. The central mediator of this response is a DNA-binding complex termed hypoxia inducible factor 1 (HIF-1), which plays a key role in the regulation by oxygen of several genes besides the erythropoietin one. The HIF-1 complex is formed in hypoxia and modulates gene expression through hypoxia response elements, while its oxygen-regulated destruction requires the von Hippel–Lindau tumor suppressor protein (pVHL).

With respect to its molecular action, erythropoietin is primarily a survival factor for CFU-Es and proerythroblasts. These erythroid progenitors require continual presence of the hormone to survive although their sensitivity to erythropoietin varies widely. According to this model of erythropoiesis based on erythropoietin prevention of programmed cell death, red cell production can be substantially and steadily expanded only through preamplification of erythropoietin-dependent progenitors. This notion is central to the clinical use of rHuEpo. When erythropoietin levels are inappropriately low, administration of rHuEpo can be effective in allowing survival of more CFU-Es and generation of erythroid precursors that subsequently mature to red cells. By contrast, when endogenous erythropoietin is present in adequate amounts and nearly all available CFU-Es are already surviving, it is very unlikely that pharmacological doses of rHuEpo can further expand erythropoiesis. Clearly, there is room for using erythropoietin in nonanemic subjects in order to expand red cell production and prevent anemia, for example, for potentiating autologous blood donation.

Evaluation of endogenous erythropoietin production and erythropoietin-deficient states

Assessment of endogenous erythropoietin production has become a routine diagnostic procedure with the availability of commercial immunoassays for serum erythropoietin. From a practical point of view, every laboratory should employ only one immunoassay and become familiar with it.

Levels found in anemic patients cannot be simply compared with normal values. In fact, as far as the
erythropoietin-generating apparatus in the kidney is efficient, serum levels increase exponentially as the hemoglobin decreases. Serum erythropoietin must therefore be evaluated in relation to the degree of anemia, and every single laboratory should determine the exponential regression of serum erythropoietin versus hemoglobin (or hematocrit) in a home-made reference population of anemic subjects, and define the 95% confidence limits.

In an individual patient, the adequacy of endogenous erythropoietin production can be easily assessed by graphic evaluation, or through the observed/predicted log(serum erythropoietin) ratio (O/P ratio).

Anemia associated with multiple myeloma or non-Hodgkin lymphoma

Anemia associated with cancer therapy

Anemia of chronic disease

Anemia of chronic heart failure

Anemia of prematurity

Anemia associated with defective endogenous erythropoietin production

Table 1 Main clinical conditions outside nephrology that may be associated with defective endogenous erythropoietin production

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Anemia associated with cancer therapy

Erythropoietin therapy is medically effective in only a portion of anemic cancer patients since the pathogenesis of cancer-related anemia is multifactorial. Seidenfeld et al. reviewed 22 clinical trials on the use of rHuEpo for treatment of anemia associated with cancer therapy. rHuEpo decreased the percentage of patients transfused by 9–45% in adults with mean baseline hemoglobin concentrations of 10 g/dl or less, by 7–47% in those with hemoglobin concentrations between 10 and 12 g/dl, and by 7–39% in those with hemoglobin concentrations of 12 g/dl or higher. Only studies with mean baseline hemoglobin concentrations of 10 g/dl or less reported statistically significant effects of rHuEpo treatment on quality of life. The conclusion of this meta-analysis was that rHuEpo reduces the odds of transfusion for cancer patients undergoing therapy. However, little information is currently available concerning prediction of response to rHuEpo in these patients.

Consequently, physicians must treat 100 patients on chemotherapy with rHuEpo to reduce the number of transfusions in approximately 11 of them. By better tailoring rHuEpo administration to patients with a high probability of response, that is, improving the prediction of response, erythropoietin therapy may become more rational and also cost-effective.

Evidence-based clinical practice guidelines for the use of rHuEpo in cancer patients with chemotherapy-associated anemia have been developed by the American Society of Clinical Oncology and the American Society of Hematology. The guideline panel found good evidence to recommend use of rHuEpo as a treatment option for patients with a hemoglobin level below 10 g/dl. The recommended starting dose was 150 U/kg thrice weekly for a minimum of 4 weeks, with consideration for dose escalation to 300 U/kg thrice weekly for an additional 4–8 weeks in those patients who do not respond to the initial dose.

Defective endogenous erythropoietin production as a strong predictor of response to rHuEpo in anemic patients with multiple myeloma or non-Hodgkin's lymphoma

Despite considerable differences in their designs, a number of European trials on the use of rHuEpo in patients with multiple myeloma or non-Hodgkin's lymphoma have shown that defective endogenous erythropoietin production is a strong baseline predictor of response to treatment. In the last study, of a total of 322 anemic patients screened for serum Epo, 241 were found of have levels ≤100 mU/ml: thus, the vast majority of anemic patients with lymphoproliferative malignancy had evidence of defective endogenous erythropoietin production, and about 3/4 of these individuals responded to rHuEpo.
Therefore, using rHuEpo in anemic patients with defective endogenous erythropoietin production appears to be a rational use of this drug.

**Erythropoietin treatment of anemia in patients with chronic heart failure**

Although heart failure is primarily a cardiac abnormality, any impairment in the remaining components of tissue oxygen supply can worsen its clinical picture. In particular, due to the role of circulating red cells in carrying oxygen from lung to tissues, anemia may significantly affect oxygen delivery. A number of studies in the last few years have shown that anemia is common in patients with chronic heart failure, and have suggested that this coexisting condition may be associated with worse prognosis. Amelioration of anemia through subcutaneous administration of rHuEpo combined with IV iron involves significant improvements in both cardiac and renal function. This appears to be a rational use of this drug, although it is an experimental treatment at present.

**Caveats**

For years erythropoietin therapy has been considered remarkably safe. In 2002, Casadevall et al. reported a series of cases of epoetin-induced PRCA in renal patients. Thus, the scientific community became aware of the fact that neutralizing antierthropoietin antibodies and pure red cell aplasia can develop in patients with anemia of chronic renal failure during treatment with rHuEpo. This topic will be covered by Nicole Casadevall herself in this educational session.

A randomized, double-blind, placebo-controlled study was designed to investigate the effect of rHuEpo treatment to maintain normal hemoglobin concentrations on survival in patients with metastatic breast cancer who were receiving first-line chemotherapy. The study was terminated early by a recommendation from the Independent Data Monitoring Committee because of an observed higher mortality in the group treated with rHuEpo. These findings are at variance with those of a previous study in anemic cancer patients treated with nonplatinum chemotherapy and randomized to receive rHuEpo or placebo. Although this latter study was not powered for survival as an end point, Kaplan–Meier estimates showed a trend in overall survival favoring rHuEpo versus placebo.

In a recent study, Henke et al. have investigated whether anemia correction with rHuEpo could improve outcome of curative radiotherapy among patients with head and neck cancer. They concluded that rHuEpo corrects anemia but does not improve cancer control or survival, and that disease control might even be impaired. In fact, locoregional progression-free survival was poorer with rHuEpo than with placebo.

These latter studies raise the question as whether cancer cells express functional erythropoietin receptors and can respond to rHuEpo with growth. However, this matter is far from being clear and ad hoc studies are urgently required.

**Conclusions**

Recombinant human erythropoietin is an important drug for many anemic patients but ‘as any drug’ has adverse effects. Rationality and active surveillance are required in order to maximize its benefits for anemic patients, to minimize adverse effects, and to avoid misuses or abuses.

**References**

Epoetin-induced autoimmune pure red cell aplasia

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Introduction

Patients have been treated with recombinant human EPO (rHuEPO or epoetin) since the late 1980s. Initially, the recombinant hormone was only given i.v. Several years later, the subcutaneous route became increasingly used, which facilitates self-administration and is believed to reduce dose requirements. In this context, epoetin-induced antibodies remained a very rare complication for many years, with only few case reports published.¹–³ A single case has also been reported in which a patient never exposed to epoetin developed PRCA due to autoantibodies against EPO.⁴ The critical issue in the development of anti-EPO antibodies in patients is that these do not only abrogate the effect of the recombinant molecule, but also neutralize residual naturally occurring EPO inducing an isolated inhibition of erythropoiesis (= pure red cell aplasia, PRCA). Since 1998, there is an upsurge of cases of epoetin-induced antibodies with severe anemia, in patients with kidney disease receiving subcutaneous treatment with epoetin.⁵,⁶ To date approximately 250 cases have been reported worldwide, the vast majority of those in association with the use of epoetin alfa outside the United States.⁷

PRCA is an isolated disorder of erythropoiesis that leads to a progressively developing, severe, isolated anemia with sudden onset.⁸ Factors known to be associated with PRCA include lymphoproliferative disorders, viral infections, in particular parvovirus B19, systemic autoimmune disease and drugs.⁹ About half of the cases of PRCA are classified as idiopathic, with no identifiable cause. The mechanisms inducing PRCA under these conditions have been shown to be of autoimmune origin. It has been demonstrated that sera from about one-half of patients show IgG antibodies suppression of in vitro growth of erythroid progenitors cells. T-cell-mediated erythroid toxicity has been shown to play a pathogenic role even in B-cell proliferations.

Diagnostic criteria for epoetin-induced PRCA are summarized in Table 1. Patients developing PRCA due to EPO antibodies have typically been on epoetin therapy for 6–18 months. The hemoglobin level then suddenly starts to decline, despite continued therapy with epoetin at the same or even increased doses.⁵ The shortest time interval between start of epoetin therapy and loss of efficacy observed in a single case was 2 months and the longest time interval 90 months.

Systematic analysis of cases reveals that patients also experience a significant drop of platelet counts, when developing PRCA. Whether this is related to direct effects of EPO in megakaryopoiesis or more complex interaction of platelet formation with erythroid precursor cells, is unknown. In any case, the decline in platelet does not seem to be clinically significant and in most cases thrombocytes do not fall below the normal range.

Definite diagnosis of EPO-antibody induced PRCA requires two confirmatory investigations; a bone marrow examination and the demonstration of anti-EPO antibodies in patient serum. These investigations should be performed in each suspected case. Cases in which the results of either the antibody test or the bone marrow examination are not available or inconclusive, should not be excluded from epidemiological surveys and also need to be reported to health authorities. In fact, databases maintained by manufacturers of epoetins contain a significant percentage of cases, for which the diagnostic work-up and evidence for epoetin-induced PRCA is incomplete.

Assays for anti-EPO antibodies

Since epoetin-induced PRCA is due to the production of neutralizing anti-EPO antibodies, the identification of these antibodies in serum samples is key to the diagnosis. Different tests have so far been developed, that all have advantages and limitations.

The radioimmunoprecipitation assay (RIPA) has been used in the initial paper that described the outbreak of cases of epoetin-induced PRCA.⁵ Briefly, the serum samples are incubated first with 125I-radiolabeled epoetin, and then with protein G, which binds IgG. The antibody/protein G complexes are captured by centrifugation, and the radioactivity of the pellet is measured. This test is a very sensitive one. So far, no false-negative result has been reported. Furthermore, follow-up of 29 patients who recovered from epoetin-induced PRCA shows that no patient had persistent signs of PRCA while the RIPA had become negative. This assay may not be able to detect the presence of low-affinity antibodies. Thus, there is a theoretical possibility to obtain false-negative results at very early stages of the disease.
The biosensor immunoassay (BIACore assay) is a sophisticated assay platform\(^\text{10}\) that has recently been applied to the detection of EPO antibodies by Steve Swanson and colleagues at Amgen. Briefly, epoetin is immobilized on the surface of a sensorchip, and serum samples are flown over the surface of this biosensor. Binding of anti-EPO antibodies is detected in real time according to mass accumulation on the biosensor surface. This test allows not only to detect the presence of anti-EPO antibodies, but also to easily determine their isotypes and their binding affinities. It is less sensitive than RIPA, with a sensitivity limit of about 100 ng/ml (S. Swanson, personal communication), but it can detect low-affinity antibodies. The major disadvantage of the biosensor assay is undoubtedly the price of the corresponding equipment (BIACore), which is so far only available in few high-tech laboratories.

Enzyme-linked immunosorbent assays (ELISAs) are quantitative methods that are widely used to detect antibodies, mostly because they are easy to implement. EPO is bound to multiwell plates and the binding of antibodies in patient serum is detected with the use of secondary antibodies. The major limitation is that depending on the washing conditions the specificity may be low (unspecific binding of antibodies to the plate) or the sensitivity may decrease (low-affinity antibodies being washed off). Increased sensitivity can be achieved by adding a second step, testing the replacement of bound secondary antibody by excess EPO. However, companies and research laboratories have tried to further increase the specificity by using a bridging ELISA. Briefly, in bridging ELISAs, the two arms of anti-EPO antibodies bridge between epoetin immobilized to the surface of a microtiter plate and biotinylated epoetin, that can be detected using a colorimetric assay. The microtiter plates coated with epoetin are thus incubated first with serum samples, and then with biotinylated epoetin, and it is this latter molecule that is detected using a colorimetric assay.

Based on the data summarized above, the RIPA is currently the ‘gold standard’ assay for detection of anti-EPO antibodies. When this assay becomes more widely used, it will be crucial not only to precisely standardize it, but also to ensure that it is implemented in a limited number of reference laboratories, in which confirmatory tests can be performed. This will be important not only for individual patient laboratories, but also for obtaining reliable epidemiological data regarding this rare disease.

While the tests described above can detect the presence of anti-EPO antibodies, they cannot assess their neutralizing capability. In vitro bioassays, the fourth assay category, are the only tests that can demonstrate the ability of antibodies to neutralize endogenous erythropoietin. They are based on the ability of the patient’s serum (or ideally of the patient’s immunoglobulins) to inhibit the growth in the presence of exogenous EPO of red blood cell precursors obtained from the bone marrow of healthy donors or of a cell line that depends on EPO for proliferation. Given the expenditure associated with this test, it does not appear to be indispensable for the diagnosis of epoetin-induced PRCA, and should probably only be performed in selected cases with an atypical clinical picture.

**Table 1** Diagnosis of epoetin-induced PRCA

<table>
<thead>
<tr>
<th>Major features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment with epoetin for at least 3 weeks</td>
</tr>
<tr>
<td>Drop of hemoglobin of 0.1 g/dl per day without transfusions of transfusion need of ~1 U per week to keep the hemoglobin level in an acceptable range.</td>
</tr>
<tr>
<td>Reticulocytes&lt;10000/mm³</td>
</tr>
<tr>
<td>No major drop of leukocytes and platelets</td>
</tr>
<tr>
<td>Minor features</td>
</tr>
<tr>
<td>Skin and systemic allergic reactions</td>
</tr>
<tr>
<td>Confirmational investigations</td>
</tr>
<tr>
<td>Bone marrow aspirate: Normal cellularity and &lt;5% erythroblasts</td>
</tr>
<tr>
<td>evidence of block of maturation</td>
</tr>
<tr>
<td>Serum: Presence of antierthropoeitin antibodies</td>
</tr>
</tbody>
</table>

**Characterization of anti-EPO antibodies**

Analysis of the anti-EPO antibodies detected in 13 patients with epoetin-induced PRCA has shown that they recognize the protein moiety of the molecule, since they bound native epoetin as well as deglycosylated epoetin.\(^\text{5}\) The epitope that is recognized by the antibodies seems to be almost always conformational, and denaturation of the protein completely abolished the binding of the antibodies in all but one of these 13 cases. In this latter case, the patient appeared to have produced antibodies against both a linear and a conformational epitope. Because of its conformational nature, the precise mapping of the epitope has not yet been successfully performed. Analysis of eight patients with epoetin-induced PRCA has shown that, in six cases, anti-EPO IgGs were mostly IgG4, while in the two remaining cases, they were mainly IgG1 (S Swanson, personal communication), which points to a role of T cells in the development of the antibody response.

**Epidemiology of anti-EPO antibody-induced PRCA**

During the first 10 years of therapy with epoetin, only three cases of antibody-associated PRCA have been described in patients treated with epoetin.\(^\text{1–3}\) While several millions of patients have received a treatment. Thus, the possibility for epoetin to induce the formation of anti-EPO antibodies was considered extremely low. However, since 1998, a significant increase in the number of cases of epoetin-induced PRCA has been found in patients with CKD.
The large majority of cases have occurred in patients receiving epoetin alfa produced by Ortho-Biotech and marketed outside the United States (i.e. in patients treated with Eprex/Erypo). Analysis of the data made available by this pharmaceutical company shows that between January 1998 and July 2003, 184 cases of antibody-mediated PRCA occurred in patients exposed to Eprex (subcutaneous route) either alone (169 cases) or in association with another epoetin (15 cases), and that 62 cases are still being investigated. During about the same period of time, Roche, the manufacturer of epoetin beta has reported eight cases of antibody-associated PRCA in patients with CKD exclusively exposed to epoetin beta. Since July 1997, six cases of epoetin-induced PRCA have been observed with epoetin alfa produced by Amgen and marketed in the US (i.e. Procrit, that is marketed by Ortho-Biotech, or Epogen, that is marketed by Amgen). Finally, Amgen has not reported any case in patients treated with darbepoetin alfa. Thus, the total number of cases of epoetin-induced PRCA to date is probably close to 250.

Surprisingly, analysis of the annual incidence of epoetin-induced PRCA per country shows very significant differences, even among different European countries; the European countries with the highest incidence of epoetin-induced PRCA being France and United Kingdom. For example, since 1998 there have been 34 cases of epoetin-induced PRCA among CKD patients reported in France and eight in Germany among CKD patient. The average annual incidence of epoetin-induced PRCA between 1998 and 2002 has been around 1.7 cases per 10,000 patients in France and 0.25 cases per 10,000 patients in Germany. The reasons for this difference are unclear. They cannot be explained by differences in market share between the different brands of epoetin in these countries. For each epoetin, the different European countries receive products that are coming from the very same factories, and thus there cannot be any difference in the manufacturing processes. Differences in the route of administration of epoetin exist, but they can only explain a minor part of the difference observed between Germany and France, even though Germany and Austria have been the two European countries with the largest proportion of dialysis patients treated with IV epoetin. Thus, it is possible that differences in storage and handling account for these differences in incidence of epoetin-induced PRCA.

Analysis of the total number of cases of epoetin-induced PRCA shows that it progressively increased to peak in 2001 and 2002. Similarly, in Europe, the annual incidence of epoetin-induced PRCA among CKD patients receiving Eprex peaked in the second half of 2001, and that it has been sharply decreasing since then. It went from about 4.5 cases per 10,000 patients in the second half of 2001 to 2.7 during the first half of 2002, 2.1 during the second half of 2002, and 0.5 during the first half of 2003. Analysis of all cases of epoetin-induced PRCA that occurred in France and Germany since 1998 also shows a similar trend; the number of cases being highest in the second half of 2001 and the first half of 2002 and only one case has been reported in 2003.

Interestingly, so far no case of epoetin-induced PRCA has been reported in cancer patients on chemotherapy, although the anemia of malignant disease has become a frequent indication for epoetin therapy. Only two cases have been observed in patients not treated for CKD-related anemia. Both were French patients with myelodysplastic syndrome. One was receiving epoetin alfa and the other one epoetin beta. Potential reasons for this apparent protection of cancer patients include shorter duration of therapy and unspecific immunosuppression.

Analysis of the route of administration of epoetin in patients who developed PRCA shows that the vast majority, if not all patients were receiving epoetin subcutaneously. In summary, therefore, the epidemiological data indicate that s.c. administration of epoetin is an important risk factor for the development PRCA but that additional factors associated with the use of the ex-US formulation of epoetin alfa played an important role.

Possible causes and risk factors of immunogenicity of epoetins

Although biopharmaceuticals are designed as copies of naturally occurring molecules, their immunogenicity is a well-recognized problem. There is probably not a single recombinant molecule used in clinical medicine, which has not been found to induce antibody formation in at least some cases. The consequences of such antibodies may vary widely. While many are clinically insignificant, others can reduce or even increase the efficacy of the recombinant molecules, and some, as in the case of epoetin, crossreact with the endogenous protein.

In general, such an immunogenic reaction can be triggered by a variety of factors, including sequence variations of the protein, differences in glycosylation, contaminants and impurities occurring during the production process, as well as components and properties of the formulation. In addition, factors such as individual predisposition, route of application and length of treatment play an important role.

The observation that s.c. application of epoetin is an important risk factor is in line with the fact that i.v. use of proteins is generally associated with the lowest risk of immunogenicity. Regarding the association with the ex-US formulation of epoetin alfa, there is no reason to believe that the amino-acid sequence of the molecule is different in this particular brand. Subtle differences exist between the carbohydrate moieties of epoetin alfa and beta, but whether they have any impact on immunogenicity remains unknown. Moreover, there is no indication that the glycosylation pattern of epoetin alfa has changed over time and could therefore explain the upsurge of cases.

The important clue apparently comes from the temporal coincidence with a change in the formulation of epoetin alfa to be sold outside the US. Upon request from the European Agency for the Evaluation
of Medicinal Products (EMEA; London, UK) the manufacturers of epoetin alfa removed human serum albumin (HSA) from the formulation, and replaced it with polysorbate 80 in order to avoid potential contamination by HIV and Creutzfeldt–Jacob disease-causing prions. The formulation of epoetin beta has always been HSA-free, but the stabilizer composition differs from that of HSA-free epoetin alfa. It has been postulated that this change in the formulation might have reduced the stability of the formulation, allowing the formation of aggregates of the molecule, in particular when handling instructions, such a cold storage, were not followed. Contamination with silicone, used to lubricate pre-filled syringes, has been considered as an additional risk factor. However, neither increased formation of aggregates has been demonstrated so far in vials of epoetin alfa, nor has a plausible explanation been provided, how such aggregates lead to a critical increase in immunogenicity.

More recently, Schellekens et al hypothesized an alternative explanation. They found that the concentration of sorbitol in the new formulation of epoetin alfa is so high that it leads to micelle formation and that epoetin molecules are integrated into the surface of these micelles. As a consequence, several epoetin molecules are presented to the recipient immune system in a regular spatial configuration, which appears to trigger the immune system. Similar phenomena have long been recognized in the immune recognition of virus, where antigens integrated into the virus wall at regular distance appear to play an important role.

It also remains possible that a contaminant present in the end product could act as an immunological adjuvant. In fact, recent investigations focus on potential release of chemicals from the rubber stoppers of prefilled syringes. These rubber stoppers were only used for epoetin alfa syringes produced by Ortho-Biotech (Eprex/Erypo), and they have meanwhile been replaced by Teflon stoppers.

To date, no patient-related risk factors have been identified that might have an impact on the development of epoetin-induced PRCA. Most patients who developed epoetin-induced PRCA did not have a history of autoimmune disease or of drug-induced immune reaction. Genotyping of 10 patients did not reveal any variation in the sequence of the endogenous EPO protein, which could have explained, why the recombinant molecule was considered as foreign, nor was there any obvious association with particular HLA haplotypes in a limited number of investigated cases (P Mayeux and N Casadevall, unpublished data).

### Natural course and response to therapy

Obviously, once the diagnosis of epoetin-induced PRCA is suspected or has been proven, epoetin therapy needs to be discontinued. As far as investigated, anti-EPO antibodies crossreact not only with the endogenous hormone, but also with all recombinant epoetin molecules, including darbepoetin alfa. Therefore switching the brand of epoetin does not improve the anemia and obscures the causality. Moreover, continued exposure to epoetin implies the risk of severe systemic immune reactions.

On the other hand, available data suggest that cessation of epoetin exposure alone is usually insufficient to induce recovery from epoetin-induced PRCA. Retrospective analysis of 47 cases of epoetin-induced PRCA showed that 10 patients did not receive any specific treatment, besides stopping the administration of epoetin. One patient died suddenly 6 weeks after the diagnosis. All other nine patients still have PRCA after a median follow-up of 12 months. In contrast, administration of immunosuppressive therapy appears to largely enhance the likelihood of recovery. Out of 37 patients who received some immunosuppressive therapy, in addition to stopping epoetin administration, anti-EPO antibodies disappeared and reticulocyte counts consistently rose above 20 000/mm³ in 78% of cases (n = 29). However, defining the optimal therapy is still somehow difficult.

Among the 37 patients who received immunosuppressive therapy, 26 received only one type of treatment, while 10 successively received two different immunosuppressive regimens and one who did not recover received five different treatments. Analysis of the different treatments is summarized in Table 2. Nine patients were treated with high doses of intravenous immunoglobulins alone, and recovery has only been

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No.</th>
<th>No. of patients who recovered (%)</th>
<th>Time before recovery (months)</th>
<th>Follow-up (months)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS (+IV Ig)</td>
<td>18</td>
<td>10 (56%)</td>
<td>1°, 2°, 2°, 3°, 3°, 3°, 3°, 3°, 6°, 18°</td>
<td>3, 3, 3, 5°, 13°, 20, 30°</td>
</tr>
<tr>
<td>IV Ig</td>
<td>9</td>
<td>1 (11%)</td>
<td>1°, 2°, 3°, 4, 5, 7</td>
<td>3, 4, 4, 4, 9, 10°, 19</td>
</tr>
<tr>
<td>CS + Cyc</td>
<td>8</td>
<td>4 (87%)</td>
<td>1°, 1°, 1°, 1°</td>
<td>3, 9°</td>
</tr>
<tr>
<td>CsA</td>
<td>6</td>
<td>6 (100%)</td>
<td>&lt;1°, &lt;1°, &lt;1°, &lt;1, &lt;1</td>
<td>3, 3</td>
</tr>
<tr>
<td>Kidney transplant</td>
<td>6</td>
<td>6 (100%)</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Anti-CD20 Ab</td>
<td>2</td>
<td>0</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>CS + IV Ig + PE</td>
<td>1</td>
<td>1</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>MMF</td>
<td>1</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*aFor patients who did not recover, follow-up being the length of time between start of treatment and last visit or start of a new treatment.

*bDenotes a patient who received only one kind of treatment IV Ig, high doses intravenous immunoglobulins; CS, corticosteroids; PE, plasma exchange; Cyc, cyclophosphamide; CsA, cyclosporin A; MMF, mycophenolate mofetil.
observed in one case. It occurred after the second course of intravenous immunoglobulins (0.4 mg/kg/day for 5 days every six weeks) and no relapse has been observed after 20 months of follow-up. In all, 18 patients have received corticosteroids alone (14 patients) or in association with high doses of intravenous immunoglobulins (four patients). The starting dose of corticosteroids ranged from 0.5 to 1 mg/kg/day. In all, 10 patients (56%) have recovered; the median recovery time being 3 months (range 1–18 months) and all but two patients being cured within 3 months. In one patient, treatment with plasma exchange, corticosteroids and high-dose intravenous immunoglobulin therapy led to recovery within 3 months. Seven out of eight patients (87%) who have received corticosteroids (started at a dose of 1 mg/kg/day) together with cyclophosphamide (given as monthly pulses in six cases and daily in two) recovered fairly rapidly (delay 1.2, 2, 3, 4, 5 and 7 months, respectively). Four of the six patients (67%) who received cyclosporin A recovered within 3 weeks. These four patients who recovered as well as one who did not recover received 200 mg/day of cyclosporin A, while the other patient who did not recover received 100 mg/day only. One patient who was treated with mycophenolate mofetil alone did not recover. Two patients who received anti-CD20 monoclonal antibodies did not recover, but follow-up was only 3 months. Six patients who received a kidney transplant recovered completely within 1 month. All these patients received induction therapy (using anti-IL2 receptor antibodies or anti-lymphocyte antibodies) and were treated with triple therapy including corticosteroids, a calcineurin inhibitor and mycophenolate mofetil.

In an attempt to identify prognostic factors independent of treatment, we focused on the 19 patients who received corticosteroids (with or without intravenous immunoglobulins), since they form the largest group. The prognosis was not correlated with age, gender, length of time receiving epoetin therapy, renal status (no dialysis, hemodialysis or peritoneal dialysis), hemoglobin levels at the time of diagnosis, anti-erythropoietin antibody titres at the time of diagnosis, or with the delay between the onset of PRCA and withdrawal of epoetin (data not shown). It is of note that in all cases with a regular follow-up of antibody titers, no increase in reticulocyte counts occurred as long as anti-erythropoietin antibodies could be significantly detected by RIPA.

**Conclusions**

Given that millions of patients are being treated with epoetins, the prevalence of this complication remains very low, but the dynamics of the increase in incidence have initially risen great concern. Meanwhile, causes and risk factors have become somewhat clearer, speculations that antibodies against epoetin are a frequent and unrecognized phenomenon have not been confirmed, and the incidence rates of epoetin-induced PRCA seem to have passed the peak. Nevertheless, clinicians need to be aware of signs and consequences of this complication.

Finally, this recent experience with anti-EPO antibodies may also have considerable implications for the future approval of epoetin preparations and other biopharmaceuticals. Irrespective of the fact that the genetic code precisely defines the amino-acid sequence of recombinant molecules, the impact on immunogenicity of any difference in the formulation and production process appears to be a real concern. In Europe, at least 10 companies have applied for approval of a follow-on biologic version (‘biogeneric’) of EPO. In view of the recent experience with a licensed product, health authorities face the question of how to specify safety requirements during phase III trials and postmarketing surveillance of these new drugs, without increasing the demands to a level that prevent their development.

**References**

Erythropoietin and erythropoiesis: polycythemas due to disruption of oxygen homeostasis

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¹Baylor College of Medicine, Houston, TX, USA; ²Hôpital de la Chaux-de Fonds, Neuchâtel, Switzerland


Oxygen sensing and erythropoiesis

An essential function of red blood cells is to deliver oxygen to tissues. Oxygen is at the center of red blood cell function and production. The function of red blood cell is to transport and deliver oxygen to tissues. Erythropoietin (Epo) is the main hormone regulating red blood cell production, and its serum level increases exponentially in response to anemia or to other hypoxic stimulus. The adaptation to hypoxia is critical for survival of organisms, and hypoxia sensing is at the center of mechanisms ensuring such adaptation. The activation of the hypoxia sensing results in the activation of an array of genes such as those implicated in glucose metabolism, vasculogenesis, iron transport, and erythropoiesis. The discovery of the hypoxia-inducible factor 1 (HIF1) by Semenza’s group and its role in the regulation of Epo, the principal agent controlling red blood cell production, provided the molecular basis for O₂-homeostasis and control of erythropoiesis. The HIF1α subunit is regulated by oxygen: in normoxia it is proline hydroxylated and binds to the von Hippel–Lindau (VHL) protein and is rapidly degraded by ubiquitinization. In hypoxia, HIF1α is stable and forms a heterodimer with HIF1ββ that activates a myriad of hypoxic-responsive genes including EPO, transferrin receptor, and VEGF. Both subunits of HIF1 are highly conserved genes between species. In mice, the analysis of HIF1α and HIF1β knockout animals have revealed that both genes were important in the embryonic development and survival. Study of the embryonically lethal HIF1β knockout embryos revealed an impaired hematopoiesis that could be partially corrected in vitro by the vascular-endothelial growth factor (VEGF). However, those changes may not reflect disruption of the hypoxia-sensing, as HIF1ββ is not regulated by hypoxia and is constitutively expressed in cells. In contrast, the HIF1α subunit level is regulated by hypoxia. We have shown that the disruption of HIF1α resulted in the block in the terminal erythroid differentiation, a phenotype that appears primarily linked to a disruption of iron metabolism.

Polycythemic disorders

Polycythemia is a physiological response to chronic hypoxia; however, congenital mutations disturbing the normal response to oxygen demands and causing polycythemias exist and their elucidations provide valuable lessons in the understanding of the mechanisms controlling erythropoiesis. The autosomal dominant polycythemia with low Epo (PFCP), caused in minority of such families by various gain-of-function EPO-receptor mutations, is an example of primary polycythemic defect caused by an exaggerated response of erythroid progenitors to low Epo levels.

Primary polycythemic disorders

Polycythemia vera: Polycythemia vera (PV) is the most common primary polycythemia characterized by clonal expansion of myeloid cells from an acquired mutation of a single stem cell. Its incidence has been estimated at 2.3 per 100 000 persons, with a median age of presentation at approximately 60 years. PV is associated with significant morbidity and mortality, including thrombotic/hemorrhagic events, and a risk of an evolution to leukemia or myelodysplastic syndrome. This contrasts with other polycythemic disorders where-in the progression to leukemia is not part of disease process and wherein the thrombotic/hemorrhagic events have a lower prevalence. The molecular basis of PV is unknown. The diagnosis of PV is based on criteria established by the Polycythemia Vera Study Group; but atypical cases may not be readily discriminated by these criteria.

PV is the result of a clonal expansion of single hematopoietic progenitor as established by assays based on X-chromosome inactivation. The PV bone marrow progenitor cells are able to form erythroid colonies in the absence of exogenous Epo in the presence of serum. A standardized in vitro assay has been developed that allow the demonstration of such ‘endogenous erythroid colonies’ (EEC). Although not performed by many routine laboratories, the demonstration of EEC is a hallmark of the disease and is useful for the differentiation of PV from secondary polycythemias and primary familial and congenital polycythemia (PFCP). Rarely patients with congenital polycythemias such as those with PFCP may form some EEC; in such cases, the use of anti-Epo and anti-EpoR neutralizing antibodies completely abolish the formation of EEC. In contrast,
the EEC in PV samples persist with the use of neutralizing antibodies, demonstrating that this PV feature is independent of Epo signaling. In serum-free cultures PV are hypersensitive to IGF-1; a hypersensitivity to other cytokines such as GM-CSF, SCF, thrombopoietin and interleukin-3 has also been reported. The expression of the thrombopoietin receptor was reported to be decreased in PV. Since the thrombopoietin receptor on platelets is the major regulator of circulating thrombopoietin, it results in an increased thrombopoietin level and enhanced platelet production. Recently, increased mRNA of a receptor named polycythemia rubra vera 1 (PRV-1) has been reported in PV granulocytes but not their progenitors. The exact function of PRV-1 in normal hematopoiesis is unclear and likely plays no significant role in PV pathophysiology since there are no differences in the amount of this protein between hematopoietic progenitors of PV patients and those of normal controls. Nevertheless, the use of PRV-1 assay may be a useful diagnostic marker of the disease. Dysregulation of the expression of a number of other genes, including Bel-xL has been observed in PV. Cytogenetic abnormalities are found in up to a third of PV patients (deletion of 20q, 13q, trisomy 8, trisomy 9 or trisomy of 1q are the most common); however, none is specific for PV and these abnormalities appear to be more common as the disease progresses. A potential localization of PV gene was recently proposed by the demonstration of an upregulation of p15 and p16 transcripts and a loss-of-heterozygosity (LOH) on chromosome 9p; duplication and other cytogenetic changes of the same chromosome 9 region were reported to be more frequent than in the expression of the thrombopoietin receptor in PV when more sophisticated techniques were used. Whether PV is due to a mutation of a single gene, or if it is a multigenic disorder or the result of epigenetic abnormalities remains to be established. However, recent demonstration that LOH of 9p does not segregate with PV in those rare families who inherit PV predisposition suggest that PV genesis results from multiple genetic events. Taken together, published data suggest that the putative PV defect affects signaling downstream from the cytokine receptors that results in a dysregulated responsiveness of PV cells to cytokine stimulation.

Primary familial congenital polycythemia (PFCP) is an autosomal dominant polycythemia characterized by low Epo, and an in vitro hyporesponsivity of the erythroid progenitors to Epo. In a minority of such families, various EPO-receptor mutations with a gain-of-function have been identified. Such mutations result in an erythropoietic defect characterized by an exaggerated response of the erythroid progenitors to low Epo levels. In other PFPC families, the molecular lesion is not known; the study of one of such PFPC pedigrees has suggested that a region in 7q22.1–7q22.2 may be associated with the PFPC phenotype, but the exact molecular defect in this family remains to be identified.

Chuvash polycythemia (CP) is an autosomal recessive high Epo disorder endemic in Chuvash province of Russia, the study that resulted in the recognition of the first congenital disorder of hypoxia sensing. CP is the only endemic polycythemic disorder first described by Polyakova in Russia among Asian-origin-inhabitants of Chuvash province in Russia. It is an autosomal recessive congenital disorder, with a normal to high serum Epo, and an in vitro hyporesponsivity of the erythroid progenitors to EPO. CP is caused by homozygosity for R200W mutation in the (VHL) gene, resulting in HIF1α upregulation and activation of hypoxic-responsive genes including EPO. Several polycythemic patients of various ethnic and racial groups worldwide were thereafter found with mutations of both VHL alleles, and homozygosity for another VHL mutation was also observed in a polycythemic Croatian boy. In a majority of patients though, the CP R200W mutation was identified at least on one chromosome. The clinical phenotype of CP is extending outside of erythropoiesis. The homozygotes have decreased lifespan mainly due to an increased incidence of strokes occurring regardless of hematocrit value. Further, there is an increased occurrence of varicose vein, and decreased blood pressure; however, there is no increase of cancers, especially those found in association with the VHL syndrome. Analysis of the haplotype in the VHL locus revealed that the R200W mutation occurred once in a single founder 20–50 000 years ago. VHL mutation is thus far the most common inherited molecular defect causing polycythemia. However, in at least half of cases of familial polycythemias with high Epo of either autosomal dominant or autosomal recessive inheritance, no VHL mutations are found. The molecular basis in such cases remains to be elucidated, but it is likely to involve the hypoxia sensing with consequences possibly similar to those observed in CP.

**Secondary polycythemias**

High Epo secondary polycythemias could be congenital or acquired. High-affinity hemoglobins due to globin gene mutations or BPG mutase deficiency, and congenital methemoglobinemia are examples of congenital secondary polycythemia. Polycythemia resulting from conditions leading to hypoxia, such as high altitude, or underlying diseases (cyanotic heart disease, chronic cor pulmonale, chronic obstructive lung disease, sleep apnea) are examples of acquired secondary polycythemia. CP shares features of both primary and secondary polycythemia.
The Hematology Journal 10 Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, such as hydronephrosis, Wilms’ tumor (adult and after globin gene mutations have been conclusively decreased delivery of oxygen to the peripheral tissues and compensatory polycythemia. BPGM mutation decreased BPG level shifts the hemoglobin oxygen dissociation curve to the left resulting in oxygen. A decreased BPG level shifts the hemoglobin configuration thereby modulating its ability to bind cells. It binds hemoglobin and allosterically changes its configuration. BPG is present in very high concentrations in red blood cells. a deficiency of the biphosphoglyceromutase (BPGM).

References
13 Prchal JT. Classification and molecular biology of polycythemias (erythrocytoses) and thrombocytosis. Hematol Oncol Clin N Am 2003; 17: 1151–1158, VI.

elevated hematocrit in the pathogenesis of PV. The best initial screening test to detect high oxygen affinity hemoglobin relies on the determination of hemoglobin dissociation kinetics and P50 (pressure of O2 where Hb is 50% oxygenated). If a co-oximeter is not available, P50 can be mathematically estimated from a venous blood gas measurement.40

A rare cause of congenital polycythemia is 2,3-bisphosphoglycerate (2,3BPG) deficiency, resulting from a deficiency of the biphosphoglyceromutase (BPGM). BPG is present in very high concentrations in red blood cells. It binds hemoglobin and allosterically changes its configuration thereby modulating its ability to bind oxygen. A decreased BPG level shifts the hemoglobin oxygen dissociation curve to the left resulting in decreased delivery of oxygen to the peripheral tissues and compensatory polycythemia. BPGM mutation should be considered in the setting of a low P50 and after globin gene mutations have been conclusively excluded.

Polycythemia in kidney and liver diseases. Polycythemia has been reported in association with kidney lesions such as hydronephrosis, Wilms’ tumor (adult and children), polycystic kidneys, and in lesions associated with the VHL syndrome (renal cell carcinoma, renal hemangioma, pheochromocytoma).41,42 Polycythemia/erythrocytosis has been frequently reported as a complication occurring after kidney transplantation. An entity known as ‘post transplant erythrocytosis (PTE)’ occurs in up to 20% of renal transplant recipients. The therapeutic effects of angiotensin-converting enzyme (ACE) inhibitors, and losartan (a specific antagonist of the angiotensin type 1(AT1)) receptor on post-transplant erythrocytosis/polycythemia in renal failure patients indirectly links angiotensin II with the regulation of erythropoiesis. AT1 receptor was detected on erythroid progenitors, suggesting that angiotensin II may play a direct role in the regulation of erythropoiesis.

Polycythemia is a known paraneoplastic manifestation of hepatocellular carcinoma in 2.5% of cases;44 polycythemia is secondary to the production of Epo. Polycythemia can also be observed in patients with obstruction of the hepatic venous outflow, the Budd–Chiari syndrome but these patients may have an overt or latent myeloproliferative disorder.


STEM CELLS

Nuclear transplantation, embryonic stem cells and the potential for cell therapy

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Nuclear transfer experiments in mammals have shown that the nucleus of an adult cell has the ability to direct the development of an entire organism, *id est* its genome is totipotent. However, these experiments did not conclusively demonstrate that the nuclei of terminally differentiated adult cells remain totipotent. It is possible that rare adult stem cells served as donors for the few surviving clones. To address this question, we have generated monoclonal mice from terminally differentiated lymphocytes that carry a single antigen receptor rearrangement in all tissues. Nuclear transfer technology may provide a powerful method for obtaining autologous cells for replacement therapy. We have demonstrated the feasibility of this concept by combining nuclear transfer with gene and cell therapy to treat the immune deficiency of Rag2 mutant mice, thus establishing a paradigm for ‘therapeutic cloning’. Moreover, we will discuss the potential use of nuclear transfer to study the role of reversible genomic (epigenetic) modifications during tumorigenesis.


Totipotency of differentiated cells

Nuclear cloning, also referred to as ‘nuclear transfer’ or ‘nuclear transplantation’, denotes the introduction of a nucleus from an adult donor cell into an enucleated oocyte to generate a cloned embryo. When transferred into a recipient female, this embryo can develop into an animal, a process termed ‘reproductive cloning’ (Figure 1). However, when grown in culture, this embryo can give rise to embryonic stem cells that have the potential to become most if not all cell types of the adult body (Figure 1). Since embryonic stem cells derived by nuclear transfer are genetically identical to the donor of the nucleus, and thus potentially useful for therapeutic applications, this process is called ‘nuclear transplantation therapy’ or ‘therapeutic cloning’.

Nuclear transplantation was developed 50 years ago in frogs to test whether nuclei from differentiated cells remain genetically equivalent to zygotic nuclei. Early attempts to clone frogs from adult cells were unsuccessful, and suggested that the nucleus of a differentiated cell could not support development of an animal. However, the generation of the clone sheep Dolly from an adult mammary gland cell demonstrated the ‘nuclear equivalence’ of at least some cells within an adult organism. This result showed that the oocyte cytoplasm can successfully reprogram the genomic modifications of an introduced adult donor nucleus to a state compatible with embryonic development.

Certain genomic modifications appear to undergo efficient reprogramming after nuclear transfer, exemplified by the reactivation of the silenced X chromosome in clones derived from female cells and the extension of telomere ends in cloned embryos. However, most DNA modifications are aberrantly reprogrammed after nuclear transfer, resulting in the dysregulation of hundreds of genes in cloned mice. This may result in the abnormalities frequently observed in cloned animals, such as placental enlargement and foetal overgrowth, respiratory distress, liver, kidney and brain defects. These defects are found in clones regardless of the donor cell type (embryonic stem cells, fibroblasts, cumulus cells, etc) used for nuclear transfer. In contrast, the efficiency of obtaining cloned animals appears to be dependent on the type of donor cell. For example, the nuclei of embryonic stem (ES) cells are 10–20 times more efficient in producing cloned animals than the nuclei of adult cells such as fibroblasts or cumulus cells, suggesting that less-differentiated cells are more easily reprogrammed.

The latter notion, together with the isolation of rare adult stem cells from a variety of tissues and reports of their developmental plasticity, raised an important question: are viable cloned animals derived only from adult stem cells selected by chance from the heterogeneous donor cell population? These cells might be similar to embryonic stem cells, which require less reprogramming and support development at high efficiency.
To prove that the nucleus of a terminally differentiated cell can be reprogrammed by nuclear transplantation, we used nuclei with a stable genetic marker that, in retrospect, enabled the unequivocal identification of the donor cell that gave rise to the clone. Using mature lymphocytes and their differentiation-specific immune receptor rearrangements as a genetic marker, we showed that fertile adult mice can be generated from terminally differentiated cells.11 Animals were produced in a modified two-step cloning procedure: ES cells were first isolated from cloned blastocysts and then injected into tetraploid host blastocysts to generate cloned mice. In this approach, mice are derived entirely from the injected ES cells, whereas the placenta is derived from the tetraploid host cells (generated by electrofusion of a normal fertilized two-cell embryo). Monoclonal mice produced from B or T cells carried the genomic rearrangement of the donor lymphocyte in every tissue. This was shown by Southern blot analyses using probes that detect the rearrangement-specific deletions at the immunoglobulin and T-cell receptor loci as well as by DNA sequencing of the respective rearrangements (Figure 2). These experiments demonstrated unequivocally that the nucleus of a fully differentiated cell can be reprogrammed to a totipotent state.

Cloning efficiency is extremely poor with terminally differentiated cells

To obtain cloned mice from lymphocytes, we had to use a modified two-step procedure that involved the explantation of cloned embryos and the isolation of ES cells. Notably, no laboratory has yet succeeded in generating adult mice by directly transferring embryos cloned from lymphocytes into recipient females. These observations suggest that reactivation of embryonic genes in explanted cloned blastocysts might be under fewer time constraints than cloned blastocysts transferred directly to recipient females. Alternatively, the culturing process of the explanted blastocysts might select for those few cells within the embryo that have undergone successful reprogramming.

The generation of cloned mice from mature lymphocytes established that the nuclei of terminally differentiated cells remain totipotent. However, cloning from lymphocytes remained extremely inefficient. The
number of cloned embryos that developed to the blastocyst stage was about ten times lower (4%) as compared to cloned embryos derived from more heterogeneous adult cell populations such as fibroblasts or cumulus cells (30–70%). This suggested that adult cells other than fully differentiated cells are the nuclei donors in most if not all successful nuclear transfer experiments. It is therefore possible that many cloned animals generated by the ‘traditional’ cloning procedure are in fact derived from the nuclei of adult stem cells or progenitor cells and not from the nuclei of terminally differentiated cells. The use of purified adult stem cells, such as haematopoietic or neural stem cells, as donors in nuclear transfer experiments will clarify whether these cells are indeed more amenable to reprogramming than heterogeneous adult cell populations. The identification of the most efficient donor cell type for nuclear transfer may also be important for the therapeutic application of nuclear transfer (therapeutic cloning).

**Therapeutic cloning**

In addition to its use in studying nuclear changes during differentiation, nuclear transfer technology has significant therapeutic potential. The concept of ‘therapeutic cloning’ is to generate an autologous embryonic stem-cell line from a patient’s cell for subsequent tissue replacement therapy (Figure 1).

Immune rejection is a frequent complication of allogeneic organ transplantation due to immunological incompatibility between donor and recipient. To treat this ‘host versus graft’ disease, immunosuppressive drugs are routinely administered to transplant recipients, a treatment with serious side effects. ES cells derived by nuclear transplantation are genetically identical to the patient’s cells, thus eliminating the risk of immune rejection and the requirement for immunosuppression. Moreover, ES cells provide a renewable source of tissue for replacement, allowing repeated therapy when necessary.

**Generation of a mouse model for therapeutic cloning**

In an attempt to establish a mouse model of therapeutic cloning, we have combined nuclear cloning with gene and cell therapy to treat a genetic disorder. Specifically, we chose the well characterized Rag2 mutant mouse that suffers from severe combined immune deficiency (SCID), a disease resembling human Omenn’s syndrome. These mice are devoid of mature B and T cells due to a mutation in the recombination activating gene 2 (Rag2), which catalyses immune receptor rearrangements in lymphocytes.

In a first step, we isolated somatic donor cells (fibroblasts) from the tails of Rag2-deficient mice and injected their nuclei into enucleated oocytes (Figure 3). The resultant embryos were cultured to the blastocyst stage and autologous ES cells were isolated. Subsequently, one of the mutant Rag2 alleles was targeted by homologous recombination in ES cells; we selected for ES cells that had successfully replaced one of the defective alleles with a repair construct, thus restoring normal gene structure to the Rag2 locus. To obtain the particular somatic cell type required for the treatment of Rag2−/− mice, these ES cells were differentiated into ‘embryoid bodies’ and further into haematopoietic precursors. To further expand these precursors, we infected cells with a retrovirus expressing the homeodomain transcription factor HoxB4 that has been shown to enhance self-renewal of haematopoietic stem cells. Resulting cells were shown by FACS analysis to express markers of haematopoietic stem cells and were transplanted into irradiated Rag2-deficient animals to treat the disease.

Initial attempts to engraft these cells were, however, unsuccessful due to an increased level of natural killer (NK) cells in the Rag2 mutant hosts. ES cell-derived haematopoietic cells express low levels of the major histocompatibility complex I (MHC-I) molecule and are thus preferred targets for NK-mediated destruction. This type of rejection is different from the classical host-versus-graft disease that is seen in allogeneic organ transplantation and thus specific to the Rag2 mutant mouse model. Elimination of NK cells by antibody depletion or genetic ablation enabled the ES cells to efficiently populate the myeloid and, to a lesser degree, the lymphoid lineages. Functional B and T cells, which had undergone proper rearrangements of their immunoglobulin and T-cell receptor alleles as well as serum immunoglobulins, were detected in the transplanted mutants.

This experiment was the first to demonstrate that nuclear transfer can be combined with gene therapy to...
treat a genetic disorder. Consequently, therapeutic cloning should be applicable to other diseases of which the genetic lesion is known, for example, sickle cell anaemia or \beta-thalassemia. Importantly, recent results have shown that human embryonic stem cells can be generated by nuclear transfer from adult cells thus demonstrating the feasibility of ‘therapeutic cloning’ in humans.14

Reprogramming of the cancer genome by nuclear transfer?

Accumulating evidence shows that tumor formation is accompanied by both epigenetic and genetic alterations of the genome.15–17 Unlike genetic changes, epigenetic changes do not alter the primary DNA sequence and are therefore reversible. Examples of such epigenetic modifications are the methylation of DNA and histones, the acetylation/deacetylation of histones, and the packing of chromatin into euchromatic and heterochromatic regions.18 Epigenetic modifications play an important role during normal development by regulating gene expression through stable activation or silencing of differentiation-associated genes. Similarly, epigenetic changes can promote cell proliferation, inhibit apoptosis, and induce angiogenesis during tumorigenesis by activating oncogenes and silencing tumor suppressor genes.16,17 For example, the p16 and VHL tumor suppressor genes are frequently silenced in human cancer by methylation of their promoter regions.19 Moreover, the treatment of tumor cells with methylation and histone modifying drugs can inhibit malignancy and this inhibition correlates with the reactivation of important tumor suppressor loci.20

Nuclear transplantation of lymphocytes has demonstrated that the epigenetic modifications associated with differentiation can be reprogrammed following nuclear transfer while the irreversible genomic rearrangements were retained in cloned animals.21 Thus, nuclear transfer provides a tool to reprogram selectively the epigenetic component of a cancer cell genome to study its effect on development and tumorigenesis.

To that end, we have performed nuclear transfer of different tumor cells to derive embryonic stem cells that were then used to assay their developmental and tumorigenic potential. Preliminary results indicate that cells from different embryonic carcinomas and those from a RAS-inducible melanoma model can be reprogrammed into embryonic stem cells. However, the developmental and tumorigenic potential of these embryonic stem cell lines in chimeric animals appears to be very different for the two types of tumors.

References

Molecular basis for stem-cell self-renewal

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All mature blood cells originate from a small population of stem cells (HSC), defined by their ability to generate two identical daughter cells (self-renewal), and to produce progeny committed to differentiate along any of the hematopoietic lineages (differentiation). In mouse, a small number of HSCs is specified around day 9.5 post conception. This population expands in fetal liver to approximately 5000 cells within 5 days, and thereafter remains relatively stable throughout the lifetime. After specification, the balance between expansion and maintenance of HSC populations depends on three potential outcomes of stem cell division: (i) a symmetrical division that generates two qualitatively equivalent daughter HSCs and results in increased HSC numbers; (ii) an asymmetrical division that enables maintenance of the HSC population along with ensuring production of mature blood cells, by generating one cell that retains stemness, and one that commences lineage commitment; and (iii) a symmetrical division that yields committed progeny only, and thus leads to extinction of the HSC clone. The classical model of HSC proliferation proposed by Till et al⁴ predicts the stochastic nature of self-renewal events. Today, there is a growing body of data suggesting that the fate of HSC progeny may also be affected by the combination of intrinsic and extrinsic factors that could act before or after HSC division. In the model proposing postmitotic fate determination, a single molecular program common to a variety of stem cells regulates self-renewal, and the fate of daughter cells is determined by extrinsic factors, such as signals provided by osteoblasts in the bone marrow microenvironment.⁵,⁶ Recent data from our laboratory, however, imply the existence of two distinct molecular programs for self-renewal: (i) SR-E promotes symmetrical divisions leading to HSC expansion, is active during embryogenesis and is transiently reactivated post-transplantation, and (ii) SR-M, that regulates asymmetrical divisions required for maintenance of the HSC pool and homeostasis. In these models, the premitotic intrinsic determinants regulate the fate decisions, and extrinsic factors regulate the switch between the two programs.

The growing therapeutic use of stem-cell transplantation and potential applications of in vitro HSC expansion have focused attention on defining extrinsic and intrinsic regulators of HSC self-renewal. Among positive regulators of HSC expansion, Flt3 ligand, Steel, and IL-11 appear to be essential,⁴,⁵ and bone morphogenetic protein⁶ and Wnt3a⁷ can provide substantial additional stimulatory effects. However, increased understanding of the intrinsic molecular mechanisms that regulate HSC divisions appears to be crucial to achieve greater expansions of HSC populations with long-term repopulating lympho-myeloid potential. Identification and enumeration of HSCs is an essential component of these studies. HSC populations, as identified by a variety of cell surface markers, their metabolic status and cycling activity, are still highly heterogeneous with respect to their ability to sustain self-renewal over successive generations.⁸,⁹ The numbers of such HSCs in samples can be determined operationally using competitive repopulation unit (CRU) assay, a limiting dilution transplantation-based assay for cells with competitive, long-term lymphomyeloid repopulation function.¹⁰ Test populations of bone marrow cells, identifiable by distinct phenotypic and/or genetic markers are transplanted into sublethally irradiated hosts in doses spanning 2–4 logs. For each recipient, the contribution of transplanted cells to repopulation of myeloid (Mac-1), B-lymphoid (B-220) and/or T-lymphoid (CD3) populations is determined 12–16 weeks post-transplantation, and mice with more than 1% of donor-derived cells in all lineages are considered to be reconstituted with transplant-derived cells. CRU frequencies in the test sample are then calculated by applying Poisson statistics to the proportion of negative recipients at different dilutions. This assay was used for evaluation of HSC numbers in all studies reported below.

Our initial focus on a role the Hox family of homeodomain transcription factors might have in regulation of hemopoiesis was based on observation that several Hox genes are highly expressed in bone marrow populations enriched for HSC content, and that their expression decreases in populations of clonogenic progenitors.¹¹ Today, there is growing body of data demonstrating that deregulated Hox gene expression has effects on hemopoietic proliferation and differentiation ranging from blocked lymphoid development to acute myeloid leukaemia.¹² A notable exception among HOX proteins is HOXB4, whose overexpression promotes HSC self-renewal without impairing the normal production of mature cells, or inducing leukemic transformation. Upon transplantation, bone marrow-derived HSCs re-expand for a short period of time, but fail to achieve numbers determined in normal mice.¹³ In contrast, retroviral overexpression of HOXB4 promotes the
in vivo HSC expansion up to, but not above, the normal HSC levels indicating that HOXB4 does not over-ride the regulatory mechanisms that maintain the HSC pool within its normal range. As observed with control HSCs, the major proportion of expansion occurs within the first weeks after transplantation. After the initial expansion phase, HOXB4-transduced HSCs exhibit proliferation activity similar to that determined for nontransduced cells, suggesting a transient nature of conditions that enable self-renewal. Most notably, HOXB4 has a unique capacity to induce 40–50-fold expansion of fully competent HSCs in short-term liquid cultures, indicating that this homeoprotein represents an intrinsic regulator of HSC self-renewal (SR-E) with therapeutic potential.

To achieve in vitro HSC expansion by direct HOXB4 protein delivery, rather than through gene transfer, we modified HOXB4 protein with the addition of the protein transduction domain from human immunodeficiency virus-derived transactivating protein (TAT). In bone marrow cells engineered to overexpress HOXB4, the magnitude of the in vitro proliferation response correlated with the levels of HOXB4 protein, and we estimated that the in vitro HSC expansion would likely require at least 10–20 nM TAT-HOXB4. TAT fusion proteins moved freely between media and intracellular compartments. However, the majority of TAT-HOXB4 protein is lost after a 4-h incubation in serum-containing media and the half-life of intracellular HOXB4, as determined by pulse-chase analysis, is only approximately 1-h. Based on these observations, we introduced TAT-HOXB4 protein in our bone marrow cultures every 3-h for 4 consecutive days, and then determined the total and clonogenic progenitor cell (MNC and CFC) numbers, and absolute numbers of HSCs. In cultures of bone marrow cells enriched for HSC content, based on Sca-1 expression and absence of lineage markers (Sca-1−/Lin−), the addition of 20 nM TAT-HOXB4 had only a modest effect on total cell yields, whereas CFCs increased approximately 2-fold compared to controls. The effect of TAT-HOXB4 on HSC numbers was evaluated using CRU assays at the beginning (t0) and end (day 4) of culture, and HSC frequencies were determined at 16–18 weeks after transplantation. During 4-day treatment, the HSC numbers in control cultures supplemented with BSA or TAT-GFP decreased to less than 50% of the input values. In contrast, there was a net 4–8-fold increase in HSCs in cultures supplemented with TAT-HOXB4, for a total 13-fold difference between the two populations. The expanded HSCs exposed to TAT-HOXB4 retained unimpaired differentiation potential, and contributed to the generation of myeloid (Mac-1+, GR-1+), B-lymphoid (B-220) and T-lymphoid (CD4+, CD8+) cells. Together, these observations provided proof of principle that net in vitro expansion of HSC is achievable using a recombinant soluble transcription factor such as TAT-HOXB4.

To further enhance the expansion of HSCs engineered to overexpress HOXB4 we next explored the role PBX1 (a homeodomain protein that cooperates with HOX proteins in DNA binding) might play in HOXB4-induced HSC self-renewal. The proliferation and differentiation potentials of HSCs engineered to co-overexpress HOXB4 and PBX1 were indistinguishable from those determined for HOXB4-transduced cells. Moreover, a HOXB4 mutant that lacks the ability to interact with PBX in cooperative DNA binding, retains the capacity to induce similar in vitro HSC expansion as wild-type HOXB4, suggesting that PBX is not required for the distinct HOXB4 activity in HSCs. To explore the possibility that the HSC-expanding capacity of HOXB4 occurs through direct, PBX-independent regulation of target genes by HOXB4 protein, we knocked down the expression of PBX1 (PBX1 K.D.) in HOXB4-overexpressing cells using an antisense cDNA approach. Mouse bone marrow cells were infected with recombinant retroviruses encoding antisense PBX1 cDNA and yellow fluorescent protein (PBX1 K.D-YFP), or HOXB4 and green fluorescent protein (HOXB4-GFP), or the combination of both viruses. In cultures initiated with a mixture of nontransduced, PBX1 K.D-YFP, HOXB4-GFP and HOXB4-GFP plus PBX1 K.D-YFP cells, the doubly transduced cells became the predominant cell type within 4–6 days of incubation, while the proportions of nontransduced control and PBX1 K.D decreased significantly. During 12-day expansion, HOXB4-PBX1 K.D clonogenic progenitors (CFCs) increased approximately 2.5-fold above the levels determined for HOXB4 cells, for a net 1600-fold expansion over input, while the numbers of control and PBX1 K.D CFCs increased only 6–7-fold above input values. Knocking down PBX1 levels alone thus generated no proliferative advantage to the transduced cells, while reducing levels of PBX1 in HOXB4-transduced cells increased their in vitro proliferation potential above the values determined for HOXB4-transduced cells.

The effect of PBX1 knock down on the repopulation potential of HOXB4-transduced HSCs was evaluated in groups of bone marrow transplantation chimeras generated by transplanting a mixture of HOXB4 cells (10% of total) and nontransduced cells (Group I), or a mixture comprising 10% HOXB4-PBX1 K.D, and 15% HOXB4, and 30% PBX1 K.D cells (Group II). In Group I recipients, the proportions of HOXB4(GFP+) PBL cells steadily increased over time to represent more than 50% of the PBL at week 12 post-transplantation. Conversely, in Group II recipients, the proportions of HOXB4(GFP+) PBL remained at the input levels, and the proportions of HOXB4-PBX1 K.D (GFP+ and YFP+) cells increased to more than 50%, while PBX1 K.D (YFP+) cells could no longer be detected at week 12 post-transplantation. Thus, the repopulation advantage conferred by PBX1 K.D occurred only in the context of HOXB4 overexpression. However, these studies did not clarify whether the competitive reconstitution advantage of HOXB4-PBX1 K.D cells occurred at the level of stem cells, or in more mature progenitors. The in vivo expansion of HOXB4-PBX1 K.D stem cells was evaluated by CRU assay, and was compared to expansion of HOXB4 cells within the Hematology Journal.
the same Group II recipient. Results of these assays showed that 14 HOXB4 HSCs regenerated only about 5% of the HSC pool when transplanted together with 11 HOXB4-PBX1 K.D HSCs, whose progeny expanded approximately 1000-fold to regenerate the HSC pool up to the levels determined for normal mice. Therefore, the competitive repopulation advantage of HOXB4-PBX1 K.D over HOXB4 cells occurred at the HSC level, suggesting that PBX1 acts as an intrinsic negative regulator of HOXB4-promoted self-renewal.

Recent studies suggest that niche capacity may determine the total HSC numbers. However, upon transplantation in myeloablated hosts HSCs regenerate only a proportion of the normal HSC pool, indicating that empty niches are not capable of inducing self-renewal divisions (SR-E), but may be required for maintenance of HSCs (SR-M). Overexpression of HOXB4 bypasses the mechanism(s) that limit HSC expansion during reconstitution and promotes SR-E. The niche capacity, however, is the dominant determinant that limits total size of the HSC pool. These models predict that the in vitro conditions that bypass the constraints imposed by niche availability, together with reduction of PBX1 levels, might enable a considerable ex vivo expansion of HOXB4-transduced HSCs. We are currently testing this hypothesis, and the preliminary results are very encouraging.

Results of the studies presented above identify HoxB4 as a true enhancer of self-renewal. HoxB4 deficiency, however, creates only a mild hemopoietic defect, suggesting that HoxB4 is not essential for SR-E and that other Hox proteins may compensate for the absence of this stem-cell regulator. A quantitative assessment of Hox gene expression in fraction of day 14.5 mouse fetal liver nor bone marrow-derived myeloid and lymphoid progenitors are severely reduced compared to WT mice. In contrast, the numbers of Bmi-1−/− fetal liver-derived clonogenic progenitors are only marginally affected compared to littermate controls. Complementation of Bmi-1 deficiency by retrovirus-mediated Bmi-1 gene transfer also revealed that HSC numbers in Bmi-1−/− fetal liver were similar to those determined in control mice. Moreover, overexpression of Bmi-1 failed to induce expansion of WT HSCs. Together, these observations suggest that Bmi-1 is not required for HSC specification and expansion, but is essential for their maintenance (SR-M).

In contrast to Hox4 genes implicated in HSC expansion (SR-E), the polycomb group gene (PcG) Bmi-1 appears to be essential for HSC maintenance (SR-M) (reviewed in Lessard and Sauvageau). Mice lacking Bmi-1 develop bone marrow failure with progressive decline in all blood cells leading to their death in early adulthood. In adult mice, both the numbers and the proliferation activity of bone marrow-derived myeloid and lymphoid progenitors are severely reduced compared to WT mice. In contrast, the numbers of Bmi-1−/− fetal liver-derived clonogenic progenitors are only marginally affected compared to littermate controls. Complementation of Bmi-1 deficiency by retrovirus-mediated Bmi-1 gene transfer also revealed that HSC numbers in Bmi-1−/− fetal liver were similar to those determined in control mice. Moreover, overexpression of Bmi-1 failed to induce expansion of WT HSCs. Together, these observations suggest that Bmi-1 is not required for HSC specification and expansion, but is essential for their maintenance (SR-M).

In summary, studies presented in this paper indicate the existence of two distinct molecular programs that regulate HSC self-renewal during expansion (SR-E) and maintenance (SR-M), and identify Hox4 and Bmi-1 genes as intrinsic determinants of SR-E and SR-M, respectively.

References


6 Bhardwaj G, Murdock B, Wu D, Baker DP, Williams KP, Chadwick K et al. Sonic hedgehog induces the proliferation...


8 Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell 1997; 91: 661–672.


17 Kros J, Beslu N, Mayotte N, Humphries RK, Sauvageau G. The competitive nature of HOXB4-transduced HSC is limited by PBX1: the generation of ultra-competitive stem cells retaining full differentiation potential. Immunity 2003; 18: 561–571.


Chronic myeloproliferative disorders: molecular markers and pathogenesis

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Myeloproliferative disorders (MPD) are a heterogeneous group of diseases characterized by increased numbers of nonlymphoid cells and/or platelets in the peripheral blood. In addition to thrombotic and hemorrhagic complications, leukemic transformation can occur. The classical definition of MPD included four disease entities, which were thought to be related: chronic myelogenous leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET) and idiopathic myelofibrosis (IMF). The Philadelphia chromosome and the BCR/ABL fusion transcripts established CML as a separate entity. The cause of the three remaining forms of MPD remains unknown, but progress has been made in defining the functional and molecular characteristics of MPD.

Sporadic MPD

The majority of patients with MPD have no family history and the disease is thought to result from somatic mutation(s) at the hematopoietic stem cell level. With the exception of some cases of ET, MPD is a clonal stem cell disorder. The phenotypic consequences of the MPD mutation(s) can be seen in altered cellular responses and deregulated gene expression. Chromosomal aberrations are likely to be linked to disease progression.

Altered responses of hematopoietic progenitors towards cytokines: BFU-E colonies that grow in the absence of added growth factors, also called ‘endogenous erythroid colonies’ (EEC), are present in virtually all patients with PV.2–4 EECs have been widely used as an auxiliary diagnostic assay to distinguish PV from secondary or familial polycythemia3 and presence of EECs is included in the new WHO diagnostic criteria for PV.5 The abnormal in vitro growth of hematopoietic progenitors in MPD inspires studies that focused on altered cytokine receptor signaling. However, no mutations in the obvious candidate genes, such as EPO, EPO-receptor (EPOR), IGF-1 receptors or the TPO-receptor (c-MPL) were detected.6–7 Increased phosphatase activity attributed to a putative membrane-associated erythroid protein tyrosine phosphatase was described in erythroid cells from PV patients. Recently, this activity was identified as the phosphatase PTP-Meg2.8 The STAT proteins are common effectors of cytokine receptor signal transduction. When the STATs have been closely examined, constitutive activation of STAT3 have been reported in 4/14 PV individuals.9 It remains to be determined whether these alterations are connected with the primary events in the pathogenesis of PV, or represent secondary changes in the course of the disease. Imatinib mesylate was shown to have a beneficial effect in patients with PV by reducing red cell mass.10 A recent report confirmed that the EEC formation in PV is inhibited by imatinib, suggesting that EPO-independent erythropoiesis may be caused by a hyperactive protein tyrosine kinase that is sensitive to imatinib.11

Deregulated gene expression in MPD

Although no mutations in the c-MPL gene were detected in MPD, markedly decreased expression of MPL protein in platelets can be observed in a subset of MPD patients.7,12 Initially, decreased MPL expression in platelets was thought to be a diagnostic marker for PV that may replace the technically demanding and time-consuming EEC assay.12 However, more recent reports found c-MPL protein to be decreased in only approximately 50% of PV, ET and IMF patients.4,13,14 Furthermore, decreased c-MPL was found in reactive thrombocytosis13 and in hereditary thrombocytemia caused by a TPO gene mutation,4 suggesting that this alteration can also be caused by a different molecular mechanism. The mRNA for polycythemia rubra vera 1 (PRV-1, also called NB1, CD177 or HNA-2a) was found to be elevated in granulocytes of PV patients (Figure 1).16 Surprisingly, analysis of PRV-1 did not reveal consistent differences in protein levels between PV and normal granulocytes.17,18 Several studies confirmed a strong correlation (range of 70–100%) between the growth of EECs and increased granulocyte PRV-1 mRNA expression in PV, ET and IMF.4,10–21 Interestingly, increased expression of the transcriptional coactivator ‘high mobility group protein A2’ (HMG-A2) was recently described as the first molecular marker for IMF.22 Both PRV-1 and HMG-A2 are markers that may prove to be valuable for the diagnosis or PV or IMF, respectively. Further gene expression studies will undoubtedly provide a more complete picture of the differences between normal and MPD cells and may reveal markers with prognostic value for predicting disease progression or complications.
Chromosomal abnormalities and other genetic alterations

Cytogenetic abnormalities are present in only a minority of MPD patients (primarily patients with IMF or PV, but rarely in ET). Deletion of chromosome 20q is the best characterized abnormality in MPD (10–15% of patients). A minimal common deleted region on 20q of 1.6 Mb was defined and a number of candidate genes within this interval are being tested for a possible role in the pathogenesis of MPD. Although the most likely genetic mechanism associated with 20q deletions is tumor suppressor gene inactivation, other mechanisms such as haploinsufficiency cannot be ruled out. Characterization of the target gene(s) within the common deleted region on 20q would greatly contribute to our understanding of clonal evolution of MPD and the mechanisms leading to leukemic transformation. Another frequent chromosomal alteration was recently described for the short arm of chromosome 9; acquired uniparental disomy resulting in loss of heterozygosity of 9p is detectable in up to 30% of PV patients and may constitute the most frequent chromosomal aberration.4 Other aberrations affecting 9p and occurring at high frequency were reported recently.25 It remains to be determined whether any of these alterations constitutes a primary event involved in the initial pathogenesis or represents secondary hits possibly linked to disease progression. Other less frequent cytogenetic abnormalities include trisomies of chromosomes 8 or 9, duplication of parts of chromosome 1q and deletions of chromosome 5 or 7.

Hereditary MPD-like syndromes

Inherited syndromes that resemble MPD offer a unique opportunity to gain insights into the molecular pathophysiology since genetic linkage analysis and positional cloning allow identifying the disease-causing genes. Hereditary MPD-like syndromes are a heterogeneous group of disorders. Mutations in the TPO, MPL, EPOR and VHL genes have been described to date. The clinical manifestations range from single lineage disease with hyperactive erythropoiesis or megakaryopoiesis to disorders affecting multiple lineages and resembling sporadic PV or ET.

Hereditary thrombocytosis (HT)

Mutations in the TPO gene can cause overproduction of TPO.26 The mutations increase the translational efficiency for the mutant TPO mRNA and result in thrombocytosis with high TPO serum levels.27 The penetrance is usually 100% and high platelet count is already present in childhood or at birth. The clinical course is mild with occasional thrombotic or bleeding complications, but without leukemic transformation. TPO mutations that cause HT have not been found in patients with sporadic ET.28 TPO mutations account for only about 20% of HT. In the remaining 80% of HT families the disease-causing gene still remains unknown. Thus, one or several additional genetic loci can cause HT. Recently, a Ser505Asn mutation in the transmembrane domain of c-MPL was reported in a family with autosomal dominant HT and eight affected family members.29 The disease phenotype is mild and no leukemic transformation was observed.

Hereditary erythrocytosis (HE)

Mutations in the EPO-receptor (EPOR) gene result in hyperactive erythropoiesis with autosomal dominant inheritance. Affected HE family members display elevated erythrocyte mass, low serum EPO levels, polyclonal hematopoiesis, and normal platelet and leukocyte counts. In contrast to PV, patients with HE usually do not display EECs, but the erythroid colonies are hypersensitive to low concentrations of EPO.20,30 To date, nine different disease-causing EPOR alleles have been described. These mutations all result in truncations of the cytoplasmic domain of the EPOR protein. As a
consequence, these EPOR are hypersensitive to EPO. Truncations of the EPOR have so far not been detected in patients with sporadic MPD. EPOR mutations account for only 10–20% of all HE. In the remaining families the disease-causing gene remains unknown. Interestingly, no mutations in the EPO gene have been observed in HE families to date. Recently, the cause for an autosomal recessive syndrome of erythrocytosis with high EPO serum levels was elucidated. An Arg200Trp mutation in the von Hippel-Lindau (VHL) gene was shown to cause the disease. The VHL protein is an E3 ubiquitin ligase that is involved in the degradation of the transcription factor HIF1alpha, the primary regulator of EPO transcription. Thus, partial loss of VHL function through point mutations leads to increased levels of HIF1alpha and overproduction of EPO.

**Hereditary multilineage MPD-like syndromes and other familial MPDs**

In several pedigrees a phenotype clearly distinct from HT and HE can be observed: affected family members display presence of EEC, clonal hematopoiesis and involvement of more than one lineage. The phenotype of this inherited syndrome is indistinguishable from sporadic MPD. The presence of clonal hematopoiesis implies that in addition to a predisposing germ line mutation, acquired somatic mutations play a role in the pathogenesis of the disease. Given the close resemblance with sporadic MPD, it is likely that elucidation of the underlying mutations in these families will be of great interest in understanding the pathogenesis of sporadic MPD.

**References**

15. Hematology Journal


Management of the myeloproliferative disorders: distinguishing data from dogma

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Optimal management of patients with a myeloproliferative disorder continues to be challenging and controversial. This situation can be attributed to at least two features of these diseases. Firstly, the disorders form an overlapping spectrum of diseases and may also mimic reactive conditions. It is therefore difficult to obtain homogeneous cohorts of patients, particularly since we currently lack any understanding of molecular aetiology. Secondly, there is a paucity of randomised trials largely because the diseases are uncommon and exhibit a chronic course. Moreover different trials frequently apply distinct diagnostic criteria that may be applied with variable rigor. Against this background, data and dogma frequently become blurred. Here, we focus on the management of polycythaemia vera (PV) and essential thrombocythaemia (ET).

Polycythaemia vera

Diagnosis

In the 1970s, the Polycythaemia Vera Study Group (PVSG) did the field an important service by developing the first set of rigorous criteria for the diagnosis of PV. With the passage of time, a number of the original criteria have been superceded by the development of newer tests. Approaches to the diagnosis of patients with erythrocytosis have been reviewed recently¹ and current diagnostic criteria are summarised in Table 1. Establishing the presence of an absolute erythrocytosis remains the cornerstone of diagnosis, and this usually requires demonstration of a raised red cell mass, although it can be assumed that a haematocrit of >0.6 in men or >0.56 in women corresponds to a raised cell mass. Secondary causes of an absolute erythrocytosis may be suggested by clinical history, a low arterial oxygen saturation, a high serum erythropoietin or renal abnormalities on ultrasound. The presence of palpable splenomegaly or an acquired genetic alteration (eg cytogenetic abnormality) in bone marrow cells are robust major criteria. Neutrophilia and thrombocytosis remain minor criteria. Splenomegaly detectable only by imaging is best considered as a minor criterion given the inter-observer and intra observer variation in assessing spleen size by ultrasound. A low serum epo and the presence of epo-independent erythroid colonies (EEC) are usually included as linked criteria since both may be present in some patients with inherited erythrocytosis.

Recently, a further set of criteria has been proposed under the auspices of the WHO,² but several concerns have been expressed about their utility. (1) It is suggested that haemoglobin >18.5 in men and >16.5 in women should be a major criterion. However, only 65% of men with a haematocrit 0.56–0.58 have a raised red cell mass, and so adopting such low haemoglobin value would result in patients without a raised red cell mass being labelled as having PV. (2) EECs are included as a major criterion. However, although useful in a specialised centre, this assay is not widely available, is laborious, expensive and poorly standardised. Moreover some patients with primary familial or congenital polycythaemia (PFCP) have EECs, and would therefore be labelled as having PV. (3) A total white cell count >12 is proposed as a minor criterion. However, this would potentially include patients with lymphocytosis. (4) Histological criteria are included as a minor criterion and described as ‘bone marrow biopsy showing panmyelosis prominent erythroid and megakaryocytic proliferation’. However, these appearances are not readily quantitated and are likely to be subject to considerable interobserver variation.

A number of potentially useful markers have been reported over the past few years.³,⁴ Patients with PV (and a proportion of those with ET or myelofibrosis) but not those with secondary erythrocytosis, have raised levels of PRV-1 mRNA in their peripheral blood granulocytes. Patients with PV (and some with ET or myelofibrosis) also exhibit impaired expression of MPL, the thrombopoietin receptor on megakaryocytes. Loss of heterozygosity on chromosome 9p has also been reported in patients with PV. However, the specificity of these tests needs to be confirmed and none are available outside specialised centres.

Clinical course

The prognosis of untreated PV in the first half of the 20th century was dismal with a median survival of approximately 18 months. However, the advent of venesection, together with antithrombotic and cyto-
Table 1 Diagnostic criteria for PV

<table>
<thead>
<tr>
<th>Major criteria</th>
<th>A1. Raised red cell mass (&gt;25% above predicted, or haematocrit ≥0.80 in men or ≥0.56 in women)</th>
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<tr>
<td></td>
<td>A2. Absence of causes of secondary erythrocytosis. (Normal arterial oxygen saturation (&gt;92%) and no elevation of serum erythropoietin)</td>
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<tr>
<td></td>
<td>A3. Palpable splenomegaly</td>
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<tr>
<td></td>
<td>A4. Acquired clonal abnormality in haemopoietic cells (excluding bcr-abl)</td>
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| Minor criteria                                      | B1. Thrombocytosis (platelets ≥400 × 10^9/l)                                                |
|                                                    | B2. Neutrophilia (neutrophils ≥10 × 10^9/l; >12.5 × 10^9/l in smokers)                       |
|                                                    | B3. Radiological splenomegaly                                                                 |
|                                                    | B4. Endogenous erythroid colonies or low serum erythropoietin                                |

A1 + A2 + either another A or two B criteria is required for a diagnosis of PV

These CV values are invariably associated with a raised red cell mass in an adult population.

Note that it is possible rarely for PV to coexist with a cause of 2° erythrocytosis.

reductive agents have greatly improved the outlook.

Patients over the age of 65 years have an overall survival similar to age-matched controls and thrombosis rates are high in these patients. Young patients have a reduced survival compared to an age-matched reference population, mainly a consequence of transformation to acute leukaemia or myelofibrosis.

The clinical epidemiology of PV has been reviewed recently. Two large Italian studies (GISP and ECLAP) have provided important insights into the natural history of PV. In both studies, overall mortality was approximately 3 per 100 patients/year with cardiovascular events and haematological transformation accounting, respectively, for 41 and 13% of deaths in the ECLAP study. Major thrombosis also occurred in approximately 3 per 100 patients/year. Age over 60 years and prior thrombosis were the most important risk factors identified for the development of thrombotic events. The ECLAP study also found that smoking, diabetes mellitus, hypertension, hypercholesterolaemia and congestive heart failure were significant predictors of survival and cardiovascular morbidity.

Clinical trials

Over the past 25 years, there have been several randomised and other trials investigating the optimal management of PV and these have been reviewed recently. In the 1970s, the PVSG-01 study randomised 431 patients to venesection alone, 32p plus venesection or chlorambucil plus venesection. Patients treated with venesection alone had a better median survival (13.9 years) than those in the other two arms (11.8 and 8.9 years, respectively). However, patients in the venesection arm showed an excess mortality within the first 2–4 years mainly caused by thrombotic events, whereas patients in the other two arms subsequently showed a higher rate of leukaemia and other malignancies. The PVSG-05 and 08 trials were designed to try to reduce the major causes of death identified in the PVSG-01 trial. In an attempt to lower the thrombotic events seen in the venesection only arm, the PVSG-05 study reduced the target haematocrit to 0.45 and compared venesection plus aspirin (900 mg/day) and dipyridamole (225 mg/day) with venesection plus 32p. The study was terminated early (median follow-up 1.2 years) because patients in the venesection/aspirin/dipyridamole arm exhibited a high incidence of major gastrointestinal haemorrhage (presumably reflecting the high doses of aspirin and dipyridamole) and no reduction in thrombotic events. The PVSG-08 study compared 51 patients receiving hydroxyurea for a median period of 8.6 years with historical controls from the venesection-only arm of PVSG-01. Although no significant differences were observed, the hydroxyurea group showed a tendency to fewer total deaths (39% vs 55%), less myelofibrosis (8% vs 13%), and more acute leukaemia (10% vs 4%). However the analysis excluded any patient in the venesection-only arm whose disease required cytoreductive therapy, and so this arm is highly selected and likely to represent a good prognosis subgroup.

In Europe, the EORTC compared 293 patients randomised to busulphan or 32P and followed for a median of 8 years. Survival at 10 years was significantly better in the busulphan arm mainly because of a lower incidence of vascular deaths. Najean and colleagues compared 292 patients below the age of 65 years randomised to pipobroman or hydroxyurea and followed for up to 16 years. No significant difference was observed in the incidence of death, thrombosis or acute leukaemia. However, patients treated with hydroxyurea exhibited a high rate of myelofibrosis, possibly related to the fact that platelet counts were significantly less well controlled. The French group also compared 461 patients aged over 65 randomised to 32P alone or in combination with hydroxyurea and followed for up to 16 years. No difference in overall survival was observed, but an increase in haematological and other malignancies were noted in patients receiving both 32p and hydroxyurea.

The most recent randomised study (ECLAP) compared 518 patients randomised to receive aspirin (100 mg/day) or no aspirin. There was no difference in overall mortality, cardiovascular mortality or major bleeding. However, treatment with aspirin significantly reduced the risk of the combined end point of nonfatal myocardial infarction, nonfatal stroke, major venous thrombosis or death from cardiovascular causes. These results suggest that low-dose aspirin can reduce thrombotic complications in patients with PV.

Although there are no randomised data, interferon and anagrelide may have a role in subgroups of patients with PV. Interferon is often favoured in young patients, since it is not thought to be leukaemogenic or teratogenic, and remains the treatment of choice when cytoreduction is required in pregnancy. Its main drawbacks are that it is expensive, requires parenteral administration and produces considerable side effects. However, the development of pegylated interferon...
allowing once-weekly dosage and may reduce side effects. Anagrelide specifically reduces the platelet count (and the haemoglobin in some patients) without affecting the white cell count. Its efficacy and side effects are discussed below, but it is generally well tolerated and there is no suggestion that it is leukaemogenic. In patients with PV, it provides an alternative to hydroxyurea or interferon for control of the platelet count.

**Recommendations for management**

Based on the limited evidence described above, current recommendations for management are outlined in Table 2. Venesection remains the central feature of initial treatment with a target haematocrit of 0.45 based on the seminal studies by Pearson and Wetherley-Mein. The ECLAP study, together with several primary and secondary prevention studies in patients without PV, argue strongly for the use of low-dose aspirin in all patients lacking a clear contraindication. The ECLAP epidemiological cohort study suggests that, as might be anticipated, patients with PV and other predictors of cardiovascular morbidity (hypertension, diabetes mellitus, smoking, hypercholesterolaemia, congestive heart failure) have an increased risk of thrombosis, and that these risk factors should be managed aggressively.

Cytoreductive therapy is clearly indicated if patients are intolerant of venesection or if they develop symptomatic splenomegaly thought to be due to disease progression. The treatment of thrombocytosis is more controversial. It has been suggested that it is unnecessary to control the platelet count in patients with PV, particularly since a clear relationship between platelet number and thrombosis has not been established. However, several lines of evidence suggest that controlling the platelet count is likely to be beneficial for patients with PV. (1) The ECLAP study strongly suggests that platelets contribute to thrombosis in patients with PV. (2) The beneficial effect of controlling the platelet count has been demonstrated by Barbui and co-workers in the closely related disorder essential thrombocythaemia (ET). (3) Treatment with cytoreductive agents reduced thrombotic events in patients with PV in the PVSG-01, 05 and 08 studies. The incidence of thromboses in the venesection only arm of PVSG-01 was 32.8% after 7.5 years, whereas among patients treated with hydroxyurea in PVSG-08 the incidence was 9.8% at the same time point. However, the target haematocrit in the early stages of the PVSG-01 trial would now be viewed as too high, and this may have contributed to the excess thrombotic events. In a separate study in which haematocrits and platelets were more tightly controlled at <0.45 and <400 × 10⁹/l, respectively, the thrombosis rate was reduced to 5.6% after a similar time of 7–8 years. (4) Two French trials have produced data consistent with the idea that a persistently raised platelet count is associated with an increased risk of myelofibrosis, a concept that accords with the link between fibrosis and growth factor production by the megakaryocyte lineage. It has been suggested that the development of myelofibrosis does not have adverse prognostic implications, but the difficulty with this assertion is the variable definition of myelofibrosis. Some patients with increased reticulin as an isolated finding can have prolonged survival, but a number of studies suggest that the development of clinically overt myelofibrosis in a patient with PV is associated with a poor prognosis.

No one of these arguments is definitive on its own, but the current balance of evidence suggests that it is sensible to control the platelet count in most patients with PV, although in young patients (>40 years) with a low risk of thrombosis, it may be reasonable to use aspirin alone. In most patients, hydroxyurea represents the first-line cytoreductive agent. Although doubts remain about possible long-term leukaemogenicity, there are no robust data that show that, when used as a single agent, it increases the risk of leukaemia (see below). Interferon-α is useful in young patients and during pregnancy. Anagrelide is particularly useful for control of the platelet count when hydroxyurea or interferon-α are unsuitable. In very elderly patients for whom regular clinic attendance is impractical, 32P or intermittent busulphan may still have a place.

**Table 2 Recommendations for management of patients with PV**

- (1) Venesection to maintain haematocrit <0.45
- (2) Low-dose aspirin (unless contraindicated)
- (3) Manage reversible thrombotic risk factors aggressively (eg smoking, hypertension, hypercholesterolaemia, obesity)
- (4) Consider cytoreduction if
  - (i) patient intolerant of venesection
  - (ii) thrombocytosis develop
  - (iii) symptomatic or progressive splenomegaly
- (5) Choice of cytoreductive therapy:
  - (i) <40 years – interferon-α
  - (ii) >40 years – hydroxyurea

*aAvoid aspirin while platelet count >1500 × 10⁹/l.

*bAnagrelide may be useful as an adjunct for thrombocytosis.

*cConsider 32P or intermittent use of busulphan if patient is very elderly and outpatient attendance is impractical.

**Essential thrombocythaemia**

**Diagnosis**

In 1976, the PVSG formulated a set of criteria for the diagnosis of ET and an amended version of this is shown in Table 3. ET remains largely a diagnosis of exclusion. Since thrombocytosis is relatively nonspecific and other positive criteria are lacking patients labelled as having ET are likely to be heterogeneous. There have been a number of attempts to develop new positive diagnostic tests and these have been reviewed recently.
Since ET is a clonal haematological malignancy, there has been considerable interest in the possibility that the pattern of X chromosome inactivation might prove diagnostically useful. A skewed pattern in peripheral blood granulocytes, together with a balanced pattern in peripheral blood T cells has been observed in several myeloid malignancies. Unfortunately, the diagnostic utility of this approach in the context of ET is limited by two observations. Firstly, approximately 50% of patients who fulfill clinical criteria for ET have a balanced pattern in their peripheral blood granulocytes suggesting that the majority of these cells are polyclonal. This observation may represent a technical limitation in that current methodologies would not detect a subpopulation of skewed cells within a population of cells exhibiting a balanced inactivation pattern. However, it also raises the possibility that at least some patients, currently labelled as having ET, do not have an acquired MPD and that other mechanisms may be responsible for their persistent thrombocytosis. Secondly, approximately 25% of haematologically normal elderly women have a skewed pattern of X inactivation in peripheral blood granulocytes with a balanced pattern in T cells. This may reflect subtle allelic differences between X chromosomes which result in a selective advantage that becomes evident with increasing age. In patients with ET X chromosome inactivation patterns are therefore of limited use as a diagnostic test, since a clonal pattern (skewed granulocytes and balanced T cells) is found in a significant proportion of normal elderly women and the presence of a polyclonal pattern (balanced granulocytes and T cells) in younger women cannot be used to exclude the diagnosis of ET.

Trephine histology has also been suggested as a useful positive diagnostic criterion. It has been reported that patients with ET can be divided into histologically distinct subgroups (true ET, prefibrotic myelofibrosis, and early myelofibrosis) with different prognoses. However, megakaryocyte morphology is notoriously difficult to assess in a reproducible manner and detailed studies of interobserver variation are lacking. It is therefore not yet clear whether this sort of histological classification is robust enough to be applied widely outside specialised centres, and the inclusion of trephine biopsy histology in the WHO criteria is regarded by many as controversial.

A number of groups have investigated the potential utility of megakaryocyte and erythroid colony assays as diagnostic tests. Thrombopoietin (TPO)-independent megakaryocyte colonies have been identified in most patients with ET (and in a proportion of those with other MPDs). Erythropoietin-independent erythroid colonies (EECs) have also been found in a subset of patients with ET. A number of studies have investigated the possibility that the presence of such colonies might be diagnostically useful. However, both assays are laborious, difficult to standardise and only available in a few specialist centres. Moreover, TPO-independent colonies have been reported in patients with reactive thrombocytosis.

Serum TPO levels are unhelpful since they are normal or raised in a variety of conditions associated with a high platelet count, including reactive thrombocytosis, ET and other MPDs. Reduced expression of MPL, the TPO receptor, has been reported in patients with ET by some groups but not by others. Increased levels of PRV-1 mRNA in peripheral blood granulocytes have been reported in approximately 50% of patients with ET. Preliminary data suggest that those patients with EECs also have increased PRV-1 levels, raising the possibility that these patients form a biologically distinct subset.

Clinical course

Four of five cohort studies have reported a reduced life expectancy in patients with ET compared to age- and sex-matched controls, although the results reach significance in only two of these studies. As with PV, thrombotic events are the major complication encountered during follow-up, with haemorrhagic events occurring less commonly. The reported frequency of thrombosis is variable since different studies have included distinct patient populations and have used varying definitions of major and minor vascular events. A number of risk factors for thrombosis have been identified. Several studies have reported that patients > 60 years or with a prior thrombosis are at high risk of thrombosis. The significance of the platelet count is less clear. Major haemorrhage is reported to be more common in patients with platelets > 1500 x 10^9/l. An increased risk of thrombosis has been suggested with platelet counts > 1000 x 10^9/l at diagnosis or in patients with a platelet count above the normal range during treatment, but other studies have not found such an association. However, the increased platelet numbers seem likely to be contributing to the thrombotic diathesis in ET patients since aspirin relieves micro-
vascular symptoms and cytoreductive therapy reduces the frequency of thromboses.

A number of other features have been claimed to correlate with thrombotic complications. These include a clonal pattern of X chromosome inactivation, reduced expression of MPL in bone marrow megakaryocytes and over expression of PRV-1 in peripheral blood granulocytes. An increased risk of thrombosis may also be associated with antiphospholipid antibodies, heterozygosity for factor V Leiden and a number of cardiovascular risk factors, including hypertension, smoking and hypercholesterolemia. In the longer term patients with ET may also develop myelofibrosis or AML. The risk of myelofibrosis is difficult to assess from published data partly because different criteria have been used to define myelofibrotic transformation. In one recent study of 195 patients with a median follow-up of 7 years, the incidence of myelofibrosis was 8% at 10 years. AML occurs less frequently than in other MPDs. The reported incident of AML/MDS is very variable because most studies are retrospective and involve small numbers of patients with different lengths of follow-up. However, several points do appear to be clear. Firstly, untreated patients can develop AML. A retrospective study of 2316 Italian patients suggested that AML/MDS occurs in approximately 1% of untreated ET patients. Secondly, patients treated with hydroxyurea alone (and no other cytotoxic agent), display a low incidence of AML/MDS (see below). Thirdly, patients who require more than one cytotoxic agent have an increased risk of AML/MDS. However, it is not clear whether this reflects the combined effect of these drugs or whether it is a consequence of particularly aggressive disease.

Clinical trials

There have been only two prospective randomised studies of the treatment of patients with ET. In the first 114 high-risk patients (age > 60 years or prior thrombosis) were randomised to receive hydroxyurea or no cytoreductive agent. Patients with a platelet count > 1500 × 10^9/l were excluded. During a median follow-up period of 27 months, patients on hydroxyurea developed significantly fewer thrombotic events ($P = 0.003$). This was the first clear demonstration that cytoreductive therapy reduces thrombotic events in patients with ET.

The second randomised study is the primary thrombocytopenia-1 trial. High risk patients (prior thrombosis, age > 60 years or platelets > 1000 × 10^9/l) were randomised to receive hydroxyurea plus aspirin or anagrelide plus aspirin. Intermediate-risk patients (age 40–60 and no high-risk features) were randomised to receive hydroxyurea plus aspirin or aspirin alone. Low-risk patients (age < 40 years and no high-risk features) received aspirin alone and were monitored. The intermediate and low-risk arms are still recruiting. The high-risk study was closed towards the end of 2003 because of an excess of adverse events in the anagrelide arms.

Preliminary analysis has suggested an increase in myelofibrotic transformation, thrombotic and haemorrhagic events, but final data are not yet available. Current evidence therefore suggests that high-risk patients should be treated with a cytoreductive agent. For other patients at a lower risk of thrombosis, the situation is less clear. The decision whether or not to use a cytoreductive agent requires weighing up two opposing risks, both of which are small — the risk of a thrombotic event and the risk of a significant drug-related side effect. Unfortunately, the frequency of these two types of event is not clear from existing data. Some studies suggest that patients aged < 60 years and with no prior thrombosis do not exhibit an increased frequency of thrombosis compared to controls. However, the number of patients studied was small, the number of events very small and the choice of an appropriate control population is difficult. Moreover, other studies have found that such patients do have a significant risk of thrombosis. With respect to the risks of treatment, these differ for the different agents. The most commonly used drugs are hydroxyurea, anagrelide and interferon.

Hydroxyurea: Hydroxyurea has emerged as first-line therapy for high-risk patients because of its efficacy, low cost and rare acute toxicity. Bone marrow suppression with cytopenia is the main short-term side effect. Leg ulcers and a variety of other skin conditions (including photosensitivity and solar keratosis) are also well recognised, especially in older patients. A common concern is whether hydroxyurea might be leukaemogenic. It is clear that some cytotoxic drugs such as 32P and chlorambucil are leukaemogenic, but the situation with hydroxyurea is less clear. Hydroxyurea has a distinct mechanism of action and does not increase the frequency of acquired mutations in adults with an MPD or sickle cell disease. Some studies have reported that 5–10% of patients receiving hydroxyurea develop AML/MDS. However, these involved small numbers of patients many of whom had also received other cytoreductive agents. Patients who receive more than one cytotoxic agent do have a significantly higher risk of developing AML/MDS although is not clear whether this association reflects an effect of the drugs or a consequence of aggressive disease. By contrast, there are now a number of studies that show that ET patients receiving hydroxyurea alone have a low incidence of AML/MDS (3–4%) and there are no data to show that this incidence is significantly different from that observed in untreated patients. Moreover, follow-up over the past 10 years of patients receiving hydroxyurea for sickle cell disease also suggests that its leukaemogenic potential is very low.

Anagrelide: Anagrelide is an imidazo quinazoline derivative originally developed as an inhibitor of platelet aggregation. It was subsequently shown to lower the platelet count in a species-specific manner and at doses lower than those which inhibit platelet aggregation. It is
effective at reducing the platelet count (to <600) in 70–80% of patients but approximately 10% are completely refractory, perhaps because of an inability to generate an active metabolite. It is a phosphodiesterase inhibitor and acts as a vasodilator and a positive inotrope. Acute side effects include headaches, palpitations and fluid retention but there is no suggestion that the drug is leukaemogenic.

Although effective at reducing the platelet count, it is important to remember that this is a surrogate end point and randomised data (such as the PT-1 trial described above) are needed to confirm efficacy with respect to clinical end-points. This caveat is underscored by a study of 37 young patients followed for a median period of 10 years. Platelet counts were reduced to <600 in over 80% of patients, but thrombotic complications occurred in approximately 20% of this cohort and major haemorrhage in a similar proportion.

**Interferon:** Interferon-α is effective at reducing the platelet count below 600 x 10⁹/l in approximately 90% of patients with an average dose of 3 million international units per day. It is not known to be teratogenic or leukaemogenic, does not cross the placenta and is often the treatment of choice during pregnancy. Its acute side effects, particularly flu-like symptoms, are a major problem and result in treatment withdrawal in a substantial proportion of patients.

**Management recommendations**

It seems sensible for cardiovascular risk factors to be reversed where possible in all patients. There is also a clinical consensus that specific treatment of patients with ET should be based primarily on the expected risk of thrombotic complications (Table 4). Patients aged over 60 years or with a prior thrombosis clearly fall into a high-risk category. The level of platelet count that confers a high risk is more arbitrary and different groups have suggested 1000 or 1500 x 10⁹/l as a threshold value. Hydroxyurea is generally accepted as first-line treatment for high-risk patients in view of its efficacy, low cost, limited short term toxicity and the fact that its leukaemogenic potential, when used as a single agent, is very low and may be nonexistent. However, hydroxyurea should be used with caution in younger patients and in those previously treated with other cytotoxic drugs.

<table>
<thead>
<tr>
<th>Table 4 Recommendations for management of patients with ET</th>
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<tr>
<td>(1) All patients: Manage reversible cardiovascular risk factors aggressively (eg smoking, hypertension, hypercholesterolaemia, obesity)</td>
</tr>
<tr>
<td>(2) High-risk patients (prior thrombosis or age &gt;60 years or platelets &gt;1000 x 10⁹/l) Treat with hydroxyurea (anagrelide or interferon-α second line)</td>
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<tr>
<td>(3) Intermediate risk patients (age 40–60 years, no high-risk features) Either enter into randomised trial, eg PT-1 intermediate-risk arm Or consider cytoreduction of other cardiovascular risk factors present</td>
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<tr>
<td>(4) Low risk patients (age &lt;40 years and no high-risk features) Low dose aspirin</td>
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There is also a general consensus that patients at particularly low risk of thrombotic events (age <40 years, no high-risk features) should receive low-dose aspirin alone (or equivalent). For the remaining intermediate risk patients (age 40–60 years, no high-risk factors), there are no good data to guide management and it is not clear whether cytoreduction is beneficial. Where possible such patients should be entered into a randomised trial. If this is not feasible it would be reasonable to limit cytotherapeutic therapy to those patients with cardiovascular risk factors. There are few data to guide the management of ET in pregnancy. It seems reasonable for patients to receive low-dose aspirin, but the decision whether to lower the platelet count is more contentious and there are conflicting reports as to whether the established factors for thrombosis in non pregnant patients can predict poor pregnancy outcome. In the absence of clear data, it seems advisable to limit the use of platelet-lowering agents to patients thought to be at high risk of thrombosis and particularly to patients with a history of previous thrombosis or foetal loss. Anagrelide and hydroxyurea should be avoided because of the possibility of teratogenic effects, although there have been reports of normal pregnancies despite exposure to hydroxyurea. Interferon-α is generally regarded as the treatment of choice and should be combined with heparin in patients at particularly high risk, with treatment continuing for several weeks postpartum.

**Acknowledgements**

Unfortunately, it has not been possible to cite many important primary papers because of a limit on the number of references. The authors have therefore cited reviews that contain the primary references.

References

4 Kralovics R, Buser AS, Teo SS, Coers J, Tichelli A, van der Maas AP et al. Comparison of molecular markers in a


FIP1L1-PDGFRA in hypereosinophilic syndrome and mastocytosis

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Introduction

Idiopathic hypereosinophilic syndrome (HES) is a heterogeneous group of hematological disorders, characterized by: eosinophilia (>1500 eosinophils/μl) for more than 6 months; exclusion of reactive eosinophilia from other causes, such as parasitic infection; and evidence of end-organ damage.1,2 Eosinophilia is the most prominent feature of this disease, other myeloid lineages, in particular neutrophils, are also often increased, and contribute to the leukocytosis observed in HES patients.2

According to World Health Organization (WHO) criteria, HES is reclassified as chronic eosinophilic leukemia (CEL) when there is evidence for clonality based on the presence of chromosomal abnormalities or X-inactivation in female patients.3 However, analysis of clonality in HES is difficult due to low frequency of chromosomal abnormalities and a striking male predominance (9:1) that precludes X-inactivation studies of clonality. Splenomegaly, hepatomegaly, anemia and thrombocytopenia have been suggested to favor a diagnosis of CEL rather than HES,4 but the lack of markers of clonality to verify this classification has limited their application. Thus, HES has remained a heterogeneous disease that is difficult to reliably distinguish from CEL.

The successful treatment of HES patients with the tyrosine kinase inhibitor imatinib (Gleevec, STI-571, Novartis Pharma AG) suggested that a significant proportion of patients with idiopathic HES had a clonal myeloproliferative disease due to acquired activating mutations in an imatinib-sensitive tyrosine kinase.5-7 The recent identification of a specific chromosomal deletion del(4)(q12q12), creating the FIP1L1-PDGFRA fusion gene provides unequivocal support for this hypothesis.5 Initial reports indicate that about half of HES cases seen in hematology/oncology practices harbor the FIP1L1-PDGFRA fusion gene. These cases of HES thus have a clonality based on the presence of the del(4)(q12q12) and should be reclassified as CEL according to WHO criteria. In addition, the detection of the FIP1L1-PDGFRA fusion gene is a positive predictor for response to imatinib therapy.8,9

Important questions that remain include the determination of the best tools to use for diagnosis and monitoring, the appropriate dose and duration of therapy with imatinib, and strategies to overcome resistance to imatinib.

The Use of imatinib to treat HES

Imatinib is a potent inhibitor of the constitutively activated BCR-ABL kinase associated with chronic myelogenous leukemia (CML), and is now FDA approved for treatment of BCR-ABL-positive CML.10-12 Imatinib is not specific for ABL, but rather is a selective inhibitor of four other tyrosine kinases, including KIT, ARG (ABL2), PDGFRβ and PDGFRα kinases.13 After FDA approval, imatinib has been tested and shown to be effective in several off-label indications based on inhibition of these other tyrosine kinases, including treatment of gastrointestinal stromal cell tumors (GIST) associated with activating mutations in KIT,12 and chronic myelomonocytic leukemias associated with the constitutively activated ETV6-PDGFRA/(TEL-PDGFRA) fusion protein.13 Imatinib has also been used to treat empirically several other malignancies, and among these, the response of patients with HES hypereosinophilic syndrome has been extraordinary. Most HES patients have rapid responses to imatinib and achieve normal eosinophil levels after 1–2 weeks of imatinib treatment as a single agent. Seven separate studies have indicated that a majority of HES patients (both male and female) respond to imatinib (Table 1).5,9,14,15 Effective doses for treatment of HES have been reported to be lower (100 mg/day) than for treatment of BCR-ABL-positive CML (400 mg/day), and some patients have been successfully treated with 100 mg per week for continuation of treatment during remission. As most HES patients respond to low doses of imatinib, the side effects have been minimal and the cost for treatment lower than for CML.

Despite the success of imatinib for the treatment of HES, relapse during imatinib treatment has been observed in at least one patient with advanced disease (progression to acute eosinophilic leukemia) after ~5 months in remission.15 Similar findings have been reported with imatinib treatment of CML, where relapse is also observed, primarily in patients with advanced disease (blast crisis).16 Thus far, there have been long-standing remission durations (>1 year) for most HES patients that have responded to imatinib.
Longer-follow-up of treated patients is required to gain insight into the long-term risks and benefits of imatinib treatment for HES, and to determine optimal dosing schedules and appropriate duration of therapy with imatinib. It will also be of interest to compare the remission duration and development of resistance to imatinib treatment in HES versus CML, especially in light of the lower therapeutic dose of imatinib in HES.

**FIP1L1-PDGFRα fusion gene in HES**

The initial reports of response of HES to imatinib were not only important as a major therapeutic advance, but were also the first indication that an aberrantly activated kinase was the cause of HES in those patients with a response to imatinib. As noted above, imatinib is known to inhibit the protein tyrosine kinases ABL (ABL1) and ARG (ABL2), as well as the type III receptor protein tyrosine kinases PDGFRα, PDGFRβ and KIT. We hypothesized that a mutation in one of these five kinases was likely to be the cause of HES cases with a response to imatinib, and that this mutated kinase would also be the molecular target of imatinib. There are no recurring cytogenetic abnormalities associated with HES that might provide a clue to the identity of the putative tyrosine kinase. Therefore, we first performed DNA sequence analysis to look for activating mutations in the context of the five known imatinib-sensitive kinases, but found no mutations. However, we ultimately identified an abnormal PDGFRα transcript in a unique HES patient with a complex chromosomal rearrangement at band 4q12, near the PDGFRα locus. The chromosome 4 abnormality in this patient included a deletion of approximately 800 kb that could not be detected by conventional cytogenetics, and resulted in fusion of 5′ sequences derived from the FIP1L1 gene to 3′ sequences, including the tyrosine kinase domain, of the PDGFRα gene (Figure 1). The FIP1L1-PDGFRα gene was subsequently detected in nine of 16 (56%) and in five of nine (55%) HES patients in two studies. Taken together, these data suggest that approximately 55% of HES patients harbor the FIP1L1-PDGFRα fusion. As the presence of the FIP1L1-PDGFRα fusion gene is indicative for the clonal origin of these cases, FIP1L1-PDGFRα-positive HES cases should be reclassified as chronic eosinophilic leukemias according to WHO criteria, and will be referred to as such for the rest of this review.

In contrast to a multitude of previously identified fusion kinase genes such as BCR-ABL, ETV6-PDGFRβ, HIP1-PDGFRβ, H4-PDGFRβ, ETV6-ABL, ETV6-JAK2 and ZNF198-FGFR1 that are each the consequence of reciprocal chromosomal translocations, the FIP1L1-PDGFRα fusion gene is created by an interstitial chromosomal deletion del(4)(q12q12). The deletion is relatively small (~800 kb) and is thus not detectable by standard cytogenetics. This probably explains why the fusion gene has not been cloned previously, and suggests the hypothesis that a similar mechanism of interstitial chromosomal deletion may be at play in other hematological malignancies and solid tumors. The breakpoints in the FIP1L1 gene are variable and are located in a 40 kb region spanning introns 7–10 of FIP1L1. In contrast, the breakpoints in PDGFRα are all located within exon 12, more specifically, within the region encoding a WW-like domain (Figure 2).

Although the identification of the FIP1L1-PDGFRα fusion protein in HES cases that responded to imatinib strongly suggested that this protein was the therapeutic...
target of imatinib, definitive evidence was obtained by the identification of a resistance mutation (T674I) in FIP1L1-PDGFRα in a patient who relapsed during therapy. Tyrosine kinase domains are highly conserved, and the T674I mutation in FIP1L1-PDGFRα is homologous to the T315I mutation in BCR-ABL, known to confer resistance to imatinib in this context. As noted below, subsequent in vitro and in vivo studies have confirmed that T674I is also an imatinib resistance mutation in the context of FIP1L1-PDGFRα.

**In vitro transformation mediated by FIP1L1-PDGFRα**

FIP1L1-PDGFRα is a constitutively activated kinase that transforms the interleukin-3-dependent cell line Ba/F3 to factor independence, indicating that FIP1L1-PDGFRα is able to activate proliferation and survival pathways in hematopoietic cells. Although it is plausible that FIP1L1-PDGFRα has a similar signaling profile than ligand-stimulated PDGFRα, it is important to realize that FIP1L1-PDGFRα is predicted to be a cytoplasmic rather than a transmembrane protein and may thus have access to a different set of substrates that the transmembrane PDGFRα kinase. In addition, the fusion protein lacks part of the juxtamembrane region that may be required for the binding of several signaling proteins (Figure 2). In this regard, FIP1L1-PDGFRα activates STAT5, but unlike the native PDGFRα, STAT5 is not activated, indicating that FIP1L1-PDGFRα is unable to activate the RAS/MAPK pathway. This may be explained by lack of access of cytoplasmic FIP1L1-PDGFRα to farnesylated RAS localized to the plasma membrane. It remains to be determined whether STAT5 is necessary for transformation mediated by FIP1L1-PDGFRα, as has been reported for the ETV6-JAK2 fusion kinase, and the relative contributions of other signal transduction pathways, including PI3K/AKT and mTOR, are also unknown. There are a number of selective inhibitors for each of these pathways, as well as mouse strains that are genetically deficient in one or more of these signal transduction intermediates, which should allow these questions to be addressed. The identification of critical downstream effectors of FIP1L1-PDGFRα-mediated transformation may also provide additional therapeutic targets that could be exploited alone, or in synergy with imatinib.

The exact mechanism by which the kinase domain of PDGFRα is aberrantly activated by fusion to FIP1L1 is currently unclear. On one hand, FIP1L1-PDGFRα shows similarity with other well-characterized fusion kinases, such as BCR-ABL and ETV6-PDGFRβ that are known to be activated by homodimerization through the BCR or ETV6 moieties, respectively. However, we have not been able to identify a homodimerization domain in FIP1L1 using homology searches. On the other hand, it is striking that each unique FIP1L1-PDGFRα fusion protein that has been cloned thus far has an interrupted juxtamembrane region as a consequence of the interstitial deletion in which the first tryptophan (W) residue of the putative WW-domain is always deleted (Figure 2). The WW-like domain of PDGFRβ is believed to be a negative regulator of kinase activity, and is part of the juxtamembrane region that serves as an autoinhibitory domain in the context of other receptor tyrosine kinases such as EPHB2 and KIT. In addition, mutations in the juxtamembrane regions of FLT3, KIT and PDGFRα result in constitutive kinase activation. Thus, the genotypic data on FIP1L1-PDGFRα in CEL patients suggests that interruption of the juxtamembrane region of PDGFRα may serve as the primary mode of kinase activation, rather than dimersization mediated by FIP1L1.

The EOL-1 cell line, derived from a patient with acute eosinophilic leukemia, forms an alternative in vitro model for the study of the FIP1L1-PDGFRα fusion protein. This human myeloid cell line harbors the del(4)(q12q12), expresses the fusion gene from the FIP1L1 promoter, and has the ability to differentiate into eosinophils, making it an in vitro system that closely resembles CEL (our unpublished results). In addition, the observation that this cell line also contains a partial tandem duplication in the MLL gene, indicates that FIP1L1-PDGFRα may cooperate with mutations in transcription factors to cause acute leukemia or progression of CEL to acute leukemia.

**In vivo models for the study of FIP1L1-PDGFRα**

To further study the transforming properties in primary hematopoietic cells in vivo, we developed a murine bone marrow transplant model of FIP1L1-PDGFRα induced hematological disease. Mice transplanted with bone marrow cells expressing FIP1L1-PDGFRα developed a short latency myeloproliferative disease characterized by splenomegaly and leukocytosis that was mainly due to elevated numbers of neutrophils and only to a lesser extend due to elevated eosinophils (up to 20%). More work is required to investigate if other factors, such as the 800 kb deletion at 4q12, or the expression pattern of FIP1L1-PDGFRα are important determinants of the specific phenotype of FIP1L1-PDGFRα positive CEL.

We have also used this mouse model to test another PDGFRα inhibitor, PKC412 (N-benzoyl-stauroporine), as an alternative treatment for CEL. We explored the ability of PKC412 to treat disease caused by FIP1L1-PDGFRα or the imatinib-resistant T674I mutant. Similar to the human disease, mice transplanted with bone marrow expressing FIP1L1-PDGFRα, were responsive to imatinib, whereas mice transplanted with bone marrow cells expressing the T674I mutant were resistant to imatinib. However, PKC412 was effective for the treatment of disease caused by the FIP1L1-PDGFRα or the T674I mutant. In control experiments, a PKC412-resistant mutant, FIP1L1-PDGFRα N659D, conferred resistance to PKC412 in Ba/F3 cells, indicating that the FIP1L1-PDGFRα fusion protein is the critical target of inhibition by PKC412. Collectively, these results further demonstrate that the use of two structurally different kinase inhibitors may be of value
to prevent or treat resistance to a given kinase inhibitor. Imatinib treatment of various cancers has clearly demonstrated that one kinase inhibitor is not sufficient for the long-term treatment of cancer, but that combination therapy will be required to overcome resistance.

**Diagnosis of FIP1L1-PDGFRα-positive CEL**

The presence of *FIP1L1-PDGFRα* is an accurate predictor of response to imatinib treatment.²⁻⁶ Thus, detection of the *FIP1L1-PDGFRα* fusion gene in HES can be used prospectively to identify patients that are expected to respond to imatinib treatment, to monitor their response, molecularly and to monitor for evidence of relapse. The fusion gene itself can be detected at the RNA level by reverse transcriptase-polymerase chain reaction (RT-PCR). Unfortunately, the deletion is not visible by standard cytogenetics (patients present with an apparent normal karyotype), but the interstitial chromosomal deletion, del(4)(q12q12), can be detected by three-color fluorescence in situ hybridization (FISH) on either interphase or metaphase nuclei (Figure 1).

Before the discovery of the *FIP1L1-PDGFRα* fusion gene, the difference between HES and CEL was made on the basis of clonality.³ It was clear that this was an artificial classification, since in most HES cases, clonality could not be analyzed. As a result, HES appeared to be very heterogeneous. Now that the detection of *FIP1L1-PDGFRα* can be used as a marker for clonality, we have gained new insights into the difference between HES (negative for the *FIP1L1-PDGFRα* fusion and other clonal markers) and CEL (positive for *FIP1L1-PDGFRα* or other clonal markers).

The study of Klion *et al.*⁹ identified elevated serum tryptase and vitamin B12 levels as a marker corresponding with *FIP1L1-PDGFRα*-positive CEL cases. In addition, splenomegaly was found to be more frequent in CEL than HES, and *FIP1L1-PDGFRα*-positive cases are characterized by a higher absolute eosinophil count than *FIP1L1-PDGFRα*-negative cases.⁹

**FIP1L1-PDGFRα in systemic mast cell disease (SMCD) with eosinophilia**

*FIP1L1-PDGFRα* is under control of the ubiquitously expressed FIP1L1 promoter, suggesting that the fusion gene could also be involved in the pathogenesis of other hematological malignancies or solid tumors. As an example of this, Pardanani *et al.*²⁶ have recently identified the *FIP1L1-PDGFRα* fusion in systemic mastocytosis with associated eosinophilia (SMCD with eosinophilia).²⁶ In light of the invariant increase in serum tryptase levels in patients with HES, and the *FIP1L1-PDGFRα* fusion, these observations have raised the question of whether *FIP1L1-PDGFRα* positive HES and SMCD with eosinophilia are in fact the same disease. Although these issues of diagnosis and classification remain to be fully reconciled, it is clear that the *FIP1L1-PDGFRα* fusion and elevated tryptase identify a group of patients with hyper eosinophilia that are imatinib responsive. In addition, it is clear that some patients with HES respond to imatinib do not have the *FIP1L1-PDGFRα* fusion. Further exploration of the genomic loci of other imatinib-sensitive kinases in these patients is warranted.

**Conclusion**

The FIP1L1-PDGFRα oncogene is expressed in a majority of HES cases, identifying them as clonal leukemias, and ‘most importantly’ as responders to imatinib therapy. A number of questions related to patient treatment, molecular mechanisms of disease, and the function of the FIP1L1-PDGFRα kinase remain unanswered. First, there is evidence that some FIP1L1-PDGFRα-negative patients respond to imatinib, and the basis for these responses is currently unknown. The leading hypothesis would be that another imatinib-sensitive kinase is mutated in these cases. Second, the mechanism for aberrant activation of the kinase activity in the FIP1L1-PDGFRα fusion protein is currently unknown, and needs to be further studied with appropriate mutants. In addition, interstitial chromosomal deletion is a novel way to generate a fusion kinase gene, and would not be detected by standard karyotypic analysis. Finally, the long-term benefit of imatinib treatment in HES/CEL remains to be determined. Only when long-term benefits with imatinib treatment are achieved, the promise of molecularly targeted therapy will be fulfilled, and it remains to be determined if this will be possible with a single kinase inhibitor, or whether combination therapy will be required.

**References**


EXPRESSION PROFILING: WHERE IS THE FIELD GOING?

Discovery of novel molecular classification schemes and genes predictive of outcome in leukemia

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Introduction and background

Over the past three decades, significant advances have been made in the treatment of acute leukemia. Through the use of modern combination chemotherapy, postinduction therapeutic intensification, and transplantation, overall survival rates in adult and particularly pediatric leukemias have improved. Yet particularly resistant forms of leukemia remain, including leukemia in infants, acute lymphoblastic leukemia (ALL) in adults, acute myeloid leukemia (AML) in adults and children, and chronic myelogenous leukemia (CML) in accelerated phase or blast crisis. While intensive therapies now yield long-term remissions in nearly 75% of children with ALL, 25% still relapse with disease that is highly refractory to current therapies. Conversely, another 25% of children with ALL who receive dose intensification are likely ‘over-treated’ and may well be cured using less-intensive regimens resulting in fewer toxicities and long-term side effects. Thus, a major challenge for the treatment of adults and children with acute leukemia is to improve and refine diagnostic and risk classification schemes in order to precisely tailor therapeutic approaches to the biology of the tumor and the genotype of the host, and, to develop more effective therapies for resistant forms of disease.

To meet this challenge, many have hypothesized that genomic technologies that measure global patterns of gene expression in leukemia patients will yield orderly and systematic profiles that may ultimately be useful for: (1) the identification of intrinsic biologic groups of acute leukemia not currently appreciated by current diagnostic techniques; (2) the development of ‘molecular classification schemes’ based on gene expression profiles; (3) the identification of novel genes that can refine risk classification; and (4) the identification of new therapeutic targets. One of the most useful applications of gene expression profiling may be to identify profiles associated with response or resistance to specific therapeutic agents in well characterized, uniformly treated patient cohorts. Such studies may provide new insights into the biologic and molecular mechanisms of therapeutic response and facilitate the development of more effective treatment regimens. A major limitation to gene expression profiling methodologies is that the technology focuses on measurement of expressed RNAs, rather than translated proteins and even more importantly, the regulated functional states of these proteins (mediated through post-translational modifications such as phosphorylation or farnesylation). While proteomics technologies promise such comprehensive protein profiling in the future, these technologies are not yet sufficiently developed to yield comprehensive profiles in which thousands of proteins can be readily analyzed, identified, and their functional activation state determined. Thus, as new comprehensive molecular technologies are developed and optimized, gene expression profiling is likely to continue to be a useful tool to identify new targets for improved diagnosis and therapeutic intervention.

Our research team at the University of New Mexico (UNM) Cancer Research and Treatment Center and our collaborators at the UNM High Performance Computing Center and Sandia National Laboratory have completed comprehensive analyses of gene expression profiles using Affymetrix oligonucleotide arrays (U-95A.v2 containing 12,625 genes/ESTs) in several retrospective cohorts of pediatric and adult leukemia patients, including: (1) 127 infants with leukemia (79 ALL, 48 AML, 57/127 with MLL rearrangements) registered to Pediatric Oncology Group (POG)/Children’s Oncology Group (COG) clinical trials; (2) 254 pediatric ALL patients registered to POG trials stratified for the presence of recurrent cytogenetic abnormalities and remission failure within each cytogenetic group; (3) 110 pediatric ALL patients with or without minimal residual disease at end-induction registered to POG trial ALinC17; and (4) 325 cases of adult AML registered to various trials conducted by the Southwest Oncology Group (SWOG). Gene expression data were correlated with a large number of biologic and clinical covariables (including AML/ALL type, cytogenetics, clinical outcome with long-term follow-up) using novel algorithms, new data visualization tools, and parallel high performance computing approaches at the UNM High Performance Computing Center and Sandia National Laboratory in Albuquerque. This report will...
briefly summarize this work in the context of a discussion of the different methodologies for computational data analysis, an extremely critical component of these investigations. Although each of the statistically defined cohorts that we have studied represents different types of leukemia, there were some consistent themes:

1. Although strong correlations exist in certain forms of leukemia, the intrinsic biologic clusters found in acute leukemias in infants, children, and older adults are often uniquely identified through gene expression profiling and are not precisely identified or defined by traditional morphologic features, phenotype, or cytogenetics.

2. Novel genes associated with specific biologic groups defined by expression arrays can be used to identify and diagnose intrinsic biologic groups.

3. The biologic groups of leukemia (clusters) identified through gene expression profiling are highly dependent on the specific cases under investigation and the statistical design of the patient cohort; this information is critical when comparing results obtained by different laboratories.

4. Using powerful, supervised machine learning algorithms in well-designed statistical cohorts, one can identify gene expression profiles and frequently novel genes that are associated with or predictive of class (cluster), outcome, or other clinical and biologic parameters. We and others have identified novel genes highly predictive of outcome in infant and pediatric ALL and adult AML.

Class discovery: identification of novel biologic classes of leukemia

A major challenge of gene expression profiling is the capability to address the informatics and computational requirements needed to analyze large, complex gene expression data sets and correlate gene expression data with a large volume of laboratory and clinical covariables. Non-supervised learning tools for hierarchical clustering of gene expression data and other clustering approaches are used for ‘class discovery’: the discovery of intrinsic biologic groups of patients based on shared patterns of gene expression. Such class discovery approaches are ideally performed without prior knowledge of the biologic and clinical aspects of the data set under analysis (morphology, cytogenetics, outcome, etc); so-called ‘unsupervised’ machine learning. Many mathematical algorithms can be used for class discovery, including hierarchical clustering, K-means, self organizing maps, and principal component analysis (PCA). However, with the exception of PCA, most unsupervised hierarchical clustering algorithms are not multidimensional enough or stable enough to resolve multiple clusters in very large data sets (>30,000 genes in >100 cases). Thus, investigators may select the number of clusters that they expect that the data will fit and determine whether the data conform to this expected pattern, or a subset of genes upon which to focus. Such preselection may introduce significant bias into the analysis. Working with the computing group at Sandia National Laboratory in Albuquerque, we have developed ‘higher order’ multidimensional clustering algorithms such as VxInsight (http://www.cs.sandia.gov/projects/VxInsight.html), a new and very powerful tool for non-supervised clustering and visualization of genomic data.¹,² VxInsight has the capacity to cluster patients or genes, using all of the gene expression data without having to select smaller subsets of genes for actual clustering, in a novel and intuitive way. Similar genes are clustered together spatially and represented in a three dimensional terrain map, where large mountains represent large clusters of similar genes, and smaller hills represent clusters with fewer genes (Figure 1). Clusters

Figure 1
that are the most similar (genes or patients) are also sited nearer to each other and farther away from less similar clusters. The analogy to real terrain allows one to memorize the landscape, which makes subsequent explorations very easy. The level of detail can be changed to ‘fly’ over the terrain, and view smaller clusters within clusters. The results of database queries can then be shown within the context of the clusters by highlighting those genes matching the queries. VxInsight also provides a simple way to select genes and immediately display web-base information for those genes. In addition, all of clinical and laboratory covariables can be loaded into the program and the visual display and clusters can be queried in real-time and reconfigured to show the interaction between the patients or genes in a cluster and these clinical and laboratory covariables. In applying VxInsight to our leukemia data sets, the raw gene expression data were transferred from the Affymetrix scanner and LIMS server to an ORACLE database. These raw data were then transformed with two methods. Log (base 2) transforms were initially used to reduce the effect of the long distribution tails on the similarity computations (which use Pearson’s correlation coefficient, R). However, we prefer to use Savage-Scoring, a nonparametric transform that decreases the influence of genes with the lowest expression levels. The transformed data were used to identify genes with similar expression profiles (all pairwise comparisons between the 12,625 rows in the gene matrix by computing Pearson’s correlation coefficient for those pairs across the 127 patients (Figure 1, inset right, top view) and also to identify patients who had similar patterns in their gene expressions (all pairwise comparisons between the 127 columns of patients where Pearson’s correlation coefficient is computed across all 12,625 genes; Figure 1, inset right, left view). The 20 highest-ranking correlations for each gene (and the five highest ranking correlations for each patient) were used to produce clusters of genes (and of patients). The close relationship between clusters of patients and the genes purportedly causing those clusters can be explored by coupling two VxInsight sessions together with a mechanism for computing gene contrasts. One obvious question might be: ‘which genes distinguish the patients in the cluster labeled white versus the cluster labeled green (Figure 1, left view)?’ To explore this question the patients to be contrasted are selected and a user contrast requested. An analysis of variance (ANOVA) is computed for each of the 12,625 genes comparing the patients in these two groups, and the contrast between the mean gene expression levels in the two groups is computed for every gene. This list is sorted and made available for display in the second VxInsight session showing those genes with contrasts between the two patient groups that have significant F-statistics. The genes having F-values in the 99 percentile are also immediately available as an HTML page, which links the gene to its description at OMIM (Figure 1, bottom).

Application of class discovery: identification of novel biologic classes of infant leukemia using VxInsight

An illustrative example of class discovery methods using either PCA or VxInsight can be seen in our analysis of infant leukemia. When VxInsight was applied to expression array data derived from our infant leukemia cohort, we discovered that there were at least three statistically significant, intrinsic biologic groups of infant leukemia and that these intrinsic biologic groups could not simply be predicted by ALL versus AML.
labels or by the presence or absence of cytogenetic abnormalities involving MLL (Figure 2). These findings were unexpected. In each cluster, an individual patient sample (and its associated gene expression profile) is represented by a pyramid (highly similar patients in each cluster will be overlapping in this ‘high level’ view). By querying VxInsight in ‘real-time,’ we can ask which cases were previously diagnosed as ALL (pyramids colored white) versus AML (pyramids colored green) (Figure 2); thus it is clear that the clusters are not defined by AML versus ALL morphology. In addition, we can perform the function in VxInsight described in the paragraph above and ask VxInsight to provide the most statistically significant genes that distinguish each of these clusters (some of the most significant of these genes are listed in the boxes adjacent to each cluster in Figure 2). Interestingly, the top cluster of cases (Figure 2) has a gene expression profile reflective of a more multipotent stem cell, perhaps at the hemangio-blast stage with expression of endothelial, stem cell, and genes associated with the earliest stages of hematopoiesis; interestingly, the outcome to current leukemia therapies was worst in this group compared to patients within the other two clusters. The left most cluster of cases (Figure 2) is virtually all ALL cases and is characterized by expression of B lineage antigens and a high frequency of MLL rearrangements, predominantly t(4;11). This cluster interestingly has activation of many viral-induced genes that could reflect either viral infection or a stress response. Finally, the third right-most cluster (Figure 2) contains 42 AML and 12 ALL cases. This set of cases has activation of many DNA repair and GST genes, methylation genes, and novel genes in the RAS signaling pathway. All of the genes identified in this cluster analysis represent potentially novel diagnostic and therapeutic targets for leukemia. Analysis of this cluster reveals that there is a subset of infant ALL cases with a gene expression profile most consistent with a myeloid profile. These findings, if validated in our current prospective cohort, have implications for classification and therapeutic targeting in infant leukemia.

Class prediction: using supervised machine learning methods to identify gene expression profiles associated with or predictive of clinical outcome and biologic parameters

A second major goal of our studies has been to identify sets of genes that could be used to predict the presence or absence of specific morphologic types of leukemia (such as AML versus ALL), gene expression profiles specific for recurrent cytogenetic or molecular genetic abnormalities seen in leukemia, and most importantly, gene expression profiles that could be used to predict for outcome and therapeutic response. Such analyses require ‘supervised’ machine learning methods in which the observer first ‘trains’ or derives a gene expression profile on a training set of cases and then subsequently ‘tests’ the predictive power of this gene expression profile on a set of previously unanalyzed ‘test’ cases. The statistical design of the patient or sample cohort under analysis and careful consideration of biologic and experimental variables that may impact the design and composition of the training and test sets are absolutely paramount for successful analyses. Obviously, if large numbers of expressed genes are to be analyzed in a large cohort, computing hardware and supervised learning algorithms must be very robust. Many of these techniques, particularly when ‘leave one out’ cross-validation is performed on large data sets, require extensive parametric studies or the solution of large matrix problems that can only be done using parallel computers. Thus, our group and others have developed fast parallel algorithms to solve these mathematical problems on the high performance massively parallel computers. Identifying gene expression profiles predictive of clinical outcome using supervised learning methods can be a very difficult and complex task. Our clinical labels for ‘continuous complete remission (CCR)’ or long term remission and ‘failure/relapse’ or ‘overall survival’ are not biologically pure or precisely and consistently defined, and are dependent on time and on numerous host and tumor factors. In addition, there is a tendency to ‘over-fit’ supervised learning algorithms and computational approaches to find predictive profiles and genes. Frequently, genes discovered by these approaches on the training set of cases cannot be validated in the test set in a statistically significant way. Thus, these methods require carefully designed cohorts, crossvalidation, and statistical analyses. Although it is best to confirm preliminary results on independent data sets, this is rarely done. Nonetheless, identification of genes and pathways associated with treatment failure is essential for understanding therapeutic resistance and the development of appropriate therapies that overcome resistance. Identification of novel genes that are predictive of outcome, if successful, would also potentially significantly enhance current risk classification schemes. Our group and others have developed and applied several different learning methods for class prediction in leukemia cohorts; two that we use frequently include Bayesian networks and Support Vector Machines. These methods will be briefly described and an illustrative application in pediatric ALL will be presented.

Class prediction using Bayesian networks

Our work with Bayesian networks have shown classification class prediction rates in excess of 90–95%. A Bayesian network is a graph-based model for representing probabilistic relationships between random variables. A Bayesian net asserts that each node is statistically independent of all its nondescendants, once the values of its parents (immediate ancestors) in the graph are known. This makes Bayesian nets an attractive framework for gene expression analysis, since they can methodically hypothesize and test gene
regulatory models (and other such relationships) using the rigorous methods of classical probability theory and statistics. One of the strengths of the Bayesian net approach is its ability to sample, evaluate, and synthesize a large number of alternative scenarios, represented by different networks with corresponding posterior probabilities. To construct a Bayesian net, cohorts were divided into training (2/3 of cases) and test (1/3 of cases) subsets; computational scientists were blinded to all clinical and biologic covariables during training, except those necessary for the computational tasks. A large number of computational experiments were performed, in order to properly sample the space of Bayesian nets satisfying the constraints of the problem.

In our work on the classification problem (ALL versus AML, long-term remission versus failure, presence of a cytogenetic abnormality or not), in the context of high-dimensional gene expression data, the inclusion of more nets than is typical in the literature appears to yield better results. This problem presents natural parallelism, and thus we can use significantly larger samples with supercomputers.

Class Prediction Using Support Vector Machines with Recursive Feature Elimination (SVM-RFE). New methods utilizing recursive feature elimination in the context of SVM are being developed for identifying robust sets of 'discriminating genes' that distinguish between distinct classes of disease or prognostic outcome. This approach, in both linear and nonlinear modifications, has proven extremely valuable. SVM attempts to define the maximal hyperplane (or corridor) between two parameters (such as long-term remission versus failure) in a gene expression data set. This corridor or hyperplane may be linear or nonlinear. Genes marking the boundaries of this hyperplane are the most discriminating. A first step in most classification models is the application of feature selection techniques to identify those unique and robust gene sets that best discriminate among the classes of interest. Recursive feature elimination (RFE) is an SVM-based method for feature selection in binary classification problems. RFE searches through the given gene space of approximately 12000 genes to find the optimal hyperplane separating the two classes. The process results in a weight being assigned to each gene. Hence, an iterative procedure can be implemented whereby, per iteration, the genes whose corresponding weight magnitudes are the smallest can be removed, and then the SVM is trained using only the surviving features/genes. In most instances of RFE in the literature, the overall training error on a given subset of genes is the sole criterion for judging performance. Other metrics of classifier assessment are rarely used. We are investigating the use of early stopping criteria, as used in neural networks, to decide as to which genes to preserve at each iteration. This technique combines the advantage of monitoring the SVM training process with respect to generalization, to avoid overtraining the classifier, as well as providing an effective way to rank relative gene importance in the resulting gene lists, analogous to the TNoM score. Use of Supervised Learning Methods for Class Prediction to Find Novel Genes Associated with Outcome in Pediatric ALL. To identify genes strongly predictive of outcome in pediatric ALL, we divided our POG ALL case control cohort (n = 254) described above into training (2/3 of cases) and test (1/3 of cases) sets and applied supervised learning methods (Support Vector Machines, Bayesian Networks, VxInsight/ANOVA, TNoM) and performed statistical analyses. Through this approach, we identified a limited set of novel genes that were predictive of outcome in pediatric ALL. Several interesting genes and novel genes were identified that we are currently further investigating. Interestingly, one of these genes termed G0 in the Bayesian analysis (see Table 1; 38653_at; NM_Hypothetical protein FLJ20154) was discovered by four different supervised learning algorithms and was ranked extremely high (top five or 10 genes) or at the top (Bayesian) with each of these very distinct modeling approaches. The degree of overlap between outcome genes detected with these different modeling algorithms was quite striking. A particularly strong set of candidate genes predictive of outcome was identified through a Bayesian network.
approach. The three genes in the strongest predictive tree identified by Bayesian networks are provided in Table 1.

Our analyses have shown that pediatric ALL patients whose leukemic cells contain relatively high levels of expression of each of these genes have an extremely good outcome while low levels of expression of these genes is associated with treatment failure. G0, a novel gene identified only as an EST and a hypothetical protein on the Affymetrix U95A chip, which we have now fully cloned and named OPAL1 I (for Outcome Prediction in Acute Leukemia number 1) conferred the strongest predictive power and was at the top of predictive lists in all supervised learning methods employed. By assessing the level of OPAL1 alone, ALL cases could be split into those with good outcomes (OPAL1 high: 87% long-term remissions) versus those with poor outcomes (OPAL1 low: 32% long-term remissions, 68% treatment failure). Detailed statistical analyses of the significance of OPAL1 expression in the retrospective cohort revealed that low OPAL1 expression was associated with induction failure, while high OPAL1 expression was associated with long-term event-free survival, particularly in males. Higher levels of OPAL1 were also associated with certain cytogenetic abnormalities (such as t(12;21) and normal cytogenetics). Although the number of cases were limited in our initial retrospective cohort, low levels of OPAL1 appeared to define those patients with low risk ALL who failed to achieve long-term remission, suggesting that OPAL1 may be useful in prospectively identifying children with low or standard risk disease who would benefit from further intensification. As a very significant validation of the importance of this novel gene in outcome prediction, we validated its predictive ability in an independent array cohort of cases developed by St Jude and reported by Yeoh et al. OPAL1 was also highly predictive of outcome in this set of cases, a critical validation step. We also found a trend between high OPAL1 and improved outcome in our retrospective cohort of infant ALL cases. OPAL1 was also predictive of outcome in T ALL (P = 0.02), as well as B precursor ALL. We have also now developed real time automated RT-PCR assays to directly assess this and other genes in pediatric ALL and these further validation studies are underway. As OPAL1 has been fully characterized, we have found that it encodes at least five different mRNAs through alternative splicing of 5′ exons. The genome also contains many pseudogenes, making specific detection of this low abundance transcript challenging.

In summary, supervised learning and computer learning algorithms are identifying new genes that may significantly contribute to the refinement of risk classification in acute leukemia and which may be further developed as diagnostic and therapeutic targets.

References

DIFFUSE LARGE B-CELL LYMPHOMA

Molecular heterogeneity of diffuse large B-cell lymphoma

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Introduction

Diffuse large B-cell lymphoma (DLBCL) comprises the largest subtype of non-Hodgkin’s lymphomas (NHL), accounting for approximately 32% of all lymphomas seen throughout the world. It is characterized by a significant spectrum of morphology, immunophenotypic features and molecular genetic alterations. Several subtypes have been identified and are listed in Table 1. Additionally, morphological variants are also recognized, although the reproducibility of these distinctions is shrouded in controversy. These are also listed in Table 1 and are included within the rubric of DLBCL in the World Health Organization (WHO) classification. This review will focus on the molecular genetic heterogeneity of DLBCL, with emphasis on what is known about disease initiation events (mostly translocations), loss of critical tumor suppressor genes, gene copy number alterations and gene expression profiles, all of which have contributed a significant improvement in our understanding of this molecularly diverse group of tumors.

Relationship to normal B-cell differentiation

Our understanding of normal B-cell ontogeny has provided the basis for understanding DLBCL. B cells originate in the bone marrow where they undergo primary rearrangements of their immunoglobulin genes prior to encounter with antigen. Those cells with successful rearrangements will exit the bone marrow and seed secondary lymphoid organs such as lymph nodes, tonsil, spleen and Peyer’s patches. At these sites, B cells will encounter antigen in the paracortex and then migrate to form follicles together with follicular dendritic cells and T cells, a process that results in the formation of germinal centers in secondary lymphoid follicles. The default program in the germinal center is cell death (apoptosis), but those cells with high-affinity receptors on their surface will survive this environment and exit the follicle to become terminally differentiated plasma cells or long-lived memory B cells. The process of refining the humoral immune response in the germinal center is known as affinity maturation, brought about the action of the somatic hypermutation apparatus. This introduces predominantly single base pair mutations into the germline sequence of the immunoglobulin heavy (IGH) and light chain sequences. Evidence of somatic hypermutation in a B cell serves as a fingerprint for that B cell having seen the germinal center microenvironment. For the most part, this molecular technique is used to distinguish naive B cells (germline IGH), from germinal center derived or postgerminal center B cells (mutated IGH). These same techniques are used to characterize DLBCLs as either germinal center or postgerminal center-derived neoplasms. The vast majority of DLBCLs show evidence of having seen the germinal center.

Chromosomal translocations

Chromosomal translocations characterize most leukemias, NHLs and sarcomas. Typically, these are balanced exchanges of chromosomal material between two partner chromosomes. In acute leukemia, translocations often result in novel fusion mRNA species and proteins that contribute to the pathogenesis of the leukemia. With few exceptions in NHLs, most translocations result in the deregulated expression of a proto-oncogene in the proximity of the chromosomal recombination site. Examples include the BCL2 oncogene at chromosome 18q21.3, the BCL6 oncogene at 3q27 and a small number of other oncogenes. The most common translocations in DLBCL are those involving the BCL6 gene. These often involve the immunoglobulin loci at 14q32, 2p12 or 22q11, corresponding to IGH, kappa light chains or lambda chain genes, respectively. However, the BCL6 oncogene is promiscuous and translocations may involve a number of other genes referred to as variant translocation partners.

The t(14;18)(q32;q21.3) involving the BCL2 oncogene is a characteristic finding in follicular lymphoma (FL). This same translocation can be found in 15–20% of de novo DLBCLs, but does not necessarily indicate a relationship to clinically silent FL. It does, however, provide insights into lymphomagenesis, as it is only seen in DLBCLs with a germinall center B cell-like (GCB) gene expression profile (see below). Similarly, translocations involving the MYC oncogene (chromosome 8q24)
Table 1 Subtypes of DLBCL

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<tr>
<th>DLBCL subtypes</th>
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<tr>
<td>Primary mediastinal large B-cell lymphoma</td>
<td>Centroblastic</td>
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<tr>
<td>Intravascular large B-cell lymphoma</td>
<td>Immunoblastic</td>
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<td>Primary effusion lymphoma</td>
<td>Anaplastic</td>
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<td>T-cell/histiocyte-rich large B-cell lymphoma</td>
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<td>Lymphomatoid granulomatosis-type large B-cell lymphoma</td>
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are typically found in cases of Burkitt lymphoma. Although a sensitive marker of this disease, it is not entirely specific. MYC oncogene alterations can also occur in de novo DLBCL, accounting for 5–7% of cases. A number of other rare chromosomal translocations are also found in DLBCL. In aggregate, chromosomal translocations are detected in approximately 55–60% of DLBCL cases, leaving a sizeable minority of cases left unexplained by this mechanism.

Tumor suppressor genes

A sizeable fraction of cases of DLBCL are characterized by the loss of function or frank deletion of tumor suppressor genes (TSG). These may occur as secondary events in clonal evolution of the tumor, or alternatively may be important events in disease initiation in a smaller number of cases. The classic example is the p53 gene on chromosome 17p13, the loss of which characterizes a small number of DLBCL cases. Others include the p16 gene at chromosome 9p21. This gene can be silenced by epigenetic mechanisms as well, including hypermethylation of the promoter leading to transcriptional inactivation. The retinoblastoma gene may also be involved in a small number of DLBCL cases. Others include the p16 gene at chromosome 9p21. This gene can be silenced by epigenetic mechanisms as well, including hypermethylation of the promoter leading to transcriptional inactivation. The retinoblastoma gene may also be involved in a small number of DLBCL cases.

It is important, but not always possible, to distinguish de novo DLBCL from transformation of FL into DLBCL (secondary DLBCL). In time, the majority of FL cases will transform into a disease most often resembling DLBCL. Most of these cases will harbor a t(14;18) and will have acquired additional alterations that contribute to histologic transformation. The majority of the cytogenetic events that underlie transformation are related to copy number alterations in a number of critical genes. The best described include p53 loss, p16 deletions or hypermethylation, MYC translocations and occasional cases with alterations of BCL6 or BCL2 genes.

Immunophenotypic heterogeneity

Clear evidence of the molecular heterogeneity of DLBCL is reflected by the spectrum of immunophenotypic patterns encountered in DLBCL. DLBCLs that appear to arise from the germinal center typically express Bcl-6 and CD10 proteins. Those that arise from postgerminal center cells may still express Bcl-6, but also characteristically express MUM-1 (IRF-4) and less often CD138. This panel of markers can be used to distinguish DLBCL of various stages of differentiation. Moreover, these markers can be used as surrogates for the gene expression profile and are predictive of outcome in this disease.

A classic example of molecular heterogeneity in DLBCL is the expression of Bcl-2 protein. Depending on the threshold used to distinguish positive versus negative cases, Bcl-2 is expressed by approximately 60–70% of DLBCL cases. Thus, expression of Bcl-2 is imperfectly correlated with the presence of the translocation, i.e. t(14;18) that is found in only 15-20% of cases. As indicated above, the presence of the t(14;18) is restricted to cases within the GCB-type of DLBCL. Most of the cases harboring this translocation also express Bcl-2 protein, albeit at lower levels than typical ABC-type of DLBCL. Uncommonly, cases can be shown to have a BCL2 translocation, but fail to express the Bcl-2 protein. These probably result from mutations resulting in premature stop codons or unstable mRNA molecules. Thus, increased BCL2 mRNA is found in a small fraction of GCB-type of DLBCL. However, the highest frequency of Bcl-2 protein-positive cases is within the subgroup of DLBCL with a gene expression profile mimicking activated peripheral blood B cells (ABC-type). Therefore, other molecular alterations must be active to explain expression of the Bcl-2 protein. The most frequently implicated mechanism is transcriptional upregulation of Bcl-2 expression resulting from constitutive activation of the NF-κB transcription factor. Elevated levels of NF-κB characterize the ABC-type of DLBCL and the BCL2 gene is a well-known target of this transcription factor. A second dominant mechanism of Bcl-2 expression in DLBCL cases lacking a t(14;18) appears to be increased copy number of the BCL2 gene. This may occur by two mechanisms, one of which appears to result from tandem duplication of the BCL2 gene. Importantly, this mechanism is mutually exclusive of the t(14;18). Increased copy number of BCL2 accounts for more cases of Bcl-2-positive DLBCL than does the BCL2 translocation. Rare cases of Bcl-2-positive DLBCL may result from somatic alterations of the 3'UTR of the BCL2 mRNA resulting in increased stability of the message. Lastly, some cases may be due to epigenetic alterations, including hypomethylation of the BCL2 promoter. Thus, expression of Bcl-2 protein may be the result of a variety of different molecular mechanisms that target the BCL2 gene and provide the tumor cells with a growth advantage.

Gene expression profiling

Recent developments in nanotechnology and completion of the first iteration of the human genome project laid the groundwork for the advent of new technology in
the era of genomic medicine. Previous studies of biological factors in NHL typically included the analysis of one or at most a few biomarkers in a given subtype of NHL and their correlation with clinical parameters. Gene expression profiling has allowed us to examine the entire transcriptome using genomewide approaches such as microarray technology, and have resulted in a major paradigm shift in the taxonomy of human tumors.

The initial publication in February 2000 created a great deal of excitement in the lymphoma world. Using a noncommercial array, the Lymphochip, investigators demonstrated that microarray technology could be used to study clinical NHL samples and that these data had an impact on predicting outcome in DLBCL. The authors used an approach of identifying gene signatures that represented groups of genes that are coordinately expressed. The study of normal B cell populations allowed the identification of two signatures that mimicked B-cell differentiation. The first was a list of genes that were characteristically expressed by normal germinal center B cells (GCB) and included genes such as CD10, BCL6, LMO2, A-myb, JAW1 etc. Importantly, BCL2 was not overexpressed in these cells as would be expected for benign germinal center B cells. The second group revealed a gene expression pattern similar to mitogen-stimulated peripheral blood B cells known as activated B cell (ABC) and included genes such as IRF-4, FoxP1, Cyclin D2, Flip, etc. Within the DLBCL data set, cases could be stratified into these two gene expression groups including GCB and ABC subtypes. Importantly, the GCB group conferred a favorable survival in comparison to the ABC group, independent of the clinical IPI factors. Thus, gene expression microarrays had been shown for the first time to be capable of determining prognosis in a human cancer (see Table 2).

This initial study of DLBCL was followed by two additional studies. The group led by Margaret Shipp published in early 2002 a study of 58 DLBCL samples. These authors analyzed cases using Affymetrix arrays and supervised learning to determine the gene list that allowed the discrimination between patients who did well following diagnosis versus those with fatal/refractory disease. This allowed the identification of 13 genes that were highly statistically different between these two widely disparate clinical groups. However, when the cellular stage of differentiation algorithm of the LLMP was applied (ie GCB versus ABC), this approach did not appear to be able to predict outcome. In retrospect, this appears to be due to the fact that the gene list chosen to identify GCB was not accurate, and failed to include the best discriminator genes for this distinction. A repeat of this analysis by members of the LLMP confirmed that within the Shipp data set, GCB versus ABC had prognostic value.

Rosenwald et al representing the LLMP published a very large confirmatory study in June 2002. It confirmed the previous studies from Alizadeh et al and validated that GCB versus ABC predicted outcome in DLBCL. This study of 240 cases of DLBCL also identified a third subgroup of DLBCL within these data, initially referred to as ‘type 3’. The nature of these cases has remained elusive, but is believed to contain several subgroups of DLBCL. This ‘type 3’ subgroup has now been renamed ‘unclassifiable’ following a study using a new statistical approach to identifying subgroups based on Bayes algorithm. Importantly, this approach allows a better discrimination of these data, with many previously identified type 3 cases being reassigned to the GCB subgroup and shrinking the size of the ‘unclassifiable’ subgroup to only one sixth of the entire group.

The LLMP study demonstrated that a small number of predictive genes could be used to construct a linear predictor score for determining outcome in patients with DLBCL. This included genes involved in proliferation, host immune response (MHC class II), the ‘lymph node’ signature comprising genes expressed by cells resident within the lymph node and germinal center signature genes (n = 16). A single gene, BMP6 was shown to have independent predictive power and thus was added to this gene list, now totalling 17 genes. This linear predictor was clearly shown to have prognostic value and was independent of the IPI clinical variables. Moreover, the distinction of DLBCL cases based on GCB versus ABC distinctions had some merit in distinguishing lymphomas with different oncogenic events. For example, the GCB group was associated with BCL2 translocations and amplification of the C-REL locus on chromosome 2p. These molecular alterations were never found in DLBCL cases classified as ABC-type. On the other hand, the ABC cases were characterized by constitutive activation of the NF-κB pathway and thus over-expressed a large number of genes identified as NF-κB targets. Bcl-2 protein was commonly expressed in the ABC group, as the BCL2 gene is a well-recognized NF-κB responsive gene. These distinctions and their impact on survival in DLBCL are shown in Table 2.

These two studies provide some important caveats concerning the use of this methodology. Firstly, a frozen tissue archive is invaluable for performing this type of research. Clinicians and pathologists alike must

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<th>Table 2 Gene expression microarray profiles</th>
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<td><strong>Germinal center B cell (GCB)</strong></td>
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<td>Clinical outcome</td>
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continue to collect such material on newly diagnosed patients in order to have the archive necessary to address the next series of questions. Secondly, the cell of origin approach to dividing DLBCL based on gene expression technology has clinical meaning. Lastly, novel gene discovery remains a real advantage of microarray studies, but we need to have more infrastructure to facilitate the clinical translation of this new knowledge. Table 1 lists DLBCL according to the new WHO classification into subtypes and morphological variants. Primary mediastinal large B-cell lymphoma (PMBCL) serves as a perfect example of the power of this new technology. For many years, PMBCL has been clinically defined. Despite this approach, there are no uniform criteria available for what constitutes a diagnosis of PMBCL. Much controversy surrounds the clinical relevance of a diagnosis of PMBCL and the best choice of therapy.14 There is little doubt that much of the controversy is due to the fact that most published series of PMBCL include a heterogeneous mix of cases consisting of DLBCLs that arise predominantly in mediastinal lymph nodes and cases of true PMBCL that arise from a normal B-cell resident to the thymus. Molecular profiling studies have now definitively established that PMBCL is a specific subtype of DLBCL with a unique gene expression profile. In two separate studies, it was confirmed that PMBCL is distinguished from either GCB or ABC-types of nodal DLBCL by hundreds of genes that are differentially expressed.15,16 Moreover, the transcriptome of PMBCL shares many features with classical Hodgkin’s lymphoma, suggesting both a clinical and biological overlap between these two disease entities. These data provide a rigorous means to distinguish difficult cases and may even suggest that rare cases of PMBCL may not present with a predominantly mediastinal localization.

Finally, a recent tissue microarray study has validated the previous gene expression studies and provided some findings to suggest that the clinical translation of this new technology into the routine laboratory and its direct application to patient care might occur at the level of immunohistochemistry.7 In other words, although new gene discoveries and targets may require the use of microarray studies using a genomewide approach, a short list of surrogate markers may be developed to both validate the mRNA expression levels at the protein level and help direct targeted therapies.

Conclusions

DLBCL represents a disease entity with a significant degree of heterogeneity. The application of molecular profiling technology to better understand the diverse spectrum of biology will allow a much better definition of distinct disease entities within the rubric of DLBCL. A clear appreciation of this biological and clinical diversity may have suggested to some that DLBCL was not a good place to start with such molecular profiling studies; however, a strong argument can be put forward that it represented the perfect place to try to introduce a molecular taxonomy into the classification of NHLs. These data, particularly when integrated with high-resolution array comparative genomic hybridization data, will facilitate the understanding of DLBCL molecular mechanisms and may hold the secret to finally realizing truly targeted therapy in NHL.

References

PET scan in the therapeutic strategy

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Radionuclide imaging has been used in the evaluation of lymphoma, particularly with gallium-67, to supplement the information obtained from computed tomography (CT). The emergence of ¹⁸F-2-fluoro-2-deoxy-D glucose positron emission tomography (FDG-PET) and its increasing availability have provided an alternative of functional imaging with potentials for improving the accuracy of tumour staging, for the assessment of response identifying both early effective responders and poor responders and, finally, for the monitoring of patients after completion of therapy with early detection of relapse.

FDG-PET: physical properties and pharmacokinetics

FDG-PET is a rapidly evolving whole-body functional imaging technique, which takes advantage of the fact that malignant tumours have an increased rate of aerobic glycolysis, compared to normal tissue. Most tumours demonstrate an increase in glucose transporters as well as increased hexokinase and decreased glucose-6-phosphatase activities, resulting in a trapping of the glucose analogue FDG. Briefly, FDG is transported into viable cells by facilitative glucose transporter molecules, where it is phosphorylated by hexokinase into FDG-6-phosphate, as glucose is normally phosphorylated into glucose-6-phosphate. Unlike glucose-6-phosphate, however, FDG-6-phosphate undergoes no further metabolism within the cell. Moreover, its dephosphorylation by glucose-6-phosphatase is a relatively slow process in comparison to that of glucose-6-phosphate. This, combined with the fact that FDG-6-phosphate cannot easily cross the cell membrane, results in entrapment of FDG-6-phosphate within viable cells. The entrapment is further potentiated in malignant cells, which typically have a greater number of glucose transport molecules (GLUT-1 and -3) at the cell surface, higher hexokinase activity and lower level of glucose-6-phosphatase.

The radionuclides used for radiolabelling in PET are positron emitters, whereas single photon emitters are used in conventional nuclear medicine imaging. An emitted positron (a positively charged beta particle, signified by β⁺) will penetrate only a few millimetres in soft tissues before combining with an electron (β⁻). The particle pair then annihilates and their masses are entirely converted into energy. This energy takes the form of two 511 keV annihilation photons, emitted at approximately 180° from each other. Detection of both annihilation photons is termed coincidence detection, and this is the principle by which PET tomographs operate. Fluorine-18, the most widely used tracer in clinical PET applications, has a half-life of 109.8 min, allowing acquisition of images over 30–120 min.

Primary staging

The accuracy of FDG-PET as an imaging tool for primary staging of lymphoma suffers from the absence of a reference criterion to which all imaging modalities can be compared. It is generally reported that FDG-PET is superior to conventional staging, that is, physical examination, laboratory tests, radiographies and CT, by 10–20%. Review of the literature reveals only a limited number of studies that have addressed this issue. Moreover, most of these studies mixed Hodgkin and different subtypes of non-Hodgkin lymphomas and compared only PET to anatomical imaging with CT.¹,² The clinically relevant question of how PET impacts on the staging of lymphoma and, above all, whether or not up or down staging leads to changes in therapeutic strategy has been addressed in some studies and variable results have been reported.³,⁴ In a prospective study,⁵ FDG-PET has been demonstrated as an efficient and non-invasive method for the primary staging of patients with untreated lymphoma. FDG-PET, CT and bone marrow biopsy were performed to investigate lymph node/extranodal localizations and bone marrow infiltration. The accuracy of FDG-PET was significantly superior to CT in detecting lymph node and extranodal lymphoma, irrespective of supra diaphragmatic or infradiaphragmatic spread of lymphoma and was found to be equivalent to bone marrow biopsy. In a preliminary analysis of our series of 50 patients with diffuse large B-cell lymphoma (personnal data), the clinical impact of PET was evaluated for primary staging as compared to that of conventional imaging and bone marrow biopsy. Staging by PET was concordant in 40/50 patients, better (up) than conventional staging in four and worse (down) in six. Among the downstaged patients, two had bone marrow involvement.
Response to first-line therapy

Obtaining a complete remission after first-line treatment is the main objective in aggressive lymphoma as it is usually associated with a longer progression-free survival. An initially bulky tumour may remain enlarged without activity because of fibrosis and/or tumour necrosis. The status of CRu (complete remission unconfirmed) reflects the unknown significance of persisting radiological abnormalities in patients who otherwise seem to be in complete remission. FDG-PET is considered as a more efficient method than gallium-67 scintigraphy because of superior resolution and sensitivity, better interpretation of residual lesions in the abdomen and because the maximum examination time is 2h. The most important question in the post-treatment evaluation of aggressive lymphoma remains: can FDG-PET identify, among patients in CR/CRu, those with active disease and potentially poorer clinical outcome? A limited number of published studies addressed this question and showed that post-treatment evaluation with FDG-PET was highly predictive of disease outcome with better accuracy than CT. Mikhaeel et al. showed that the relapse rate of aggressive lymphoma was 100% for positive PET after treatment and 17% for negative PET, leading to a 1-year progression-free survival of 0% for the PET-positive group, compared to 83% for the PET-negative group. Spaepen et al. confirmed, on a larger series of 93 patients, the important role of FDG-PET in the post-treatment evaluation. All patients with a positive PET relapsed early. No false-positive results were noted; however, a negative PET could not exclude minimal residual disease since 11 of the 67 patients with a negative PET relapsed. Nevertheless, progression-free survival was significantly longer in this group than in patients with a positive PET (median progression-free survival: 404 versus 73 days). Of interest, in another series, the majority of relapses in FDG-PET-negative patients occurred outside the residual mass site.

Early response to first-line treatment

Since more aggressive, but also potentially more toxic treatments, are now available, there is an increasing interest in the treatment monitoring of patients with aggressive lymphoma (see Figures 1 and 2). The clinical parameters incorporated in the international prognostic index (IPI), which has become an established tool for risk stratification, grossly reflect the biological heterogeneity of the disease. In this respect, the duration of a complete remission might be significantly more influenced by the chemosensitivity than by the initial IPI factors; consequently, an early evaluation during treatment, leading to an alternative treatment might improve outcome. Several studies have pointed out the prognostic importance of 67Ga scans performed in aggressive lymphoma patients at mid-treatment. Notably, Janicek et al. have shown that 70% of patients who had a negative interim 67Ga scans midway through standard induction treatment remained disease-free whereas only 25% of patients who were 67Ga-positive at the same therapeutic point had a durable remission. More recently, several studies have established that interim FDG-PET scans after 2–3 cycles of chemotherapy provided valuable information regarding early assessment of response and survival. Romer et al. documented the extent and time cause of changes in FDG metabolism in response to chemotherapy in 11 patients. Conventional chemotherapy induced a rapid decrease in FDG-uptake at 7 days after...
treatment. However, FDG uptake at 42 days correlated better with outcome than the 7 day parameter. Jerusalem et al.11 have shown on 28 patients, who underwent an FDG-PET after a median of 3 cycles of chemotherapy, that persistent uptake was predictive of progression-free survival and overall survival. However, it has to be outlined that the number of studied patients was rather small and that the population was mostly heterogeneous regarding histology and status (first line or relapsing patients). Mikhaeel et al.14 published a series of 23 patients with newly diagnosed aggressive non-Hodgkin’s lymphoma who underwent a PET scan after 2–4 cycles of chemotherapy. No relapse was observed in patients with no residual disease compared with a 87% relapse rate in those with persistent PET positivity. Spaepen et al.12 have shown the important prognostic value of mid-treatment FDG-PET in the first-line therapy monitoring of 70 aggressive non-Hodgkin’s lymphoma. At mid-treatment, 33 patients showed persistent abnormal FDG uptake and none of them achieved a durable complete remission, whereas 37 showed a negative scan. Out of the 37 patients, 31 remained in complete remission. Comparison between groups indicated a statistically significant association between FDG-PET findings and progression free survival and overall survival. By multivariate analysis, FDG-PET at mid-treatment was a stronger prognostic factor for progression-free survival and overall survival than IPI. More recently, we have confirmed13 the early (after 2 cycles) prognostic impact of FDG-PET in terms of response and survival on a series of 50 patients, 96% of them with diffuse large B-cell lymphoma. After the first two cycles, 32 patients were considered PET-negative and 18 PET-positive. After induction treatment (4 cycles), 31 out of 32 PET-negative patients were considered in complete remission on the basis of conventional diagnostic methods, out of whom 28 remained in complete remission at the last follow-up. Conversely, only six out of 18 PET-positive achieved complete remission and four of them remained with this status. Two-year survival differed significantly between the two groups of PET-positive and PET-negative patients. To note, the prognostic impact of early PET was observed in both the lower-risk (low and low-intermediate) and the higher-risk (high-intermediate and high) patients, as defined by IPI.

Kostakoglu et al.14 have recently reported the prognostic value of FDG-PET after only one cycle of chemotherapy and compared this information with the post-treatment scan. In 23 patients, positive FDG-PET obtained both after the first cycle and at completion of therapy was associated with a shorter progression-free survival, compared to negative PET. Results obtained after the first cycle correlated better with progression-free survival than those obtained at the end of treatment leading to consider, on this small and heterogeneous series that FDG-PET had greater sensitivity and positive predictive value after the first cycle than after the last one.

Prognostic impact before high-dose therapy (HDT) followed by autologous stem cell transplantation (ASCT)

The superiority of HDT/ASCT over conventional salvage chemotherapy in relapsed aggressive lymphoma has been shown by the randomized PARMA trial on patients who remained chemosensitive.15 A later retrospective analysis suggested that the benefit of HDT/ASCT over chemotherapy was more pronounced in the higher-risk patients defined on the basis of IPI.16 Moreover, it has been shown that HDT/ASCT as consolidation therapy for patients in first complete remission, offered a survival benefit in the higher-risk patients.17 However, discrepancies have been observed between trials and additional prognostic factors are needed. The value of FDG-PET to predict clinical outcome after HDT/ASCT has been established in a few published series.18,19 The larger and more recent study20 assessed the prognostic value of FDG-PET after salvage chemotherapy before HDT/ASCT on 60 patients with induction failure or relapsing chemosensitive lymphoma. In all, 30 patients had a negative FDG-PET before HDT/ASCT and only three of them relapsed. By contrast, among the 30 patients with a residual uptake before HDT, 26 had relapse. Comparison between the two groups indicated a statistically significant association between FDG-PET findings and progression-free survival and survival. Consequently, in the near future, such an information should extend the concept of chemosensitivity used for the selection of patients who will benefit from HDT/ASCT.

Combined PET-CT systems

Recently, integrated PET-CT scanners have been made available. They were initially designed to improve attenuation correction of conventional PET images: indeed, deep foci of uptake are physically attenuated by...
the surrounding tissues before the photons can reach the detector, leading to a loss of sensitivity. The addition of a CT gantry to the PET scanner allows to obtain high-resolution cartographies of attenuation coefficients (air, soft tissue, bone) and improves substantially the image quality. In addition, it furnished an excellent anatomical reference to help interpretation of foci of uptake (Figure 3): for example, it may help to distinguish a reference to help interpretation of foci of uptake quality. In addition, it furnished an excellent anatomical soft tissue, bone) and improves substantially the image resolution cartographies of attenuation coefficients (air, a CT gantry to the PET scanner allows to obtain high-resolution cartographies of attenuation coefficients.

FDG-PET. In a recent study, integrated PET-CT has been shown to provide additional diagnostic information in 41% of 49 patients referred for suspected non-small-cell lung cancer, beyond that provided by conventional visual correlation of PET and CT imaging slices. No such study has been published in aggressive lymphoma, up to now.

Conclusion

FDG-PET has a number of potential advantages for refining and improving the management of lymphomas. Today, the most promising advantage appears to be in the early prediction of response to chemotherapy and evaluation of the residual masses. Other important areas, which require further assessment, are its value in refining prognostic indices at staging and its role in follow-up. The added value of the new combined PET-CT scanners requires evaluation in each of this setting. The ultimate aim is to improve management by identifying—in association with other established prognostic factors—those patients who can be cured with minimal treatment and those for whom conventional treatment will fail and who may benefit of innovative approaches.

References

9. Janicek M, Kaplan W, Neuberg D, Canellos GP, Shulman LN, Shipp MA. Early restaging gallium scans predict outcome in poor-prognosis patients with aggressive non-


Dose intensity or monoclonal antibody in first-line treatment

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The first demonstration of cure in patients with aggressive lymphoma was presented more than 25 years ago with the first reports of the CHOP and C-MOPP regimens.1 The CHOP regimen became the ‘gold standard’ for treating patients with diffuse large B-cell lymphoma (DLCL) and none of the so-called third-generation regimens showed better results in randomized trials,2–4 because of its ease of administration and its lack of severe toxicity in most patients, and because the results were widely reproducible.5 In all these trials, CHOP was given for eight cycles with standard doses of cyclophosphamide (750 mg/m²), doxorubicin (50 mg/m²), and vincristine (1.4 mg/m² to a maximum of 2 mg), even if the dose of corticosteroids was slightly variable (from 40 mg/m² to 100 mg flat), with cycles given every 3 weeks. However, in all studies, this CHOP regimen was only associated with a 60–70% complete response (CR) rate and 30–35% 10-year survival. So, in order to improve these results, one should increase the CR rate, particularly by decreasing the number of patients refractory to chemotherapy, and decrease the relapse rate.

All studies on first-line chemotherapy allow the description of a group of patients with a very poor outcome, a 5-year survival less than 25%. This high-risk group corresponds to patients with either low CR rate, high relapse rate, or both. Tentative solutions to improve the outcome of these patients have been diverse and without positive results. If HDT in first CR may decrease the risk of relapse in a subgroup of patients, it does not increase the CR rate.6,7 Adding more drugs or increasing doses to increase the CR rate have been considered and have usually failed.4,8 Whatever the reason, failure to respond or relapse, the survival of these patients when treated with CHOP chemotherapy is unsatisfactory. In all, 5- and 10-year survival are approximately 20%, presenting an opportunity to improve on these results. Even in those with good prognosis, survival is around 50–70%, also allowing numerous possibilities to improve outcome.

High-dose therapy with autologous transplant in first line

The use of high-dose therapy with autologous stem-cell transplant has been the only major progress made since the introduction of CHOP, but its use in first-line patients remains controversial. Several studies have compared cohorts of young patients with aggressive lymphoma and adverse prognostic factors treated either with standard chemotherapy or high-dose therapy with autologous transplant (HDT) and have failed to demonstrate improved efficacy.7 Early HDT did not increase the complete remission (CR) rate and failed to improve outcome, except in the sequential trial presented by Gianni.9,10 HDT in slow responders also failed to improve the outcome.11 However, in a group of young patients with poor prognosis according to the International Prognostic Index, HDT in CR resulted in prolonging survival.8 Finally, there are evidence that HDT in first partial response is associated with a longer time-to-progression and longer survival even if this has not been demonstrated in randomized studies.12 If the place of HDT in relapsing patients is currently accepted, its place in first-line patients is still debated more than 10 years after the first trials were reported. The conclusion of all these study is that HDT in first-line patients may only decrease the relapse rate and in only a small group of patients, and that it is not the recommended regimen for all patients.

Increasing dose-intensity of chemotherapy

Several attempts have been made to increase the CR rate and decrease the relapse rate by adding new drugs to the CHOP regimen, such as in the MACOP-B regimen, the m-BACOD regimen, and the ProMACE-CytaBOM regimen. The first phase II results of these regimens suggested benefit in comparison to historical controls, but randomized trials showed comparable results to those observed with CHOP.5,4,13,14 Only 10 randomized studies showed a statistically significant improvement for survival compared to CHOP (Table 1). Three of these 10 studies used a regimen with a short interval (2 weeks) between cycles15–17 and one increased the dose of CHOP.17 Five studies showed a statistically significant longer event-free survival or disease-free survival and a statistically significant longer overall survival, while the others showed a benefit only for event-free survival (three studies), overall survival (one study), or cause-specific survival (one study). All the other published randomized studies either showed a worse outcome or a similar outcome for patients treated with the experimental arm.

If the addition of more drugs to the CHOP regimen has been frequently attempted and failed to show any benefit, few studies have addressed the potential of
Table 1  Randomized studies showing a statistically significant benefit over CHOP (or CHOP-like) regimen in aggressive lymphomas.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Setting</th>
<th>CHOP</th>
<th>Experimental arm</th>
<th>Number of patients</th>
<th>End point</th>
<th>Event-free survival</th>
<th>Overall survival</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Increasing chemotherapy dose intensity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carde et al.\textsuperscript{28}</td>
<td>Unfavorable subtypes</td>
<td>CHVmP</td>
<td>CHVmP-VB</td>
<td>141</td>
<td>5 years</td>
<td>—</td>
<td>29 versus 53%</td>
</tr>
<tr>
<td>Linch et al.\textsuperscript{29}</td>
<td>Aggressive</td>
<td>CHOP</td>
<td>PACEBOM</td>
<td>459</td>
<td>8 years</td>
<td>—</td>
<td>CSS 49 versus 59%</td>
</tr>
<tr>
<td>Pfreundschuh et al.\textsuperscript{15}</td>
<td>Aggressive, &gt; 60 years</td>
<td>CHOP</td>
<td>CHOP-14</td>
<td>738</td>
<td>49 months</td>
<td>39 versus 47%</td>
<td>45 versus 59%</td>
</tr>
<tr>
<td>Pfreundschuh et al.\textsuperscript{16}</td>
<td>Aggressive, young, low risk</td>
<td>CHOP</td>
<td>CHOEP</td>
<td>762</td>
<td>49 months</td>
<td>63 versus 73%</td>
<td>81 versus 86% ((P = 0.13))</td>
</tr>
<tr>
<td>Tilly et al.\textsuperscript{17}</td>
<td>60–69 years, aaIPI &gt; 0</td>
<td>CHOP</td>
<td>ACVB + sequential consolidation</td>
<td>635</td>
<td>5 years</td>
<td>29 versus 39%</td>
<td>38 versus 46%</td>
</tr>
<tr>
<td>Wolf et al.\textsuperscript{30}</td>
<td>Aggressive</td>
<td>CHOP</td>
<td>MACOP-B</td>
<td>236</td>
<td>6.5 years</td>
<td>30 versus 42%</td>
<td>41 versus 54%</td>
</tr>
<tr>
<td><strong>High-dose therapy in first line</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gianni et al.\textsuperscript{31}</td>
<td>Young DLCL</td>
<td>MACOP-B</td>
<td>Sequential HDT</td>
<td>98</td>
<td>55 months</td>
<td>49 versus 76%</td>
<td>55 versus 81% ((P = 0.09))</td>
</tr>
<tr>
<td>Haioun et al.\textsuperscript{18}</td>
<td>Young, aaIPI = 2 or 3</td>
<td>ACVB + sequential consolidation</td>
<td>ACVB + CBV and autologous transplant</td>
<td>236</td>
<td>8 years (DFS)</td>
<td>39 versus 55%</td>
<td>49 versus 64%</td>
</tr>
<tr>
<td>Intragumtornchai et al.\textsuperscript{31}</td>
<td>&gt; 56 years, aaIPI = 2 or 3</td>
<td>CHOP</td>
<td>CHOP, ESHAP and HDT</td>
<td>58</td>
<td>4 years</td>
<td>15 versus 38%</td>
<td>30 versus 51% ((P = 0.25))</td>
</tr>
<tr>
<td><strong>Combining with monoclonal antibody</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coiffier et al.\textsuperscript{23}</td>
<td>Elderly, DLCL</td>
<td>CHOP</td>
<td>CHOP rituximab</td>
<td>399</td>
<td>2 years</td>
<td>38 versus 57%</td>
<td>57 versus 70%</td>
</tr>
<tr>
<td>Habermann et al.\textsuperscript{26}</td>
<td>Elderly, DLCL</td>
<td>CHOP</td>
<td>CHOP rituximab, w/wo rituximab maintenance</td>
<td>540</td>
<td>2 years</td>
<td>Interpretation problems due to maintenance rituximab</td>
<td></td>
</tr>
</tbody>
</table>

DLCL = diffuse large-cell lymphoma; DFS = disease-free survival; HDT = high-dose therapy with autotransplant; CSS = cause-specific survival.

Studies with longer event-free and overall survivals are shaded.
increasing doses of standard drugs. A recent study by the GELA compared eight cycles of CHOP to ACVB (doxorubicin, cyclophosphamide, vindesine, bleomycin, and prednisone) plus sequential consolidation in 635 elderly patients (60-69 years old) with aggressive lymphoma and at least one adverse prognostic parameter according to the International Prognostic Index. The results showed a similar CR rate (56 versus 58%) but a statistically longer event-free survival (39% in the ACVB group and 29% in the CHOP group) and longer overall survival (46% compared to 38%, respectively) at 5 years.17 These results were comparable to a previous study from the GELA, in which ACVB was compared to M-BACOD and resulted in better outcome in patients with adverse prognosis.18

Recently, Blayney et al.19 present a phase II study of CHOP-DI (for dose intense), a regimen with the same drugs of CHOP, but with increased doses of doxorubicin and cyclophosphamide, and with courses repeated every 2 weeks.19 These modifications resulted in a dose of 320% for cyclophosphamide and 190% for doxorubicin (Table 2). The 5-year failure-free and overall survivals were 41 and 60%, respectively, suggesting an improvement of 10–15% over results usually obtained with standard CHOP. However, this is a phase II study and, even if the number of patients was large, bias in patient selection may be present. Nevertheless, two randomized studies had either increased doses or decreased intervals between cycles and confirmed that these alterations were feasible and associated with a better outcome.15,17 The CHOP-14 study from the German Non-Hodgkin’s Lymphoma Study Group used moderately increased doses (Table 2) but showed a better event-free survival and overall survival than for patients treated with standard CHOP, even if the increase in survival was moderate (10% at 4 years).15

The GELA studies used another approach, with four alterations of the CHOP regimen: a sequential consolidation after induction with the modified CHOP regimen, a shortening of the interval between courses to 2 weeks, an increase of doses, and a repeat of nonhematotoxic drugs on day 5 of each course.20 The ACVB regimen was used as standard arm in several randomized studies since 1987 and had shown its superiority over M-BACOD and CHOP for patients with poor risk lymphoma, and equivalence with HDT in patients with good risk lymphoma.17,18,21,22 These studies show that the CHOP regimen may be improved by increasing doses and decreasing intervals between cycles. Even if these regimens are associated with a higher hematological toxicity, particularly neutropenia, the infectious risk is not significantly increased, and may be reduced by the use of G-CSF. However, if moving from CHOP to one of these regimens as the future gold standard regimen in patients with aggressive lymphoma was feasible a few years ago, it may be less clinically relevant currently because of the breakthrough of the combination of rituximab and chemotherapy.23

Combining chemotherapy and monoclonal antibodies

R-CHOP combines the CHOP regimen every 3 weeks for eight cycles with rituximab given on day 1 of each cycle. R-CHOP was associated with a statistically significant higher CR rate, lower progression rate during chemotherapy, lower relapse rate, longer event-free survival, longer disease-free survival, and longer overall survival (Figure 1).24,25 This regimen has been shown to

**Table 2** Comparison of different regimens with increased doses or reduced interval between courses

<table>
<thead>
<tr>
<th></th>
<th>CHOP</th>
<th>CHOP-DI</th>
<th>ACVB</th>
<th>CHOP-14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophosphamide</td>
<td>250</td>
<td>800</td>
<td>600</td>
<td>300</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>16.7</td>
<td>32.5</td>
<td>37.5</td>
<td>25</td>
</tr>
<tr>
<td>Vincristine</td>
<td>0.47</td>
<td>0.7</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Vindesine</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bleomycin</td>
<td></td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>95</td>
<td>143</td>
<td>150</td>
<td>150</td>
</tr>
</tbody>
</table>

Doses are given in mg/m²/week.
decrease the refractoriness to chemotherapy associated with bel-2 protein expression.\textsuperscript{24,25}

In the last American Society of Hematology meeting, two studies on R-CHOP were presented as abstract. The first one from the intergroup/ECOG included elderly patients and randomized them between CHOP +/- rituximab, then for responders between rituximab and rituximab, then for responders between rituximab and rituximab. The major differences with the GELA study were the maintenance therapy, a lowest dose of rituximab (once every two cycles of CHOP), and a reduced number of CHOP +/− R (six cycles in most of the patients). Thus, the two studies are not really comparable. Median follow-up was also shorter for the American study. Currently, the main conclusion of this study is that patients receiving rituximab, either initially or as maintenance, had a longer progression-free survival.

A very interesting study was presented by Sehn \textit{et al.}\textsuperscript{28} on the population of patients with DLCL treated in British Columbia. Cancer patients, in this case, DLCL patients, are treated according to rules determined by the Cancer Agency. It decided in March 2001 to modify the treatment of DLCL patients from CHOP to R-CHOP. Patients treated for the 18 months before and after this decision were included in this analysis. They had the same characteristics, but 9% of the prerituximab group received R-CHOP and 15% of the post-rituximab group did not receive rituximab. Analyses were conducted on the intent-to-treat rule. This study confirmed a statistically significant benefit for R-CHOP in terms of progression-free and overall survivals for the whole group or young or elderly patients. It was the first study showing a benefit in young patients. Even if it was not a randomized study, it is a bias-free study because it was based on the population of patients with DLCL in British Columbia.

R-CHOP has become the reference regimen for patients with diffuse large B-cell lymphoma and for other B-cell lymphoma subtypes. However, several questions remain to be answered regarding the combination of chemotherapy and monoclonal antibodies: is CHOP the best regimen in combination with rituximab? Do other monoclonal antibodies have the same effect as rituximab? What is the necessary number of rituximab infusions? May we increase the benefit by using higher doses of chemotherapy? May we increase the benefit by giving maintenance treatment?

In conclusion, current results suggest that CHOP may soon be considered a historical standard because we have several means of improving the results by increasing doses, decreasing intervals between cycles, and combining new drugs with different mechanisms of action. But the best combination is not yet known and efforts have to be made to design appropriate randomized studies that will allow us to establish the next gold standard. Entering patients into prospective trials remains best clinical practice.

\textbf{References}


26 Habermann TM, Weller EA, Morrison VA, Cassileth PA, Cohn JB, Dakhil SR et al. Phase III trial of rituximab-CHOP (R-CHOP) vs. CHOP with a second randomization to maintenance rituximab (MR) or observation in patients 60 years of age and older with diffuse large B-Cell lymphoma (DLBCL). Blood 2003; 102: (abstract 8).


PLATELETS

Platelet receptor signalling

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Introduction

Platelets are archetypical signalling cells. Lacking a nucleus and with only a limited capacity for protein synthesis they have to carry out their various functions by rapid and diverse reactions. In order to do this, they contain a very high ratio of signalling molecules and membrane receptors to cell mass compared to other cells. Most of the mechanisms used and types of receptor involved are common to other cell types. What distinguishes platelets is the rapidity and flexibility of their responses. How these are finely regulated by complex feedback controls is also critically important for avoiding bleeding to death on the one hand or developing a life-threatening thrombus on the other. Attempts to shift and rebalance these pathways in disease constitute the main approaches to prophylaxis and treatment.

Platelet receptors can be classed according to type and signalling mechanism. As with other cells, the G-protein coupled, seven-transmembrane class form a major group and will be dealt with first. A second, smaller but still important, group are those receptors that link to tyrosine kinase activation. A third important group are integrins, where signalling is still under active investigation. Other groups include GPI-anchored receptors, such as those involved in complement regulation, as well as the tetraspanins, which may have a role in integrin signalling. Miscellaneous receptors with various signalling mechanisms on platelets include those linked to cation channels such as P2X1.

Receptor categories and signalling mechanisms

G-protein coupled, seven-transmembrane class

Major platelet receptors in this class can either have agonist or antagonist effects on platelet activity and include receptors for thrombin, PAR1 and PAR4; for ADP, P2Y1 and P2Y12; for thromboxane A2; TXA2/PGH2, for prostaglandins; PGI2, PGD2, PGE2, lipid receptors including the platelet-activating factor (PAF-1) receptor and the lysophosphatidic acid receptor, chemokines receptors including CXCR4, CCR1, CCR3 and CCR4, as well as receptors for serotonin, A2a-adenosine receptor, β2-adrenergic receptor, vasopressin and dopamine receptors (Figure 1).

The signalling pathways from several of these receptors have been analysed fairly thoroughly and, although some problems remain, overall activation mechanisms have been established. These depend on the particular G-proteins that are coupled to a particular receptor. Major classes are:

- Gia, linked to PAR1, PAR4, P2Y12, z2A-adrenergic receptor, PAF-1 receptor, lysophosphatidic acid receptor and sphingosine-1 phosphate, signalling to decrease adenylylcyclase activity.
- Gqz, linked to PAR1, PAR4, P2Y1, thromboxane A2 receptor, 5HT2A receptor, PAF-1 receptor and V-1 vasopressin receptor, signalling to activate phospholipase Cβ2 activity.
- Gsa, linked to PGI2, PGD2, and PGE2 receptors, A2a-adenosine receptor and β2-adrenergic receptor, signalling to increase adenylylcyclase activity.
- Gil2, linked to PAR1, PAR4, P2Y12 and thromboxane A2 receptors, signalling to increase Rho kinase, pp60csrc, and pp72syk activity.
- Gil2z, linked to PAR1, PAR4, thromboxane A2 and lysophosphatidic acid receptors, signalling to increase Rho kinase, pp60csrc, and pp72syk activity.
- Gil6z, thought to be linked to the thromboxane A2 receptor, signalling to activate phospholipase Cβ2 activity.
- Gzzx, linked to the z2A-adrenergic receptor, signalling to increase adenylylcyclase activity.
- Ghxz, linked to the thromboxane A2 receptor, signalling to activate phospholipase Cβ2 activity.
- Gβγ from Gi and/or Gq signalling to activate phospholipase Cβ2 and Cβ3 and PI3 kinase γ activities.

As can be seen from this list, the main function of these pathways leads to phospholipase Cβ activation and hence to liberation of diacylglycerol (DAG) and IP3 from membrane lipids with release of Ca2+ from stores and activation of protein kinase C as major consequences. On the other hand, the effects on adenylylcyclase whether positive or negative regulate platelet function via cAMP-dependent kinase levels.
Tyrosine kinase receptors

These are receptors that act mainly by activating a cascade of tyrosine kinases/phosphatases and include in particular GPVI and FcγRIIA but also probably GPIb complex, as well as FceRI, and CD226, JAM-1, ICAM-2, and CD47. PECAM-1, which contains ITIM rather than ITAM domains, downregulates platelet activity by activating tyrosine phosphatases rather than kinases. Tyrosine kinase receptors where the kinase is an integral part of the cytoplasmic domain are also a well-known group, generally activated by dimerisation via ligand binding. A well-known representative present on platelets is the thrombopoietin receptor, CD110, but other members present on platelets include the Tie-1 receptor, the insulin receptor, and the platelet derived growth factor receptor (Figure 2).

Although many of the pathways from these receptors are still poorly understood and they may synergise with signalling from other receptors, there are a few that have been well investigated and where the main molecules involved and their inter-relationships are broadly known. The GPVI signalling pathway has many similarities with intensively studied immune cell pathways. GPVI is a member of the Ig superfamily and is present in the platelet membrane as a dimer associated with Fcγ via a salt bridge. Clustering of GPVI/Fcγ leads to a change in kinase/phosphatase balance so that Fcγ is phosphorylated on tyrosines within two immune tyrosine-containing activation motifs (ITAM) domains. This allows the binding via SH2 domains and activation via (partly) auto tyrosine phosphorylation, of the syk tyrosine kinase, which is critical for platelet activation. Syk then goes on to phosphorylate a number of other substrates in a cascade process. One of the important substrates of syk is linker for activation of T-cells (LAT), which is an ‘adaptor’ molecule (Figure 3). This means a molecule that participates in signalling cascades by providing an appropriately situated assembly point or scaffolding structure but does not itself have kinase or phosphatase activity. LAT is a membrane-associated molecule and its N-terminal extends across to the outside of the membrane. When it is phosphorylated, it provides a scaffold on which many signalling proteins can assemble and interact. These include SLP76, grb2 and PLCγ2. PLCγ2 is phosphorylated and activated via the Tec family kinases Btk or Tec. PLCγ2 can then cleave membrane lipids with liberation of diacylglycerol and IP3 releasing Ca2+ from stores and activating protein kinase C as major consequences.

Signalling from tyrosine kinase receptors of the cytokine and growth factor class use different signalling pathways involving Janus kinase (JAK) members. Two of these have been detected in platelets, JAK2 and TYK2, and are phosphorylated in platelets treated with thrombopoietin. There is also evidence for the presence of STAT molecules in platelets, which in nucleated cells signal to the nucleus downstream of JAKs. The Tie-1 receptor has been shown to be present on platelets and is the receptor for angiopoietin-1 on other cells. Insulin and PDGF receptors are also members of the cytokine class of receptors present on platelets and are thought to signal by similar mechanisms.

Integrins

Platelet integrins include the major fibrinogen, von Willebrand factor and fibronectin receptor αIIbβ3, the
collagen receptor $\alpha_2\beta_1$, the vitronectin receptor $\alpha_5\beta_1$, the fibronectin receptor $\alpha_3\beta_1$, and the lamnin receptor $\alpha_6\beta_1$. The $\alpha_5\beta_3$ receptor is also present on the surface of activated platelets. Signalling from integrins is less well understood than that from the other classes mentioned above but starts to be investigated more profoundly. $\alpha_{IIb}\beta_3$ is a major platelet receptor and is involved in platelet–platelet aggregation via fibrinogen as well as interplatelet adhesion via VWF under high shear conditions. Integrin signalling involves both inside-out as well as outside-in components since resting integrins are generally thought to be inactive. This is now well substantiated by recent structure determinations and modelling studies. Inside-out signalling and activation of $\alpha_{IIb}\beta_3$ involves phosphorylation of the cytoplasmic domains at both Ser/Thr and Tyr sites causing conformational changes that cause the outer domains also to change conformation as well to the ligand binding state. These changes are stabilised by disulphide isomerase activity switching disulphide bonds. Internally this affects associations with the cytoskeleton and the organisation of focal adhesion sites and induces tyrosine phosphorylation of a range of signalling proteins including focal adhesion kinase and cortactin. Tyrosine phosphatases are also activated and down-regulate tyrosine kinase signals from other receptors shortening and sharpening the signal wave. $\alpha_{IIb}\beta_3$ occupancy is also critical for later events such as clot retraction where fibrin–$\alpha_{IIb}\beta_3$–cytoskeletal interactions pull together edges of a wound to prevent blood loss and aid healing. Less is known about signalling via other integrins but the same basic mechanism is thought to apply. $\alpha_5\beta_1$ is a major collagen receptor involved in platelet adhesion to this ligand. Recent results also show that it needs to be activated to bind collagen and uses disulphide isomerase activity to switch resting and activated states. What little is known about signalling pathways is based upon studies with GPVI and Fc$\gamma$ knockout mice or humans lacking GPVI and indicate that $\alpha_2\beta_1$ is able to signal at least to src tyrosine kinase and probably further to syk. In the case of both $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$, there is evidence for activation of syk via alternative mechanisms involving the ITAM domains of moesin/ezrin/radixin family proteins associated with the integrins. Src was shown to be activated by direct interaction with the $\beta_1$ cytoplasmic domain.

**Other receptors**

$P2X_1$ This is one of the major purinergic feedback receptors activated by ATP and antagonised by ADP. However, since ATP is also released when platelets are activated $P2X_1$ seems to play an important role in these responses. Binding of ATP causes conformational shifts allowing $P2X_1$ to function as a $Ca^{2+}$ channel contributing to induction of the platelet shape change and formation of small platelet aggregates. $P2X_1$ stimulation also showed synergistic effects with other weak platelet agonists and its inhibition reduced platelet responses to stronger platelet agonists.

**Second messengers**

**Calcium**

Calcium plays a critical role in platelets, as in other cells and because of that is tightly regulated. Levels of calcium in the platelet cytoplasm control many enzyme activities. Calcium concentration is regulated in several different ways: it is increased on stimulus via release from storage organelles (endoplasmonic reticulum) in response to IP$_3$ formed by phospholipase C from phosphoinositides or it is admitted from the platelet exterior by calcium channels that may be agonist or calcium dependent. Calcium is removed from the cytoplasm by pumping back into storage organelles or by pumping to the platelet exterior. Raised calcium levels are detected by the hTRPC1 (human canonical transient receptor potential 1) protein, leading to pumping out of platelets by plasma exchange $Ca^{2+}$-ATPases (PMCA) and back into storage organelles by sarco/endoplasmonic reticulum $Ca^{2+}$-ATPase (SERCA) isoforms. The $Ca^{2+}$ concentration is regulated in resting platelets at 30–50 nM level via activation of the PMCA and SERCA by cAMP-dependent kinases. Many kinases are dependent on $Ca^{2+}$ for activation such as the classic ($\alpha$, $\beta$ and $\gamma$) members of the protein kinase C family and proteinases such as calpains. Plasma membrane removal of calcium could also be carried out by $Na^+/Ca^{2+}$ exchange, but there is little evidence that this actually occurs.

**Protein kinase C family**

Protein kinase C isoenzymes (PKC) are a family of Ser/Thr kinase isoenzymes that are activated in platelets downstream of phospholipase B or C production of DAG and IP$_3$. Most PKCs require DAG or phorbol esters for activation. The classical $\alpha$, $\beta$ and $\gamma$ PKCs all require $Ca^{2+}$ as well. The so-called novel forms $\delta$, $e$, $\eta$, and $\theta$ do not need $Ca^{2+}$. The isoforms $\alpha$, $\beta$, $\delta$, $\epsilon$, $\eta$, and $\theta$ are all present in platelets, whereas others are either absent or only present in very low amounts. There is a lot of evidence for a wide variety of roles for PKCs in platelet activation from secretion through to aggregation with effects on actin assembly and activation of integrin being major routes. Pleckstrin, a major substrate for PKCs is the prototype for the ‘pleckstrin homology domains’ and how membrane association is regulated by phosphorylation. Stimulation of PKC also leads to activation of ERK (MAPK) signalling pathways, one of the response pathways from several platelet receptors. PKC have also been reported to have a role in megakaryocyte differentiation.

**Phospholipase A$_2$**

Thromboxanes are well known to play important roles in platelet function and are produced from arachidonic acid which itself is cleaved from membrane phospho-
lipids by phospholipase A₂ activity. Thromboxanes are formed from arachidonic acid by cyclooxygenase-1 (COX-1), the target of irreversible inactivation by aspirin. Because platelets lack a nucleus they cannot replenish COX-1, which is why aspirin is selectively effective against them. Platelets contain two types of phospholipase A₂, group IIA sPLA₂ and cPLA₂. Group IIA sPLA₂ is stored in platelet granules and released in active form when platelets are activated. Its physiological function has still not been established but as with some other platelet granule components it may play a role in defence against microorganisms. cPLA₂ is thought to be the enzyme involved in the release of arachidonic acid following platelet activation by strong agonists such as thrombin and collagen. cPLA₂ activity is regulated by calcium levels and by phosphorylation. It contains two phosphorylation sites at Ser505 and Ser727 both of which are fully substituted in response to thrombin by a p38 member of the SAPK (Stress activated protein kinase) family.

**Phosphoinositide-specific phospholipase C (PLC)**

PLC has an essential role in platelet response via receptors. These calcium-dependent enzymes hydrolyse PIP₂ to DAG and IP₃ as mentioned above. DAG is rapidly inactivated by phosphorylation to phosphatidic acid by DAG kinase. Soluble IP₃ is rapidly dephosphorylated to the inactive inositol 1,4-biphosphate by IP₃ 5-phosphatase and then recycled as inositol after removal of the other phosphate groups or may be further phosphorylated to IP₄ which is thought to have other second messenger roles. While PLCβ enzymes are mainly stimulated by the α-subunits of the Gq family of G-proteins, activated βγ-dimers are also involved. PLCγ is activated by tyrosine phosphorylation and contains SH2 and SH3 domains. It has a major role in signalling during platelet activation by collagen and VWF and immune complex binding to the FcγRIIA receptor. The activation of PLCδ enzymes and their role in platelets are poorly understood. All types of PLC contain an N-terminal pleckstrin homology (PH) domain involved in membrane targeting.

**Phospholipase D (PLD)**

PLD hydrolyses lipids such as phosphatidylcholine to produce PA and choline. The PA may act as second messenger or be converted to lysophosphatidic acid or be converted to DAG by a PA-phosphohydrolase, in the latter case providing a sustained second wave of DAG following the initial burst from PLC activity. PLD has been shown to be activated in platelets in response to several agonists, but it still seems relative unimportant for signalling compared to the other phospholipases.

**cAMP- and cGMP-dependent pathways**

Platelet activity is regulated not only by agonist-driven pathways but also by antagonists, and in the circulation the latter have an important role in maintaining platelets in a resting state despite the trauma they are exposed to in passing through narrow vessels at high shear. Thus, endothelial cells express CD39/ecto-nucleotidase-1, which remove ADP from the circulation and prevent platelet activation. Nitric oxide (NO) is also an important platelet downregulator and acts by raising sguanylyl cyclase activity, which produces cGMP from GTP. The cGMP then regulates cGMP-dependent protein kinase (PKG) activity, which has several identified substrates including VASP, IP₃ receptor and PLCβ. The PKG system is intimately associated with the cAMP-dependent protein kinase (PKA) system and they affect one another in ways that are not completely elucidated. PKA activity is regulated via receptor-mediated stimulation or inhibition of adenylate cyclase activity affecting cAMP synthesis from ATP. The receptors linked to stimulation are those for prostaglandins and adenosine while those decreasing adenylate cyclase activity are the ADP receptor P2Y₁₂, the z₂-adrenergic receptor and the EP₃ prostaglandin receptor. PKA has a wider range of identified substrates than PKG and they include Filamin-1 and 2, GPlibβ, MLCK, VASP, RapIb, IP₃ receptor, various Ca²⁺ pumps and PLCβ. Phosphorylation of filamin and GPlibβ affects membrane-cytoskeletal association and may be one of the ways to reduce platelet responses. The effect of phosphorylation on the other substrates may be a direct one on signalling. Stimulation of Ca²⁺ pumps is certainly a major way of reducing platelet sensitivity. Cyclic nucleotide phosphodiesterases (PDE) also affect these pathways by hydrolysing cAMP and cGMP. Three major isoenzymes, PDE2, PDE3 and PDE5, are found in platelets. cAMP is hydrolysed by PDE2 and PDE3 and cGMP is hydrolysed by PDE2 and PDE5. PDE are the targets for a wide range of inhibitors. Since sildenafil, the active ingredient of Viagra, inhibits PDE5 in platelets there was some concern that platelet function might be enhanced as a side effect and that it could be the reason behind reports of increased myocardial infarction in men taking this drug. The overall impression, despite some controversy, is that this is not the case. Dipyridamole, another inhibitor of PDE5, has been used alone or in combination with other drugs like aspirin in prophylaxis against cardiovascular diseases. Mice lacking PKG had much higher platelet–platelet and platelet–endothelium interactions after ischaemia showing that PKA cannot compensate for its absence.

**Crosstalk between receptors and their pathways**

In reality, platelet receptors never act alone but different ones are activated quasi-simultaneously leading to interacting pathways and the final stages of platelet activation. Thus, during adhesion under shear, the first receptor is the GP Ib complex, from which the signalling pathways are adequate to bring integrins such as z₂β₁ and z₃β₃ into play. Platelet arrest via GP Ib and contact with collagen almost immediately brings GP VI into
action to amplify activation of integrins. Both these steps are accompanied by release of granule contents with feedback to their receptors. ADP, ATP, serotonin, calcium from dense granules and fibrinogen, VWF, thrombospondin and fibronectin from α-granules are all involved in amplifying the haemostatic response and after the first layer of platelets adhere tightly on the exposed subendothelium already start to add subsequent layers of platelets on the way to forming a thrombus which may be necessary to prevent blood loss from a severed vessel. Thromboxane formation from arachidonic acid and thrombin generation from prothrombin also start from the very early stages. Platelet adhesion via GPIb is enough to induce procoagulant activity but is also amplified by the following stages. Thrombin also feeds back to its platelet receptors, PAR1, PAR4 and GPIb to amplify platelet activation and reacts with fibrinogen to form fibrin, which has an essential role in stabilising the thrombus and in clot retraction.

What limits thrombus growth? What is the difference between haemostasis and thrombosis?

As mentioned in the Introduction, there is a delicate balance between too little and too much haemostasis, bleeding and thrombosis. Because this is so important for the maintenance of life, there are many regulatory mechanisms controlling it and many built in redundancies. In evolution, bleeding to death was most likely more of a driving force than thrombosis, so that there are many alternative ways of activating platelets and it is seldom that the lack of one is sufficient to lead to major problems. Thrombus growth is regulated by a balance between activating signals and inhibitory ones and slows and eventually stops when the activating ones are not sufficient to ensure propagation or when the inhibitory ones become predominant. Thus, for example, on the outer surface of a thrombus, αIIbβ3 is no longer activated and neither fibrinogen nor VWF adhere avoiding further recruitment of platelets via GPIb on passing platelets interacting with the attached VWF or by subsequent binding of fibrinogen to αIIbβ3. This weakening of the signalling in the outer platelets is most likely due to the effects of activating signals being cancelled out by inhibitory ones such as those from NO and PGI2 released from the damaged or activated endothelial cells surrounding the injury site. In thrombosis either the platelets themselves are in a more sensitive state and can therefore be activated to a higher degree, or the endothelium is not compensating adequately. Of course, the type of injury involved in thrombosis is different from that of normal haemostasis and in life-threatening situations exposes large areas of highly reactive collagen under very high shear conditions, leading to over response from the platelets.
Measuring platelet function?

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Introduction

Human platelets are small and discoid in shape, with dimensions of approximately 2.0–4.0 × 0.5 μm, and a mean volume of 7–11 fl.¹ They are the second most numerous corpuscle in the blood normally circulating at between 150 and 450 × 10⁹/l. Platelets are anucleated cells derived from megakaryocytes and typically circulate for 10 days.¹ Their shape and small size enables the platelets to be pushed to the edge of vessels, placing them in the optimum location required to constantly survey the integrity of the vasculature.

Upon vessel wall damage, platelets undergo a highly regulated set of functional responses including adhesion, spreading, release reactions, aggregation, exposure of a procoagulant surface, microparticle formation, and clot retraction.² All of these platelet responses function to rapidly form a haemostatic plug that occludes the site of damage to prevent blood loss. When there is a defect in any of these functions and/or platelet number, haemostasis is usually impaired and there may be an associated increased risk of bleeding. In contrast, marked increase(s) in platelet number or reactivity can lead to inappropriate thrombus formation. Arterial thrombi can also develop within atherosclerotic lesions resulting in stroke and myocardial infarction, two of the major causes of morbidity and mortality in the Western world.³ Antiplatelet therapy can therefore be beneficial in the treatment and prophylaxis of arterial thrombotic conditions, but must be carefully administered without increasing the risk of bleeding to an unacceptable level.¹

The main use(s) of platelet function tests have been traditionally to identify the potential cause(s) of abnormal bleeding, to monitor prohaemostatic therapy in patients with a high risk of bleeding and to ensure normal platelet function either prior to or during surgery.⁴ However, they are increasingly being utilised to monitor the efficacy of antiplatelet therapy and to potentially identify platelet hyperfunction to predict thrombosis.³ For a full list of potential clinical uses of platelet function tests, see Table 1.

Quality control, blood sampling and anticoagulation

Platelet function testing presents many problems in ensuring that accurate and meaningful results are obtained. Firstly, unlike with coagulation tests, there are no widely available internal or external quality control materials available. Most assays are performed on fresh blood and so many laboratories either establish normal ranges using control volunteer blood and/or...
assay known normal samples in parallel to ensure that each test/reagent is viable. Many platelet function tests such as aggregometry remain poorly standardised. Normal platelet function is also largely calcium dependent, so anticoagulation of the blood sample through calcium chelation immediately presents a problem. Most laboratories utilise tests that require citrated blood samples within an narrow time window (<2 h). Although this is convenient within a coagulation laboratory, the quality, handling, temperature and age of the blood sample can cause significant artefacts in platelet analysis. Platelets are inherently prone to artefactual activation but also to desensitisation. It is important that these are minimised during phlebotomy, anticoagulation, sample transit and handling within the laboratory. There are a number of published guidelines that can help to minimise platelet activation and platelet aggregation.\textsuperscript{10,11} These include using a light tourniquet, a needle of at least 21 gauge, a nontraumatic venepuncture with smooth blood flow, discarding the first few millilitres of blood drawn, using polypropylene or siliconised glass tubes/syringes, immediate gentle mixing with anticoagulant, minimising delays from sampling to analysis, keeping all tubes at room temperature, checking that the blood tube is not over- or under-filled and avoiding unnecessary manipulation of the sample.\textsuperscript{10,11} Flow cytometry is a particularly sensitive technique for measuring platelet activation, but these simple measures should also be implemented when performing any platelet function testing.

### Approach to diagnosing platelet dysfunction

When investigating a suspected bleeding disorder, it is essential to obtain a detailed clinical and family history and perform a physical examination before choosing the laboratory tests to be undertaken.\textsuperscript{12} In particular, a recent drug history is critically important. Some drugs may cause a transient haemostatic defect, so repeat or deferred testing is often necessary. There are various characteristic clinical features that can be used to distinguish a platelet or primary haemostatic disorder from a coagulation or secondary haemostatic defect.\textsuperscript{12}

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<th>Table 1</th>
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<td>New tests are more reliable and sensitive than the BT?</td>
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<td>Responsiveness to panel of agonists</td>
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<td>Platelet release markers, for example, bTG PF4</td>
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Primary haemostatic disorders are characterised by immediate bleeding after injury, whereas delayed bleeding is more characteristic of a coagulation type defect. Petechiae, epistaxes and menorrhagia are more likely in patients with a primary haemostatic disorder, whereas haemarthroses and intramuscular haematomas are more common in coagulation disorders.12

Once the clinician suspects a platelet defect, it is important to exclude thrombocytopenia as a primary cause of bleeding by performing a full blood count often in conjunction with a blood-film examination, particularly if abnormal platelet flags are given on the haematology counter. The blood film can give valuable information about platelets including their size, granule content and number.

The bleeding time (BT), developed by Duke in 1910,13 was the first in vivo test of platelet function and was still regarded as the most useful test of platelet function until the early 1990s.9 The bleeding time is the time taken for bleeding to stop after an incision is made into the skin, usually into the anterior surface of forearm. The test has been refined and standardised particularly with the use of a sphygmomanometer cuff and a spring-loaded template device to make standard-sized cuts within the skin. Normal times are usually between 2 and 10 min whereas, severe platelet defects can result in a BT >30 min. However, despite its apparent simplicity, the test is poorly reproducible, invasive, insensitive and time consuming.14 In particular, the BT does not seem to correlate with the bleeding tendency within individual patients and it is widely considered that an accurate bleeding history is a more valuable screening test. The clear advantage of the BT is that it does study natural haemostasis, does not require expensive equipment or a laboratory, and is not prone to variables associated with blood sampling and anticoagulation.

Platelet aggregometry was developed in the early 1960s and soon became regarded as the ‘gold standard’ of platelet function testing.15 This is still the most widely used test for identifying and diagnosing platelet function defects and can be performed within commercially available multichannel aggregometers. Citrated blood is normally centrifuged to obtain platelet-rich plasma (PRP) which is stirred within a cuvette incubated at 37°C between light source and a detector. Upon addition of various concentrations of a panel of agonists (eg collagen, ADP, thrombin ristocetin, adrenaline, etc), the platelets aggregate and light transmission increases. Classical platelet responses to each agonist can then be measured including lag phase, shape change and primary and secondary aggregation. Parameters measured include the rate (slope) of aggregation and the maximal amplitude (%) or percentage of aggregation after a fixed period of time. There is no doubt that aggregation will remain an important clinical test within the specialised laboratory as many platelet disorders are easily diagnosed. But the clinical significance of mildly abnormal aggregation to weak agonists remains

<table>
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<th>Table 3 List of new platelet function tests</th>
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<td>Cone and Plate Analyser (CPA) or IMPACT device</td>
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<td>Flow Cytometry</td>
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<td>ICHOR-Plateletworks</td>
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<tr>
<td>Hemostasis analysis system</td>
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<tr>
<td>Hemostasus device</td>
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<tr>
<td>Platelet Function Analyser (PFA-100)</td>
</tr>
<tr>
<td>Ultegra (RPFA) – rapid platelet function analyser</td>
</tr>
<tr>
<td>Thrombotic Status Analyser (TSA) Gorog thrombosis test</td>
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<tr>
<td>Platelet-Stat</td>
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undefined. Furthermore, aggregometers also test platelets under relatively low shear conditions and in free solution within PRP, conditions that do not accurately simulate primary haemostasis. Also, it is logistically impossible to perform platelet aggregation on all patients with a suspected platelet defect and tests need to be performed within 2h of blood sampling.

Because of the many disadvantages of the BT and aggregometry, several alternative automated technologies have been developed, which attempt to simulate haemostasis in vitro. It is impossible to mention all of these in this brief review, so I will focus only upon four types of analyser that are currently available.

The Platelet Function Analyser – PFA-100

The PFA-100 is a relatively simple bench-top instrument that simulates high-shear platelet function within disposable test cartridges. Citrated blood is aspirated under constant negative pressure from the sample reservoir through a capillary and a microscopic aperture (147 μm) cut into a membrane. The membrane is coated with either collagen/epinephrine (CEPI) or collagen/ADP (CADP). The presence of these platelet activators and the high shear rates (5000–6000 s⁻¹) under the standardised conditions result in platelet adhesion, activation and aggregation resulting in formation of a platelet plug within the aperture. The test is simple to perform, rapid (with maximal closure times (CT) of 300s) and can test relatively small volumes (0.8 ml/ cartridge) of citrated blood up to 4h from sampling. However, as with any other laboratory tests of platelet function, there are recommended PFA-100 good practice guidelines that are required to maintain optimal performance. These include daily instrument QC checks, ensuring the quality of blood sampling, anticoagulation and checking for cartridge batch to batch variation. Various reports have shown that the test is reliable with near identical normal ranges reported from many different laboratories. However, it is recommended that each laboratory should establish their own reference range using normal blood samples taken into identical citrate anticoagulant that is utilised within the use’s institution, that is, either 3.8% (0.129 M) or 3.2% (0.105 M) buffered trisodium citrate. Typical normal ranges obtained with 3.2% trisodium citrate are 55–112s for CADP and 79–164s for CEPI (Oxford Haemophilia Centre, 2002 normal range). Although widespread experience is increasing (≥170 Medline articles, February 2004), how exactly the test should be incorporated into normal laboratory practice remains to be fully defined. The PFA-100 is sensitive to many acquired variables (eg dietary and drug effects) that influence platelet function borderline CTs either side of the upper normal ranges can also be difficult to interpret, given that the reported CVs of a normal sample have been reported as 10%. It is important that repeat or deferred tests are performed if drug ingestion is strongly suspected.

The PFA-100 appears superior to the bleeding time and is therefore recommended as a replacement screening test. Many additional studies are in progress to assess whether the PFA-100 can reliably predict either thrombotic or bleeding complications in different patient groups.

The Cone and Plate(let) Analyser (CPA)

Another test that has recently been developed is based upon the adhesion of platelets to the extracellular matrix under physiological conditions. The original Cone and Plate (CPA) device tested whole blood platelet adhesion and aggregation on a plate coated with extracellular matrix (ECM). The CPA has now been developed into a fully automated commercial instrument called the IMPACT (Diamed, Switzerland) and now utilises polystyrene plates instead of the ECM. This modification facilitated the commercialisation of the test. The test is now fully automated, simple to operate, uses a very small quantity of whole blood (0.12 ml) and displays results in 6 min. The instrument contains a microscope and performs staining and image analysis of the platelets adhering and aggregating upon the plate under an applied shear rate of 1800 s⁻¹. The software enables storage of the images of each analysis and records a number of parameters including surface coverage, average size and a distribution histogram of the adhered platelets. Comparative analysis of the
polystyrene and ECM plates shows a good correlation both in normals and VWD. No platelet adhesion occurs on plastic plates in samples from patients with either Glanzmann’s thrombasthenia or afibrinogenemia. The adhesion of platelets to the plate is absolutely dependent upon plasma VWF, fibrinogen binding to the plastic surface, GpIb and Gp IIb/IIIa and platelet activation events. Recent data suggest that the instrument is a reliable tool for the diagnosis of platelet defects. The instrument has only just become commercially available so widespread experience is limited.

Flow cytometry

One of the biggest advances in platelet function analysis has been the application of flow cytometry. This technique requires access to an expensive instrument and specialised training to perform. A number of largely research applications continue to evolve into clinically useful tests particularly as many of the reagents, antibodies and dyes are now commercially available. A list of currently available diagnostic flow cytometry assays are shown in Table 4. Most laboratories prefer to analyse platelets within whole blood. Only small quantities of blood are required and providing the venepuncture and analysis technique(s) are standardised, platelets can be analysed in their resting state. For recommendations how to perform flow cytometric analysis, refer to recent review articles.10,11

Table 4 List of clinically useful platelet function tests by flow cytometry

<table>
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<tr>
<th>Flow cytometry platelet function test</th>
<th>Examples</th>
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<td>Glanzmann’s thrombasthenia, Bernard-Soulier syndrome, storage pool disease, Scott syndrome, HIT</td>
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<tr>
<td>Quantification of receptor density</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Signal transduction</td>
<td>Calcium measurement, intracellular phosphorylation</td>
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</table>

The Ultegra rapid platelet function assay – RPFA

The Ultegra-RPFA (Accumetrics, Inc., San Diego, CA, USA) is a turbidimetric-based optical detection system that measures platelet-induced aggregation as an increase in light transmittance. This modified platelet aggregometry device was originally developed as a near-patient testing instrument in order to provide a simple and rapid functional means of monitoring anti-Gp IIb/IIIa therapy with various antiplatelet drugs (eg abciximab). The disposable cartridges contain fibrinogen-coated beads and a platelet activator. The instrument simply measures changes in light transmission automatically and thus the rate of platelet/bead aggregation. The test appears to correlate well with conventional platelet aggregometry. This represents a significant advance as the test can be performed reliably within different institutions/clinics without requiring either transport of sample, blood handling or processing, time delay or specialised personnel to perform the test. The test has recently been adapted to potentially measure the effectiveness of either aspirin or clopidogrel therapy within modified cartridges.

Summary

The last guidelines for platelet function testing were written in the late 1980s.9 Given the advances in this field and the introduction of potentially useful additions to our existing portfolio of platelet function tests, work is currently in progress to rewrite these guidelines. The development of reliable, sophisticated but simple to use whole blood tests that attempt to simulate in vivo haemostasis provides the ability to screen samples rapidly before applying our existing portfolio of tests.4,5,8 Certainly, the general consensus is that the in vivo bleeding time should be replaced. Many of the simpler platelet function tests could be potentially utilised as point of care instruments for assessing bleeding risk and monitoring antiplatelet therapy. Platelet function testing will therefore become increasingly utilised outside of the specialised laboratory. Although this represents an important advance, validation, reliability and quality control testing of these tests will also become an increasingly important issue. It is highly likely that in the near future the platelet genome and proteome will also be defined resulting in many exciting advances in the field, which could also have significant impact upon the diagnosis and management of patients with either Haemostatic or Thrombotic defects.

Acknowledgements

I thank Dr David Keeling and Professor Steve Watson for carefully reading the manuscript.
References

16 Jilma B. Platelet function analyzer (PFA-100): a tool to quantify congenital or acquired platelet dysfunction. *J Lab Clin Med* 2001; **138**: 152–163.
Antiplatelet agents

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The essential role played by platelets in the pathogenesis of arterial thrombosis is well established: therefore, platelets represent a major target of therapeutic interventions aiming at decreasing the incidence and severity of cardiovascular and cerebrovascular accidents in patients at risk. There are three families of agents that inhibit platelet function, with proven clinical efficacy: (1) cyclooxygenase inhibitors, such as aspirin; (2) ADP receptor antagonists, such as the thienopyridine compounds ticlopidine and clopidogrel; (3) glycoprotein (GP) IIb/IIIa (or integrin αIIb/β3) antagonists. All these drugs are used during coronary interventions and in the medical management of acute coronary syndromes, while only aspirin and the thienopyridine compounds are used in long-term prevention of cardiovascular and cerebrovascular events.

Both aspirin and the thienopyridines selectively inhibit a single pathway of platelet activation: aspirin affects the arachidonate-thromboxane A2 (TxA2) pathway by irreversibly inhibiting cyclooxygenase-1 (COX-1),1 while the thienopyridines affect the ADP pathway, by irreversibly blocking the ADP receptor P2Y12.2 In contrast, GPIIb/IIIa antagonists block the binding of adhesive proteins to GPIIb/IIIa on activated platelets, which represent the final common and essential pathway for platelet aggregation,3 thereby inhibiting platelet aggregation irrespective of the type and number of platelet agonists that triggered platelet aggregation. Despite their selective mechanism of action, which does not counteract the many alternative pathways for platelet activation, aspirin and thienopyridines display a good antithrombotic activity: this is explained by the fact that both the arachidonate-TxA2 pathway and the ADP pathway contribute to the amplification of platelet activation and are essential for the full aggregation response of platelets to platelet agonists.2

Despite the fact that the currently available drugs inhibiting platelet function have a good risk-to-benefit ratio, they suffer some drawbacks, which justifies the unceasing search for agents that can further improve the clinical outcome of patients with atherosclerosis through greater efficacy and/or safety. In this short review, I shall summarize the experimental evidence of the efficacy and safety of the currently available drugs inhibiting platelet function, their limitations and, finally, consider the new drugs that are being developed and the potential new targets for future antiplatelet agents.

Aspirin

Aspirin is the most widely used inhibitor of platelet function. A single dose of 160 mg completely abolishes platelet COX-1 activity; the same effect can be achieved with daily doses of 30–50 mg.1 Since aspirin acetylates COX-1 in all tissues, including endothelial cells, where the enzyme converts arachidonic acid into the natural platelet agonist prostacyclin, for several years there has been the concern that the potential antithrombotic effect of aspirin could be blunted, or even overcome, by the theoretical prothrombotic effect associated to the parallel inhibition of prostacyclin.4 The search for low doses of aspirin that could completely inhibit platelet COX-1 while sparing endothelial cell COX-1 has been intensive for several years. However, based on the compelling evidence in the literature, one can safely conclude that doses of aspirin that inhibit both platelet and endothelial cell COX-1 are antithrombotic, indicating that the protective effects of platelet inhibition largely prevail over the theoretically prothrombotic effects of endothelial cell inhibition. It must be noted that high doses aspirin might have antithrombotic effects that are independent of platelet COX-1 inhibition, including increased fibrilolytic activity,5 depression of prothrombin synthesis,6 improvement of endothelial function,7,8 and the well-known anti-inflammatory effects.

The meta-analysis of the Antiplatelet Trialists’ Collaboration (ATC) (now renamed Antithrombotic Trialists’ Collaboration) demonstrated an approximately 25% reduction of vascular death, myocardial infarction or stroke for antiplatelet therapy, primarily aspirin, versus placebo in patients with acute or previous cardiovascular or cerebrovascular events.9 A more recent analysis from the same group suggested that the indications for aspirin use should be expanded to primary prevention in populations at high risk, such as those with diabetes, peripheral vascular disease, carotid stenosis and end-stage renal disease.10

Of paramount importance are the results of the ISIS-2 trial, which showed that aspirin reduces the mortality from acute myocardial infarction (AMI) to an extent that is similar to that of the thrombolytic agent
streptokinase. Considering that aspirin is very cheap and extremely safe, this finding could have the greatest impact on cardiovascular mortality worldwide than any other, albeit very important achievement in this field.

**Thienopyridines**

**Ticlopidine**

Ticlopidine (250 mg b. i.d.) is an efficacious antithrombotic agent in patients with claudication, unstable angina, peripheral artery bypass surgery and cerebrovascular disease. It is associated with a high incidence of neutropenia (about 1%), which is usually reversible upon discontinuation of treatment; however, in few cases, it is irreversible and potentially fatal. Patients must be periodically monitored, especially in the first three months of treatment, to detect this harmful complication. Another potentially life-threatening complication of ticlopidine therapy is thrombotic thrombocytopenic purpura (TTP).

**Clopidogrel**

Clopidogrel represents an advance in antiplatelet therapy because, compared to ticlopidine, its use is not complicated by neutropenia. It must be noted, however, that TTP is still a harmful, albeit rare, complication of clopidogrel treatment. Clopidogrel (75 mg daily) was compared to 325 mg aspirin in the CAPRIE trial, which enrolled patients at risk of ischemic events due to previous MI, ischemic stroke or peripheral artery disease (PAD). The trial showed an 8.7% relative risk reduction of the major end points (MI, ischemic stroke and vascular death) in patients treated with clopidogrel compared to patients treated with aspirin. The absolute risk reduction was only 0.9% and the number needed to treat (NNT) 115 (95% CI, 58-8647), which, given the high cost of the drug, would render its cost-effectiveness in this type of patients at best unattractive. Subset analysis of the CAPRIE study showed that the benefits of clopidogrel over aspirin are more pronounced in high-risk patients with diabetes or prior revascularization.

**GPIIb/IIIa antagonists**

**Intravenous GPIIb/IIIa antagonists**

Abciximab is the Fab fragment of a humanized murine monoclonal antibody, 7E3, which binds irreversibly to the platelet GPIIb/IIIa complex. Given as an intravenous bolus, followed by a continuous infusion to maintain >80% receptor occupancy, it reduces the complications associated with percutaneous coronary interventions and improves long-term survival in patients undergoing coronary angioplasty (EPIC and EPILOG studies), in patients with refractory angina (CAPTURE study) and in patients undergoing coronary artery stenting (EPISTENT). A recent study by Kastrati et al showed that patients with low or intermediate risk, who had been treated with aspirin and a 600 mg loading dose of clopidogrel before stent implantation, do not have additional benefit from abciximab infusion. This important study, in addition to be of great practical value, emphasizes the importance of the two amplification pathways, the arachidonate/TxA2 and the ADP pathway, in platelet aggregation and thrombus formation.

Abciximab use is associated with bleeding complications, because the drug induces a thrombasthenia-like syndrome in patients who are being treated with other antithromostatic agents, such as aspirin, heparin and thrombolytics. It also induces thrombocytopenia, which is probably immune-mediated, in a small percentage of patients (0.4–1.6% had platelet counts < 50 × 10⁹/l in the above-mentioned studies). Eptifibatide, a synthetic cyclic heptapeptide based on the KGD motif of the snake venom barbourin reversibly blocks fibrinogen binding to GPIIb/IIIa. It has been shown to be safe and effective in emergency coronary interventions (IMPACT-II study) and unstable angina (PURSUIT study).

Tirofiban, a non-peptide reversible antagonist of GPIIb/IIIa, was shown to be effective to prevent early adverse events, without improving long-term outcomes, in patients undergoing angioplasty (RESTORE study) or with unstable angina (PRISM, PRISM-PLUS studies).

**Oral GPIIb/IIIa antagonists**

The results of five large trials (EXCITE, OPUS, SYMPHONY, SYMPHONY II, BRAVO studies) with four different oral GPIIb/IIIa antagonists not only failed to demonstrate a reduction in long-term prevention of recurrent ischemic events, but showed that the use of oral GPIIb/IIIa antagonists was associated with an increase in the rate of all-cause mortality and bleeding complications. The reasons for this catastrophic failure of oral GPIIb/IIIa antagonists are still uncertain and reflect our insufficient understanding of their pharmacodynamics and pharmacokinetics.

**Combined anti-platelet therapy**

Theoretically, inhibition of the two main amplification pathways of platelet aggregation, the ADP and the arachidonate/TxA2 pathways, is superior to inhibition of either pathway alone in preventing thrombus formation. As a matter of fact, dual antiplatelet therapy with aspirin plus ticlopidine is superior to aspirin alone, and also to aspirin plus warfarin, in patients undergoing coronary stent implantation. The combination of clopidogrel and aspirin seems to be at least as effective.
as the combination of aspirin and ticlopidine. The CURE study showed that the addition of clopidogrel to aspirin reduced by 20% the incidence of vascular end points in patients with unstable angina or non-ST segment elevation MI.\textsuperscript{1,18} The PCI-CURE substudy showed that also patients undergoing percutaneous revascularization benefit from dual antiplatelet therapy.\textsuperscript{19} Finally, the CREDO trial showed that dual antiplatelet therapy should be continued beyond the usual 30 days, because, after 1 year treatment, patients in dual therapy experienced a 27% relative risk reduction in death, MI and stroke compared to patients who were assigned to aspirin alone after the first 30 days of treatment with clopidogrel and aspirin.\textsuperscript{20}

As previously mentioned, Kastrati \textit{et al}\textsuperscript{17} recently showed that patients with low or intermediate risk, who had been treated with aspirin and a 600 mg loading dose of clopidogrel before stent implantation, do not have additional benefit from abciximab infusion, demonstrating the combined inhibition of the arachidonate/TxA2 and the ADP pathways can accomplish a very good antithrombotic effect.

Unfortunately, combined therapy with thienopyridines and aspirin is associated with an increased risk of hemorrhagic complications, which can require blood transfusion, especially in patients undergoing coronary revascularization.

\section*{Limitations of aspirin and thienopyridines: the problem of drug resistance}

In the last few years, the problem of ‘aspirin resistance’ has been largely emphasized in the medical literature, although its definition and probably even its real existence are still uncertain. Less well known, but certainly better characterized is the problem of ‘clopidogrel resistance’.

\subsection*{Aspirin resistance}

The term ‘aspirin resistance’ has been given different definitions by different researchers\textsuperscript{21–23} and I think that an effort should be made in trying to propose a universally acceptable definition of this phenomenon. A short list of definitions that have been given to ‘aspirin resistance’ follows, with my personal considerations relative to each of them.

(1) \textit{Failure of aspirin to prevent thrombotic events}: This phenomenon should be termed ‘treatment failure’: it can be observed with any kind of treatment and is expected to be particularly frequent for drugs, like aspirin and all other anti-thrombotic agents, that are used to prevent multi-factorial diseases, such as those associated with vascular occlusions. Therefore, this definition of ‘aspirin resistance’ is unacceptable.

(2) \textit{Failure of aspirin to inhibit platelet function in vivo or in vitro}. This is also an unacceptable definition of ‘aspirin resistance’. Platelet function \textit{in vitro} is measured by the bleeding time, while platelet function \textit{in vitro} is usually measured by the light transmission aggregometry or by the more recent, global techniques that evaluate primary hemostasis, such as the PFA-100 system. All these techniques, albeit to different degrees, are inaccurate, poorly reproducible and, most importantly, sensitive to a large variety of variables. Among these variables, platelet TxA2 production, which is the pharmacological target of aspirin, is usually of marginal importance. The only exception in this regards, could be represented by the study of platelet aggregation induced by arachidonic acid, which is transformed into TxA2 by the aspirin-sensitive enzyme COX-1.

(3) \textit{Failure of aspirin to inhibit TxA2 production}. This is the only acceptable definition of ‘aspirin resistance’, because it reflects the real pharmacological target of the drug; however, it has been used very rarely in the studies published so far.

Although most of the evidence of ‘aspirin resistance’ is based on studies that chose the wrong parameters to detect it, there are indeed reports of patients relatively insensitive to aspirin inhibition of TxA2 production, who seem to be less well protected from vascular events;\textsuperscript{23} whether this effect is due to negative interaction with other drugs, such as ibuprofen,\textsuperscript{24–27} or also to other variables, it is presently unknown and should be the subject of future, well-designed controlled studies.

\subsection*{Clopidogrel resistance}

The problem of drug resistance is certainly more relevant for thienopyridines than for aspirin. Ticlopidine and clopidogrel are prodrugs, which need to be metabolized by the liver in an active metabolite with antiaggregating activity.\textsuperscript{12} Therefore, their pharmacological effect can be detected only several hours after their first administration and, more importantly, the plasma levels of the active metabolite, and, consequently, the degree of inhibition of platelet aggregation induced by ADP, may vary widely among subjects. In published studies, about 50% of the patients were either clopidogrel non responders or low responders.\textsuperscript{28} Interindividual variability in platelet inhibition by clopidogrel correlated well with the metabolic activity of the hepatic cytochrome \textit{P}450, which activates the prodrug to its active metabolite.\textsuperscript{31} Whether polymorphisms of the clopidogrel target, P2Y12, play additional roles in modulating the individual response it is presently unknown.\textsuperscript{29} Interference with clopidogrel metabolism by other drugs that are frequently given to patients with atherosclerosis, such as atorvastatin,\textsuperscript{30,31} can increase the number of patients who are resistant to clopidogrel, although this is still a controversial issue.\textsuperscript{32} The extent of the aggregation response \textit{in vitro} to ADP has been used to define ‘clopidogrel resistance’ in all studies that have been published so far. It must be noted that the aforementioned general pitfalls of \textit{in vitro} platelet aggregation apply not only to studies of ‘aspirin
resistance’ but also to those of ‘clopidogrel resistance’. In addition, although ADP is the most appropriate aggregating agent in this context, because clopidogrel antagonizes the ADP receptor P2Y12, it must be noted that platelets express also a second ADP receptor, P2Y1, which causes the initial wave of ADP-induced platelet aggregation. Since the extent of residual, P2Y1-dependent platelet aggregation induced by ADP vary widely among patients with congenital P2Y12-deficiency or normal subjects in whom P2Y12 function had been completely blocked in vitro by saturating concentrations of specific antagonists, ADP-induced platelet aggregation may not be the most suitable test to measure the individual response to clopidogrel. A better and more specific test would be measurement of the extent of ADP-induced inhibition of adenyl cyclase, which is uniquely mediated by P2Y12.

Development of new antiplatelet drugs

New drugs for old targets

Nitric oxide-releasing aspirin is a new chemical entity obtained by adding a nitric oxide-releasing moiety to aspirin. NCX-4016 is the prototype of this family of molecules. Both aspirin and nitric oxide moieties of NCX-4016 contribute to its effectiveness, the latter occurring via both cyclic guanosyl monophosphate-dependent and -independent mechanisms. In vitro studies have shown that NCX-4016 inhibits platelet aggregation induced by aspirin-sensitive (arachidonic acid) and aspirin-insensitive (thrombin) agonist. Human studies have shown that nitric oxide-aspirin is less toxic than aspirin on the gastrointestinal tract.

New, selective TxA2 receptor antagonists are being developed, which could counteract the effects of endogenous agonists of TxA2 receptors contributing to endothelial dysfunction, despite aspirin treatment, in patients with atherosclerosis.

New P2Y12 antagonists include a thienopyridine-like compound, CS-747, which generates an active metabolite, R-99224, in vivo that irreversibly inactivates the platelet P2Y12. CS-747 appears to be more potent than ticlopidine and clopidogrel and has a rapid onset of action. Perhaps more interesting, are compounds that directly and reversibly antagonize P2Y12. AR-C69931MX is a potent, short-acting P2Y12 antagonist, which, after intravenous administration to healthy volunteers or patients with acute coronary syndromes, was well tolerated and achieved a substantially greater P2Y12 receptor blockade than clopidogrel. A direct, orally available and reversible P2Y12 antagonist, AZD6140, is currently under clinical evaluation.

New drugs for new targets

New platelet targets for potential antithrombotic drugs include several receptors and effectors that are important for platelet function. They have recently been reviewed. The ideal antithrombotic drug should selectively target the ‘bad’ fibrin and platelet aggregates of pathologic thrombi, without affecting the ‘good’ fibrin and platelet aggregates of the hemostatic plug. The search for such a drug, with an ideal benefit-to-risk ratio, has been unsuccessful so far and chances are high that it will be elusive also in the near future, because, based on our current knowledge, it is difficult to identify a pathogenic mechanism operating selectively during pathologic thrombosis that is not involved also in normal hemostasis. Perhaps the only exception is represented by P2X1, an ATP receptor that is expressed on the platelet surface, which seems to be important for platelet thrombus formation at high shear. Recent in vitro and in vivo studies have shown that the higher the shear forces, the more relevant is the role of P2X1 in the formation of platelet aggregates. Since shear forces are highest at sites of severe pathological stenosis caused by atherosclerotic lesions, one can hypothesize that agents that interfere with P2X1 function could preferentially prevent thrombus formation at sites of atherosclerotic plaques, without significantly affecting the formation of the normal hemostatic plug in the microcirculation, where shear forces are lower.

References

9. Antiplatelet Trialists’ Collaboration. Collaborative overview of randomised trials of antiplatelet therapy-I: Prevention of death, myocardial infarction, and stroke by...
prolonged antiplatelet therapy in various categories of patients. 


CONTROVERSIES IN TRANSFUSION COMPONENT THERAPY

Haemovigilance in the United Kingdom and Europe

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The inclusion of a talk on haemovigilance under the heading of ‘Controversies in transfusion component therapy’ may seem at first surprising. The importance of systematic surveillance of adverse effects of transfusion has been accepted for almost a decade and the European Directive on Blood Safety1 will make implementation of such schemes mandatory. But there remain questions to be answered: how do we ensure that a complete picture of transfusion hazards is obtained; how do we interpret data on adverse events in the context of transfusion epidemiology; and, most importantly, how can evidence from haemovigilance be best used to ‘align efforts with risks’2 when resources are allocated to blood safety initiatives?

Haemovigilance schemes in Europe have developed in a number of different ways,3 but some common themes have emerged. Not all schemes collect data on errors unless they result in harm, and clinical practice is not encompassed by the EU Directive. However, the risk of errors resulting in ABO-incompatible transfusion is a key message, and schemes that report on no-harm errors and near-misses, such as Serious Hazards of Transfusion (SHOT) in the UK, can reveal clues as to the root causes. The importance of system failures in healthcare is becoming increasingly recognised throughout the world, and was highlighted in the report of an expert group chaired by the CMO for England, Professor Liam Donaldson.4 Lessons may be learned from other industries such as aviation, where the benefits of adverse event and near-miss reporting within an open, learning culture have been well documented.5

The SHOT scheme, launched in 1996, was one of the first transfusion adverse event reporting systems to be established. The scheme is confidential, professionally led and currently voluntary, though strongly endorsed by the UK Chief Medical Officers’ ‘Better Blood Transfusion’ initiative.6 Strategic direction comes from a multidisciplinary steering group with wide representation from UK Royal Colleges and professional bodies; funding is provided by the UK blood transfusion services. SHOT is now in its seventh year and has accumulated a robust body of evidence on serious transfusion hazards in the UK.7 In the first 6 years, 1711 initial reports were received and 1630 follow-up questionnaires analysed. Categories of adverse events collected by SHOT are shown in Table 1.

Analysis of 1630 questionnaires received from 1996–1997 to 2001–2002 is shown in Figure 1. Since the inception of SHOT it has been clear that the most frequent transfusion hazard is ‘incorrect blood component transfused’ (IBCT), that is, a patient receiving a blood component intended for another person or not meeting appropriate requirements. Haemovigilance schemes that include this category have found similar results.8,9 In total, 64% of questionnaires analysed by SHOT (1045/1630) have related to such episodes, due to avoidable system failures throughout the transfusion chain. Many events involve multiple errors (Figure 2); closer analysis of these reveals that the majority occur in clinical areas (Figure 3) with 17% relating to incorrect prescription, sampling or request and 51% to errors in collection of blood from storage areas and administration at the bedside. In all, 28% of errors occurred in hospital transfusion laboratories.

Fortunately, 883/1045 (84.5%) patients receiving incorrect blood components survived with no ill effects; nevertheless, over the course of 6 years, 15 deaths (11 due to major ABO incompatibility and three to inappropriate transfusions resulting in fluid overload) were attributed wholly or in part to transfusion errors while 69 patients suffered major morbidity.

The professional response to SHOT’s early findings was the production in 1996 of guidelines from the British Committee for Standards in Haematology on the administration of blood components,10 which have been widely adopted in hospitals. A National Comparative Audit conducted in 2003 under the auspices of the Royal College of Physicians indicated that a policy on blood administration was in place in 100% of hospitals surveyed. However, implementation of guidelines and policies into practice presents a greater challenge, and the same audit indicated that, in 5014 transfusion episodes in 169 hospitals, 10% of patients were not wearing identity bands during transfusion, clinical observations were carried out within the first 30 min of the start of transfusion in only 53% and in only 75% was there a reason for transfusion recorded in the notes (F Regan, personal communication).

Transfusion-related acute lung injury (TRALI) is one of the most controversial and complex complications of transfusion, characterised by respiratory distress and hypoxaemia associated with transfusion of
plasma-containing blood components and in the absence of fluid overload or cardiac failure. TRALI is likely to be under-recognised, and completeness of reporting is confounded by the nonspecific clinical picture, the lack of an accepted definition and absence of a conclusive diagnostic test. In institutions with a high index of suspicion of TRALI, its incidence has been estimated at 1:5000 plasma-containing components.

Reports of TRALI to SHOT were constant at an average of 14 (range 11–19) per year for the first 5 years, but rose to 26 in year 6, with increasing awareness of the condition. While taking into account uncertainties regarding diagnosis and imputability, the accumulation of 103 reports of TRALI to SHOT over 6 years and its implication in 25 deaths and 67 cases of major morbidity has led to its recognition as the most important cause of transfusion-associated mortality and morbidity.

Table 1 Adverse events reportable to SHOT

<table>
<thead>
<tr>
<th>Category of adverse event</th>
<th>SHOT definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incorrect blood component transfused</td>
<td>Patient transfused with a blood component or product that did not meet the appropriate requirement or was intended for another patient</td>
</tr>
<tr>
<td>Acute transfusion reaction</td>
<td>Adverse reactions occurring up to 24h following transfusion, excluding those due to ICBT</td>
</tr>
<tr>
<td>Delayed transfusion reaction</td>
<td>Clinical adverse reactions (not simple serological reactions) occurring more than 24h following transfusion of blood components</td>
</tr>
<tr>
<td>Transfusion related acute lung injury</td>
<td>Acute dyspnoea with hypoxia and pulmonary infiltrates within 24h of transfusion, with no other apparent cause</td>
</tr>
<tr>
<td>Transfusion-associated graft-versus-host diseases</td>
<td>Development of the classical symptoms of fever, rash, liver dysfunction and pancytopenia occurring 1–6 weeks post-transfusion, without other apparent cause. Diagnosis supported by skin/marrow biopsy appearances and/or presence of circulating donor lymphocytes</td>
</tr>
<tr>
<td>Post-transfusion purpura</td>
<td>Thrombocytopenia 5–12 days post-transfusion associated with antibodies in the patient directed against the human platelet antigen (HPA) system</td>
</tr>
<tr>
<td>Transfusion-transmitted infection</td>
<td>Post-transfusion infection in which</td>
</tr>
<tr>
<td></td>
<td>● the recipient had no evidence of infection pre-transfusion</td>
</tr>
<tr>
<td></td>
<td>and either</td>
</tr>
<tr>
<td></td>
<td>● at least one component was donated by a donor with evidence of the same transmissible infection or</td>
</tr>
<tr>
<td></td>
<td>● at least one component was shown to have been contaminated with the infective agent</td>
</tr>
<tr>
<td>‘Near-miss’ event</td>
<td>Any error which, if undetected, could result in the determination of a wrong blood group, or issue, collection or administration of an incorrect, inappropriate or unsuitable component but which was recognised before transfusion took place</td>
</tr>
</tbody>
</table>

Figure 1 Analysis of Questionnaires from 1996–1997 to 2001–2002.

Figure 2 Multiple errors in IBCT cases from 1996–1997 to 2001–2002.

Figure 3 Analysis of Multiple errors.

Reports of TRALI to SHOT were constant at an average of 14 (range 11–19) per year for the first 5 years, but rose to 26 in year 6, with increasing awareness of the condition. While taking into account uncertainties regarding diagnosis and imputability, the accumulation of 103 reports of TRALI to SHOT over 6 years and its implication in 25 deaths and 67 cases of major morbidity has led to its recognition as the most important cause of transfusion-associated mortality and morbidity.

Reporting of TRALI has been variable in Europe; prior to 2002 it was not included as a category in the French Haemovigilance database. Its precise pathogenesis is not defined, but it appears usually to be triggered by passive transfer of HLA or granulocyte antibodies from the donor. Implicated antibodies arise as a result of pregnancy or transfusion. Hence, it has been suggested, although not proven, that its occurrence could be reduced by sourcing plasma only from male untransfused donors, an option that is currently being implemented in Denmark and also in the UK as part of an overall strategy to improve plasma safety. The use of pooled solvent–detergent-treated plasma appears also to protect against this complication. Options for reducing the risk of TRALI from platelets include suspension of platelet pools in ‘male plasma’, screening female
plateletapheresis donors for leucocyte antibodies, and the use of platelet-additive solutions.

Other immunological reactions to transfusion, such as haemolytic reactions and anaphylactic/anaphylactoid and allergic responses, are unpredictable, and many may be unavoidable. The European Haemovigilance Network is working towards common definitions that will facilitate assessment of the risks of these complications. Different haemovigilance systems currently portray very different patterns; for example, in France, 78% of reported adverse events relate to immunological reactions but these include the whole spectrum from mild febrile nonhaemolytic reactions and alloimmunisation to anaphylaxis and acute intravascular haemolysis. Some consistencies may nevertheless be found. It is notable that of 104 severe allergic or anaphylactoid reactions reported to SHOT over 6 years, 81 (77%) related to plasma-containing components, which constitute only 22% of components transfused. Scrutiny of the French data yields similarly striking findings; of 2566 anaphylactoid reactions reported in 1997 and 1998, 788 related to red cells, 1651 to platelet concentrate and 285 to FFP giving an incidence of 20 per 100 000 red cells issued, 100 per 100 000 platelets and 200 per 100 000 FFP. There are few firm indications for administration of FFP and clinical audit findings in the UK suggest that it may often be administered inappropriately.

There has been a changing and evolving emphasis on other transfusion hazards as more data accumulate and practices alter. The first three SHOT reports highlighted the importance of transfusion-associated graft-versus-host disease (TA GvHD) as a rare but universally fatal reaction in immunocompromised transfusion recipients. In all, 12 deaths were reported between 1996 and 1999. The British Committee for Standards in Haematology (BCSH) Transfusion Task Force responded by producing guidelines on gamma-irradiation of blood components for the prevention of TA-GvHD, and in 2000 collaborated with the National Blood Service and Department of Health to produce a patient information leaflet and card. Despite these precautions, there remains room for improvement in procedures to ensure provision of irradiated components for vulnerable patients, as failures continue to be reported to SHOT in the IBCT category. It is notable that the incidence of TA GvHD in the UK has fallen since introduction of universal leucodepletion in 1999; however, gamma-irradiation remains the only accepted method of prevention.

Since surveillance began in the UK in 1995, only eight instances (affecting 14 patients) have been reported of confirmed transmission of viruses for which mandatory testing is performed. It must, however, be acknowledged that haemovigilance schemes do not lend themselves to capture of late complications of transfusion where the causal relationship may be overlooked. Over the same time period, SHOT received 26 confirmed case reports of transfusion transmitted infection due to bacterial contamination, of which 22 were due to platelets. These resulted in six deaths, of which five were in platelet recipients. The importance of bacterial contamination of blood has also been highlighted by other haemovigilance schemes, and these findings have led to the implementation by blood services of measures aimed at reducing this risk.

How complete is the picture of adverse events of transfusion as captured by haemovigilance? In 2001–2002 over 90% of UK hospitals where blood is transfused stated in response to a survey that they participated in SHOT, but of these only 49% reported incidents. Experience in institutions where the culture of reporting to SHOT is well established suggests that adverse events remain under-recognised and under-reported elsewhere. Numbers of reported IBCT incidents have increased year-on-year (Figure 4) and show no sign of reaching a plateau. While a true increase in incident numbers cannot be ruled out it is more likely that, with the appointment in most hospitals of Specialist Practitioners of Transfusion (SPoTs), errors are more likely to be uncovered and reported. It is tempting to assume that making haemovigilance mandatory will ensure that all events are captured. Experience from France suggests that this is not the case. Since full implementation of haemovigilance in France in 1996, mandated by law, the overall reporting rate has been stable nationally at 230 per 100 000 blood components transfused, but there is wide variation between different regions, from 110 to 460 per 100 000, indicating that not all incidents are reported.

Clinicians will be encouraged to report incidents if they perceive that by doing so they are contributing to an evidence base that will be used to effect change and to facilitate learning. They will be deterred by cumbersome reporting systems, lack of time and resource, lack of feedback and, above all, by fear of blame. The importance of cultivating an environment in which errors are seen as failures of systems rather than of individuals cannot be over-emphasised.

Gathering of data on adverse events is now well established across much of Europe and some consistent messages are emerging. But haemovigilance is but one piece of the blood safety jigsaw and it is important to recognise its limitations when evaluating transfusion risks. Scant data are available on transfusion practice to enable this information to be interpreted and as yet it is

Figure 4 Comparison of initial reports of incidents.
impossible to know whether particular patient groups or particular clinical circumstances are over-represented. In addition to knowledge of the risks of blood transfusion, we need better knowledge of the transfused patient population, their age profile, diagnoses and survival, if informed decisions are to be made about allocation of resources to transfusion safety.

Haemovigilance schemes have no powers to implement change, but can make informed recommendations based on evidence. Public perceptions of risk, outcomes of litigation, political imperatives and even media campaigns are also forceful pressures influencing decision-making bodies. In England, a successful class action was brought in 2001 by recipients of blood contaminated with HCV prior to the implementation of mandatory testing for the virus. In giving judgement, Mr Justice Burton defined blood as a ‘product’ under the Consumer Protection Act of 1988 and found the producer, that is, the blood service, to be liable if, whether avoidable or not, ‘the existence of the defect was known or should have been known in the light of accessible information’. This statement has led to an unrealistic expectation that the blood supply must be made totally safe whatever the cost, and that even minimal residual risk is unacceptable. There is therefore pressure on blood services to minimise the risk of transfusion transmitted infection by implementation of state-of-the-art screening tests and pathogen inactivation processes.

Discussions on the relative risks of transfusion hazards in the UK are dominated by the need to implement precautions to reduce the possibility of transfusion transmission of variant Creutzfeldt–Jakob disease (vCJD). The consequences of these initiatives have been a three-fold increase in the cost of cellular blood components and a potential risk of blood shortages due to increasingly stringent donor exclusion. A delicate balance exists between blood safety and availability; the risks are different for individual patients and the calculations involved are complex.

Perhaps the greatest contribution that haemovigilance schemes can make is to encourage the allocation of resources such as education and technology nearer to the bedside, where blood transfusion can be made safer without threatening sufficiency. Efforts to reduce errors in blood administration have no ‘downside’ in terms of supply; on the contrary, there are other potential benefits, as blood transfusion can be a model for other clinical activities, and initiatives in hospitals to ensure ‘right blood to right patient’ can equally well apply to ‘right drug’, ‘right procedure’ or even ‘right food’! Moreover, appropriate and thoughtful use of blood can reduce unnecessary exposure to hazards, but we must not be so energetic in our attempts to limit blood transfusion that we replace one set of risks with another. Outcome data are needed to better determine transfusion triggers, and the relative risks and costs of blood-conserving strategies need also to be evaluated.

References

Intravenous immunoglobulins: too many indications?

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As of today, a large number of diseases of different origin form a long list of indications for i.v. immunoglobulins (IVIG). Most synthesized drugs fail to match up to such a widespread beneficial use as does this stable plasma product. In this time of advancement on all the frontiers of clinical science, Dorland’s Medical Dictionary copes with the burgeoning terminology by distinguishing three types of indications: (i) indicatio causalis that aims at treating a disease afforded by its cause, (ii) indicatio curativa or morbi, an indication as to treatment afforded by the nature of the morbid processes observed and (iii) the indicatio symptomatica, an indication as to disease afforded by the symptoms that may arise. More recently, the term labeled indication has arisen to designate agency-acknowledged use of a drug, in this case IVIG-preparations to treat a given disease (Table 1). Is its usage in all these disease conditions justified?

To deal with this question, one needs to include medical, pharmaceutical, socioeconomical and legal aspects into the answer. IVIG are and always will remain blood products from donor plasma pools as the present educational message will not include intravenously administered monoclonal or engineered antibodies currently introduced at a fast rate to medical practice. It was in 1981, when the first intact, fully functional IVIG preparation was approved by the Federal Drug Agency of the USA as at that time already, approval of the then named ‘Immunoglobulin Swiss Red Cross’ preparation, later to become Sandoglobulin, would not have been possible without the favorable advice of the American Red Cross Blood Transfusion Service in Bethesda, MD, USA. Approval agencies in Europe and in Japan differed in their strategies for stable blood products in the early 1980s. Today, the European Agency for the Evaluation of Medicinal Products (EMEA) (www.emea.eu.int) has caught up on laws that govern their use and European Guidelines and Ethical Committee directives are now available for guideline; the EudraVigilance system, the new pharmacovigilance system in the European Union, will encompass stable blood products.

IVIG preparations and sale

In the developed world, there are ~30 different IVIG preparations available covering the current estimated annual transfusion amount of ~40 tons of IVIG. For the designation of this amount, many reports use the term ‘annual need’, but quantification of a need in health provision is difficult. The purpose of this contribution is to provide for a deeper insight in compelling indications as compared to prescribing IVIG on less solid ground, not precluding life-saving transfusion in the latter situation at all. Once packed in a vial, IVIG preparations become trade-named, agency- and commercially registered products. Each manufacturer’s IVIG preparation and among them each production lot, is a unique product carrying its own specific evidence-based indications. If produced by national health authorities or nonprofit organizations, their status is the one of a generic drug. If produced by commercial plasma fractionationing companies (e.g. Baxter, Bioplasma AG, Grifols, Octapharma, Biotest), current standard policies vary among several possibilities: (i) plasma from nonremunerated donors purchased from blood donor centers is used for production (ii) plasma is used from paid donations. In both cases, products currently priced in developed countries around 30–40 USD per gram are obtained. (iii) The producing fractionation plant might be located in the country of the donors or some countries might send their plasma abroad to reimport the final product. The extraction process of the IVIG from plasma pools has remained similar since 20 years. The technically driven, scientifically oriented and highly regulated plasma fractionation industry realizes that its backbone technology, cold ethanol fractionation, maintains its reduction property for viral load. The current average IVIG consumption in the developed world of ~40 tons contrasts to a demand of the whole world (6 billion people) of 120–240 tons with a theoretical volume of plasma required to meet the needs of 40 to 80 million liters. It is not excluded that the future will see some companies charging only modest royalties such as with AIDS drugs and/or granting licences for production and/or distribution. In addition, some countries with lower gross national products might want to produce their own IVIG preparations (for example; Serbia, Argentina). Such topics are vociferously discussed at annual meetings of the plasma fractionation industry.

The distribution of IVIG to the market is multifaceted. A direct link of the producer with hospital pharmacies is now complemented with IVIG brokers who are specialized in watching out for the best buy on
the international market weighing availability, efficacy, safety and prize. Some patients who depend on IVIG for the rest of their lives, might want to buy IVIG for self-administration from package delivery firms some of which distribute the preparations on credit card on-line sales; so far, business with on-line drug suppliers is prohibited.

Attribution of a single patient to a nosological entity, which is an indication for IVIG

Precision of diagnosis yielding a given nosological entity is a prerequisite before we can envisage prescription of IVIG for a particular patient. This process of identifying a disease by its signs and symptoms must encompass such procedures as clinical history making and examination of the patient, laboratory analysis, imaging and pedigree analysis. Very soon, genetic marker analysis, such as in certain immunodeficiency states, might further confirm or refute a given diagnosis but for the patients suffering from diseases as shown in Table 1 no such requirement is yet defined.

To establish any given indication for a drug, the study protocols transgress stride through different stages: animal studies, clinical studies phase I, II and III. Agencies now set the standards for such studies (Figure 1). With IVIG the animal study phase is often bypassed. This is so because using a human IgG preparation injected into an animal will provoke the test animal to produce anti-human IgG, which will result in immune complex disease. To prepare isologous IVIG of any animal to study a veterinary disease, serving as a model for the human variety is too expensive and might remain inconclusive. Rare exceptions exist mostly for situations in which animal experiments of short duration might add evidence for action mechanisms.

Clinical studies point the way to labeled indications and serve approval for new preparations

The first placebo-controlled clinical study with IVIG was carried out in Zurich. Four major procedures are brought together: Phase I trials make a tentative IVIG treatment administered to a small number of patients. Phase II trials aim to test effectiveness, to identify its optimum dose and to control side effects; usually 15–20 patients take part in a study of phase II whereas, with phase III, hundreds of thousands of patients would have to be tested, randomized with placebo and with the best known reference treatment. Phase III studies therefore are not feasible with IVIG because of its limited supply and they are replaced by close postmarketing surveillance protocols, including proof of efficacy now implemented in numerous hospitals around the world.

Finally, phase IV trials aim at controlling the effects of IVIG in the long term, measuring effectiveness, the nontoxicity and nontransmittal of infectious agents—number of patients taking part in phase IV trials is variable.

Successful studies

What opened the door to successful studies with IVIG? It is a mixture of plausible reasons: (i) Single case descriptions with successful outcome paved the way for the now acknowledged or even labeled indications based on phase II studies with idiopathic thrombocytopenic purpura. Kawasaki disease, myasthenia gravis and dermatomyositis as examples. (ii) The solid rationale of deviating complement activation to innocuous targets made the prescription of adjuvant IVIG in transplanta-

Table 1

<table>
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<tr>
<th>Incentive</th>
<th>Disease</th>
<th>Indication</th>
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<tbody>
<tr>
<td>Immunisation</td>
<td>Viral, bacterial, immunodeficiencies</td>
<td>labeled</td>
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<tr>
<td>Immunomodulation</td>
<td>ITP</td>
<td>labeled</td>
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<td></td>
<td>Kawasaki</td>
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<td>Guillain-Barré</td>
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<td>Myasthenia</td>
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<td></td>
<td>Lyell syndrome</td>
<td>doubtful</td>
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<tr>
<td></td>
<td>Organ transplantation</td>
<td>Not yet labeled</td>
</tr>
<tr>
<td></td>
<td>Retinopathy</td>
<td>Not labeled</td>
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Figure 1 How federal and state agencies support development of the major stable plasma product, i.v. immunoglobulin. Please read counter-clockwise starting with 1.
recommend IVIG treatment in sepsis. The forum agrees that sceptical conclusions have been drawn by the experts of maglobulinemic patients of primary or secondary origin. Infection events in appropriately substituted hypogammaglobulinemic patients ranges. And, (iii) the endeavour to avoid efficacious yet side effect-compromised drugs with more precise immunosuppressive protocols. The best dosage of IVIG in many diseases in not known. IVIG is mostly administered in dosage of 0.4 g/kg body weight for 5 consecutive days. A total dosage of 2 g IVIG/kg applied during 2 days only, however, may act more rapidly, but might result in a higher rate of adverse effects. The 0.4 g/kg dosage stems from the initial observation of Imbach et al. in ITP, but half or double a dose have not been tested for efficacy over the 0.4 g in most diseases. The question is relevant for prize and for availability reasons.

**Study failures**

Failures come from rationale that is not strong enough or from study inclusion of patients without paying enough respect to subclassification of major diseases diagnosis. Thus, subclassification of chronic dysimmune neuropathy allows distinct syndromes to be recognized and helps resolve problems of heterogeneity and overlap. Distinction between these subgroups is of immediate practical relevance to patient management. Although steroids are beneficial for most of the subgroups, this is not so for both the pure motor syndromes, which should be treated with IVIG.8

There are quite a few study failures on record when researchers wanted to explore the solid rationale of antiinfectious (antiviral, antibacterial) properties of IVIG. The rationale to treat sepsis patients with IVIG is enhanced even when looking at the damming up of infectious events in appropriately substituted hypogammaglobulinemic patients of primary or secondary origin. Sceptical conclusions have been drawn by the experts of the International Sepsis Forum. The forum agrees that some small, encouraging IVIG-sepsis trials exist but states that evidence remains unconvincing enough to recommend IVIG treatment in sepsis—the German Sepsis Society supports this statement. Sepsis has remained a nonlabeled measure, that is, IVIG are not indicated in sepsis.9

With multiple sclerosis (MS),10,11 treatment attempts have changed dramatically over the last decade, treatment has shifted its base to recent immunobiological findings. Advances in biotechnology, improvement in clinical trial design and development of magnetic resonance imaging have successfully been introduced and established immunomodulatory therapies, such as interferon-beta and glatiramer acetate. One might look at these successful compounds as the leftovers of broader endeavours, which remained unsuccessful albeit based on convincing immunological concepts, endorsed with convincing animal models and promising results from phase I/II studies. Whereas IVIG or IgM-enriched IVIG promote remyelination for neurocytes in vitro,12 this property is apparently not strong enough to help MS patients in vivo. The future will show whether during dosage – or time point of administration – failures of IVIG in MS might become improved.

With the precious stable plasma product IVIG that should remain in sufficient supply for those in need, some workers have tried to answer the question posed at the outset (is usage in all these disease conditions justified?) from another end, that is, for which disease are IVIG prescribed in daily practice according to the single diagnosis. Table 2 lists the result of a study conducted by the Marketing Research Bureau. Looking at this table, one comes to the conclusion that not each labeled indication is adhered to and that physicians may prescribe alternate medicine before recurring to IVIG.

Current shortage of IVIG hence proud pricing comes from three major tracks: (i) Supply of sufficient plasma satisfying the quality requirements of fractionation plasma; track-keeping from the fractionation plasma to the single individual donors (feasibility for lookbacks). Confinement of drug promotion for unlabeled indication to patients where national expert panels agree. (ii) In response to the information explosion, the reluctance of many physicians against evidence-based medicine might be justified insofar as strict confinement of IVIG transfusion to labeled indications would prevent discoveries such as the recent strong experience-based practice to use IVIG in solid organ transplantation.7 (iii) The strategy of WHO similar to the UNAIDS program should include IVIG in a policy that promotes international partnership and advocacy, direct support to countries, simplified and standardized instruments to identify patients, deliver IVIG, and track progress; develop measures to ensure a reliable supply of effective medicines and diagnostics; and rapid identification and dissemination of new knowledge to improve programme quality.

Study failures with any drug supposed to benefit the patients at the outset must be seen as a result of either conclusive lack of evidence that the drug is efficient or that the chosen dosage, time point and way of administration (i.v., s.c., p.o.) were set at inefficient ranges.

**Table 2**

<table>
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<tr>
<th>IVIG Therapy and Prophylaxis against New Infectious Agents and Autoimmunity</th>
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<td>IVIG promotes remyelination for neurocytes in vitro.</td>
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Quite a few diagnoses eligible to treat with IVIG relate to rare diseases. Because IVIG are used extensively, they are not officially orphan drugs but with rare diseases large phase II/III randomized placebo-controlled studies will find it difficult to reach statistical power for drawing conclusions.

True placebo control for IVIG may be attained with IVIG-preparations devoid of the putative efficient specific antiviral antibody. Thus, a phase I/II randomized, placebo-controlled trial to assess the efficacy of IVIG containing high anti-West Nile Virus (WNV) antibody titers in patients with or at a high risk for progression to WNV-encephalitis and/or myelitis is currently under way under sponsorship by the US-National Institute of Allergy and Infectious Diseases (NIAID). This shows that the initial lack of market interest of the plasma fractionation industry for a product that traditionally has been injected through the intramuscular route can be complemented by sponsoring of state agencies. It should be avoided that economic reasons or lack of interest of pharmaceutical companies for market access, may hamper development of otherwise, anti-infectious hyperimmunoglobulins.

The physician interested for updated practice might consult www.clinicaltrials.gov in order to inform patients about the latest developments. Thus, entering the abbreviation IVIG into the search field for clinical trials yields, at the time of writing this, there were four studies to recruit volunteers, two of which were West Nile Virus (WNV) infection prophylaxis/treatment, as well as studies that assess the molecular background of immunodeficiency, such as in patients suffering from Wiskott–Aldrich syndrome, adenosine deaminase (ADA) deficiency, Janus Associated Kinase (JAK3) deficiency, common variable immunodeficiency (CVID) and other immunodeficiencies and the auspices of the National Human Genome Research Institute, NHGRI.

Before prescribing IVIG, the physician may adhere to simple algorithms, as one exemplified in Figure 2, to decide on proposing potential benefit to the patient.

Acknowledgements
I acknowledge Mr Christian Langenegger for drawing the figures.

References
GENES SWITCHING THEM ON AND OFF

Switching off oncogenic signals in chronic myeloid leukaemia

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\textsuperscript{1}Imperial College London, London, UK


Introduction

Chronic myeloid leukaemia (CML) is a haematological disorder characterised by the malignant proliferation of bone marrow stem cells. Underlying the aetiology of this disease is a reciprocal chromosomal translocation involving chromosomes 9 and 22 [t(9;22)(q34;q11)] (reviewed in Deininger et al.\textsuperscript{1}). The derivative chromosome 22q\textemdash known as the Philadelphia (Ph) chromosome\textemdash bears BCR-ABL, an oncogene generated by the fusion of sequences from two genes, BCR and ABL. The malignant phenotype of CML is caused by Bcr-Abl, the abnormal tyrosine kinase encoded by this fusion gene. In this review, we consider the options for 'switching off' the oncogenic signal that originates with Bcr-Abl. These include small molecule inhibitors of the tyrosine kinase domain of Bcr-Abl and RNA interference approaches for silencing BCR-ABL gene expression. We also describe potential therapies that exploit key molecular targets in intracellular signalling pathways downstream of Bcr-Abl.

Targeting Bcr-Abl

The tyrosine kinase domain

The ability of Bcr-Abl to transform cells derives mainly from its tyrosine kinase activity.\textsuperscript{2} Consequently, the uncontrolled, intrinsically active and perpetually 'switched on' protein domain responsible for this catalytic function has been identified as a prime target for molecular therapy. Imatinib mesylate (Glivec or Gleevec), a small molecule inhibitor of the tyrosine kinase domain of Bcr-Abl, has proved to be effective in the treatment of CML. Developed by Novartis Pharma (Basel, Switzerland), imatinib is a synthetic 2-phenylaminopyrimidine that binds to the ATP-binding site within the kinase domain of Bcr-Abl (Figure 1). \textit{In vitro}, imatinib was found to inhibit the autophosphorylation of relatively few protein tyrosine kinases, including Bcr-Abl, Abl, the platelet-derived growth factor receptor (PDGFR), the Kit receptor and the ABL-related gene product (Arg) (reviewed in Capdeville et al.\textsuperscript{3}). Effects on other protein tyrosine kinases were negligible. Such a remarkable degree of specificity probably accounts for the favourable clinical profile of imatinib and, in general, few side effects associated with its use. Positive results from phase I and II clinical trials led to its rapid adoption as a first line treatment for CML.\textsuperscript{3,4}

A serious problem associated with the use of imatinib was recognised early on in its clinical life (reviewed in Gambacorti-Passerini et al.\textsuperscript{5}). Whereas patients in advanced phase CML frequently achieve a haematological response upon first receiving imatinib, it is rare for these responses to be sustained for any length of time. Disease progression invariably follows despite continued treatment with imatinib. This unfortunate outcome is due to acquired resistance to the drug. Although multiple mechanisms have been identified that can account for this phenomenon in cell lines, including amplification of the BCR-ABL gene and overexpression of the multidrug resistance P-glycoprotein,\textsuperscript{6} it is becoming increasingly clear that the major cause of resistance in patients is the selection of sub-clones that express mutant forms of Bcr-Abl. A large and growing number of point mutations within the Bcr-Abl kinase domain have been identified that allow cells to evade the cytotoxic effects of imatinib. The mutant Bcr-Abl oncoprotein expressed by these cells remains kinase active even in the presence of therapeutic doses of the drug. Recently, a subset of mutations in the ATP binding site ('P-loop') of the kinase domain has been shown to be associated with a significantly worse prognosis than mutations elsewhere in the domain.\textsuperscript{7} This ominous development has highlighted the value of molecular monitoring in identifying patients who are most likely to undergo disease progression while being treated with imatinib. There are good reasons to suppose that imatinib mesylate will probably be the first of many small molecule inhibitors of tyrosine kinases to emerge as candidate therapeutic agents. A second generation of compounds, based on a core pyrido-[2,3-d]pyrimidine structure, has already been synthesised. Like imatinib, these inhibitors act by occupying the ATP-binding pocket of tyrosine kinase domains (Figure 1). Although they were originally identified as inhibitors of Src-family kinases, members of this group were found to be even more potent inhibitors of Bcr-Abl \textit{in vitro} than imatinib. Moreover, the pyrido-[2,3-d]pyrimidines bind to the
regulators, S6K1 and 4E-BP1, from being phosphorylated. This kinase preventing its downstream substrates, the translational mTOR, the mammalian target of rapamycin. Rapamycin inhibits maybe inhibited with compounds such as wortmannin and LY294002. These are phosphorylated by Bcr-Abl leading to its activation. PI3K signalling via this mitogenic cascade. (7) Phosphatidylinositol-3 kinase (PI3K) pathway is activated by Bcr-Abl. Mek inhibitors reduce signalling (6) Mek-1/2 (MAPK or ERK Kinases). The Ras-Raf-Mek-Erk pathway, zoledronate, prevents both farnesylation and geranylgeranylation. Farnesyl pyrophosphate and farnesyl pyrophosphate. By inhibiting this pathway, zoledronate, prevents both farnesylation and geranylgeranylation (catalysed by the enzyme geranylgeranyl transferase (GGT)) of Ras. The Ras-Raf-Mek-Erk pathway is activated by Bcr-Abl. Mek inhibitors reduce signalling via this mitogenic cascade. (7) Phosphatidylinositol-3 kinase (PI3K) is phosphorylated by Bcr-Abl leading to its activation. PI3K signalling may be inhibited with compounds such as wortmannin and LY294002. (8) mTOR, the mammalian target of rapamycin. Rapamycin inhibits this kinase preventing its downstream substrates, the translational regulators, S6K1 and 4E-BP1, from being phosphorylated.

Figure 1 Molecular targets that afford opportunities for ‘switching off’ the oncogenic signal that originates with Bcr-Abl. Each target is marked with a ringed number. (1) The ATP-binding site of the tyrosine kinase domain of Bcr-Abl. The tyrosine kinase activity of Bcr-Abl may be inhibited by small molecule inhibitors, such as imatinib mesylate and pyrido-[2,3-d]pyrimidine compounds. (2) The substrate binding site of the tyrosine kinase domain of Bcr-Abl. This represents an alternative target for inhibiting the tyrosine kinase activity of Bcr-Abl by means of compounds, such as adaphostin, that compete with protein substrates for occupancy of this site. (3) BCR-ABL mRNA. Translation of Bcr-Abl may be transiently ‘silenced’ using double-stranded siRNA. (4) Farnesyl transferase (FT). Inhibitors of FT, such as SCH66336 and tipifarnib, suppress Ras signalling by preventing the attachment of an isoprenoid farnesyl group to Ras. Farnesyl groups are essential for the normal functioning of Ras since they anchor these G-proteins to the plasma membrane. (5) The mevalonate pathway responsible for generating intracellular pools of geranylgeranyl pyrophosphate and farnesyl pyrophosphate. By inhibiting this pathway, zoledronate, prevents both farnesylation and geranylgeranylation. (6) Mek-1/2 (MAPK or ERK Kinases). The Ras-Raf-Mek-Erk pathway is activated by Bcr-Abl. Mek inhibitors reduce signalling via this mitogenic cascade. (7) Phosphatidylinositol-3 kinase (PI3K) is phosphorylated by Bcr-Abl leading to its activation. PI3K signalling may be inhibited with compounds such as wortmannin and LY294002. (8) mTOR, the mammalian target of rapamycin. Rapamycin inhibits this kinase preventing its downstream substrates, the translational regulators, S6K1 and 4E-BP1, from being phosphorylated.

ATP-binding site of Bcr-Abl in a different manner to imatinib which may prove to have therapeutic value. A looped region of this binding pocket controls the activation state of the kinase by adopting ‘open’ or ‘closed’ conformations depending upon the phosphorylation of a key tyrosine residue (Y393). When Y393 is phosphorylated, the activation loop assumes the open conformation that allows protein substrates to bind. The kinase becomes inactive upon dephosphorylation of Y393 when the activation loop folds into the closed conformation that makes the catalytic site inaccessible for substrate binding. Significantly, imatinib can only bind to and ‘capture’ this inactive, closed conformation of Bcr-Abl. In contrast, the pyrido-[2,3-d]pyrimidines bind to the ATP-binding pocket of Bcr-Abl irrespective of the conformation of its activation loop. One compound in this group, PD173955, has been co-crystallized with Abl protein in which the activation loop was folded into the open conformation.9 In addition, PD173955 antagonises the kinase activity of Abl regardless of its phosphorylation state. This indiscriminate quality may account for the superior potency of pyrido-[2,3-d]pyrimidines in inhibiting Bcr-Abl relative to imatinib and also why certain Bcr-Abl-resistant mutants, including the aforementioned P-loop mutants, are sensitive to these compounds.8 One particular Bcr-Abl kinase domain mutation, however, substitution of isoleucine for threonine at position 315 (T315I), confers resistance not only to imatinib but to as many as 13 pyrido-[2,3-d]pyrimidine compounds, all having different substituents.8,10 This mutation introduces a hydroxyl side chain that presumably sterically hinders both classes of inhibitor. Since pyrido-[2,3-d]pyrimidines also potently inhibit Src-family kinases, it is possible that this activity may contribute to their antileukaemic effect. The pyrido-[2,3-d]pyrimidines could potentially surpass imatinib as therapeutic agents for the treatment of CML if they are successfully developed in clinical grade for future trials.

Both imatinib mesylate and the new pyrido-[2,3-d]pyrimidines act by inhibiting the phosphorylation of protein substrates by the tyrosine kinase domain of Bcr-Abl. Protein substrates continue to associate with Bcr-Abl in the presence of these drugs, but their phosphorylation is not catalysed because ATP, the source of phosphate for the kinase, has been displaced from its binding pocket. An alternative means of ‘switching off’ the tyrosine kinase activity of Bcr-Abl is exemplified by the mode of action of a class of synthetic compounds known as tyrphostins. Rather than antagonising the binding of ATP, tyrphostins act by interfering with the binding of substrate molecules to Bcr-Abl’s tyrosine kinase domain (Figure 1). Adaphostin (NSC 680410),11 one of the most promising tyrphostins, has been shown to be selectively toxic for leukaemic cells, inhibiting formation of CML granulocytic colony-forming units (CFU-G) but having no effect on normal CFU-G. Consistent with its different mode of action within the tyrosine kinase domain of Bcr-Abl, adaphostin could also induce cell death in an imatinib-resistant cell line. Moreover, the combination of imatinib mesylate and adaphostin proved more cytotoxic than either agent alone, indicating that the pairing of these drugs may have therapeutic benefit. Like the pyrido-[2,3-d]pyrimidines, adaphostin has yet to undergo a clinical trial.

Inhibiting BCR-ABL gene expression

Several attempts were made in the past to ‘switch off’ or silence expression of the BCR-ABL oncogene with antisense oligonucleotides (reviewed in Barnes and Melo12), but this approach has failed to deliver a viable molecular therapy. Recently, however, the newer silencing technology of RNA interference (RNAi) has been tried as a means of inhibiting expression of BCR-ABL13,14 (Figure 1). In one study,13 BCR-ABL-transfected cell lines and primary cells from CML patients were transfected with siRNAs directed against the e14a2-fusion sequence of BCR-ABL. Bcr-Abl mRNA levels were reduced by up to 79% in these cells 24 h post-
transfection. A similar strategy was employed by a second group who found that Bcr-Abl protein expression could be reduced by up to 93.9% after two consecutive rounds of transfection. This latter report demonstrates one of the intrinsic problems of this approach, namely that siRNAs are transient and do not persist within cells. Since Bcr-Abl has a long half-life (>48 h) cells need to be repeatedly exposed to RNAi in order to significantly deplete them of this oncoprotein. It remains to be seen whether RNAi treatment for CML ever proves to be feasible. Even if the many practical problems can be overcome, its routine use as a molecular therapy is still a very distant prospect.

Arsenic trioxide has also been shown to downregulate Bcr-Abl levels by inhibiting its mRNA translation. Alternatively, expression of Bcr-Abl may be inhibited post-translationally through the use of chemical agents that destabilise the oncoprotein and promote its proteolysis. Examples of these are geldanamycin or 17-AAG, and histone deacetylase inhibitors such as SAHA and LAQ8244.

Targeting cell signalling pathways downstream of Bcr-Abl

Ras/MAPK signalling The Ras family of small guanine nucleotide-binding proteins (G-proteins) are key intracellular signalling molecules. Ras binding to Raf-1, a serine–threonine kinase, initiates the mitogen-activated protein kinase (MAPK) cascade (reviewed in Steelman et al.). The terminal kinases in this pathway activate transcription factors that promote the transcription of genes involved in cell division. In CML, Ras signalling is stimulated by Bcr-Abl via intermediate ‘adapter proteins’. Autophosphorylation of tyrosine 177 on Bcr-Abl creates a binding site for one of these adapter molecules, Grb2 (Figure 1). Bound Grb2 associates with a second adapter, the ‘son of sevenless’ (SoS) protein, to form a complex that activates Ras by functioning as a guanine nucleotide exchange factor (GEF). In addition, adapter proteins Shc and Crkl, both of which are known substrates of Bcr-Abl, are also capable of activating Ras. The combined effect of these adapters in Bcr-Abl-expressing cells is to subject Ras to abnormal and sustained activation.

Ras proteins transduce signals within an area of the cytosol immediately adjacent to the plasma membrane. In order to fulfil this function, molecules of Ras must undergo prenylation, the attachment of isoprenoid groups that serve to fasten them to the membrane. A key enzyme involved in this post-translational modification is farnesyl transferase that catalyses the covalent attachment of farnesyl groups to the C-terminus of Ras (Figure 1). Pharmaceutical companies have recognised farnesyl transferase as a prime target for molecular therapy and continue to screen compounds capable of inhibiting this enzyme. Their efforts to date have yielded chemical agents that are collectively known as farnesyl transferase inhibitors (FTIs). Two FTIs in particular, SCH66336 (Schering-Plough, Kenilworth, NJ, USA) and tipifarnib (formerly R115777; Johnson & Johnson Pharmaceutical Research & Development, Titusville, NJ, USA) have shown potential as antileukaemic agents.

SCH66336 selectively and potently inhibits the growth, in methylcellulose, of primary cells from CML patients. The growth of bone marrow cells from healthy individuals was only modestly inhibited by a dose of SCH66336 that was 10-fold higher than that required to completely inhibit the growth of the CML cells. In an in vivo murine model of blast crisis CML, the onset of acute leukaemia was delayed by SCH66336 treatment of syngeneic Balb/c mice injected with Bcr-Abl-transformed Ba/F3 cells. Furthermore, SCH6636 inhibited the proliferation of imatinib-resistant cell lines and reduced colony formation by primary cells obtained from CML patients who were unresponsive to imatinib. These latter findings, together with the observation that SCH66336 sensitises imatinib-resistant cells to imatinib-induced apoptosis, suggest that the combination of SCH66336 and imatinib may prove useful for treating patients who have developed resistance to monotherapy with imatinib.

The second FTI, tipifarnib, has been studied in a phase II clinical trial involving 22 patients with CML, eight with myelofibrosis and 10 with multiple myeloma. Tipifarnib was administered orally, at a dose of 600 mg twice daily for 4 weeks every 6 weeks. The drug exhibited modest activity, inducing complete or partial haematological responses in only seven (33%) of the CML patients. Four of these patients also had minor cytogenetic responses. Responses were transient, the median duration being 9 weeks. Of the seven patients who responded, six were in CP and a seventh was in accelerated phase. None of the six BC patients achieved a haematological remission. This last finding is in contrast to the results of an earlier phase I trial in which partial haematological responses were achieved by two of three CML BC patients. Favourable responses to tipifarnib were accompanied by a reduction in the plasma level of vascular endothelial growth factor (VEGF) indicating a possible role for this cytokine in either the pathogenesis or evolution of CML. Further studies will be required to determine whether the antileukaemic activity of tipifarnib is due to an inhibitory effect upon VEGF. Although both SCH66336 and tipifarnib have potential as antileukaemic agents, they suffer from a limitation common to all FTIs, which is that inhibition of farnesyl transferase fails to ensure that Ras will remain unprenylated. Ras can be prenylated, and hence attached to the plasma membrane where it assumes its functional state, via an alternative pathway that makes use of geranylgeranyl transferase-1 (Figure 1). Zoledronate, a heterocyclic imidazole developed as a third-generation bisphosphonate for the treatment of bone disorders, inhibits both farnesylation and geranylgeranylation by antagonizing the intracellular mevalonate pathway that generates geranylgeranyl pyrophosphate and farnesyl pyrophosphate. It was found to be a potent inhibitor of the growth of two human Ph+ leukaemia cell lines in vitro, and to prolong survival of nonobese diabetic/severe
combined immunodeficient (NOD/SCID) mice that had been injected with the BV173 CML line. Moreover, a synergistic effect upon survival could be demonstrated in mice that had been treated with both zoledronate and imatinib indicating that this combination of drugs may be efficacious in the treatment of CML. In our laboratory we have shown that zoledronate is active against imatinib-resistant CML cell lines and that the combination of zoledronate and imatinib has additive to synergistic effects. There is, however, uncertainty about the bioavailability of zoledronate and whether effective serum concentrations can be achieved in vivo. Against this must be set the high affinity of zoledronate for mineralized bone that tends to concentrate this compound in bone marrow. Based on these in vitro and in vivo findings, it has been recommended that zoledronate should be evaluated for activity against Ph+ leukaemia in a phase I clinical trial.

As described earlier, Ras signalling via the serine–threonine kinase, Raf-1, activates the MAPK pathway (Figure 1). The substrates of Raf-1 are the MAPK kinases, MEK-1/2 (MAPK or ERK Kinase). Three inhibitors of MEK, PD098059, PD184352 (Parke-Davis, Ann Arbor, MI, USA) and U0126 (DuPont, Merck, Wilmington, DE, USA), have been shown to exert an antiproliferative effect upon CML cell lines. In addition, the combination of PD184352 and imatinib was found to produce a synergistic cytotoxicity. It remains to be seen whether these results can be duplicated in an in vivo setting. Moreover, since these agents fail to discriminate between normal MAPK signalling and abnormal Bcr-Abl-mediated MAPK signalling, it is possible that cytotoxic effects may occur in normal cells. Further research will indicate whether MEK inhibitors have therapeutic potential.

PI3-kinase/AKT/mTOR signalling

Bcr-Abl activates phosphatidylinositol-3 kinase (PI3K), an enzyme that catalyses the phosphorylation of phosphoinositid lipids (reviewed in Steelman et al.17). Substrates of PI3K, such as AKT, activate other molecules in turn, to generate an intracellular signalling cascade. Two inhibitors of PI3K, wortmannin and LY294002 (Lilly, Indianapolis, IN, USA) have been shown to enhance the antileukaemic effect of imatinib against CML progenitors in in vitro colony-forming assays. However, the physical properties of these drugs make them unsuitable for clinical applications. Wortmannin is highly unstable in aqueous solution and LY294002, although possessing greater stability, lacks potency.

Although signalling via the PI3K/AKT cascade is known to be essential for Bcr-Abl oncoprotein.27 the downstream intermediates and terminal effectors of this pathway have remained elusive. A recent report demonstrated that the mammalian target of rapamycin (mTOR), one of AKTs substrates, is a potential target for the molecular therapy of CML (Figure 1). Inhibition of mTOR with rapamycin, led to enhancement of the imatinib-induced killing of a murine cell line transformed with Bcr-Abl. Furthermore, the combination of rapamycin and imatinib proved effective at inducing cell death in an imatinib-resistant form of this cell line in which the mechanism of resistance was overexpression of Bcr-Abl. The translation of target genes is regulated by mTOR via its immediate substrates, the translational regulators, S6K1 and 4E-BP1. It seems likely, therefore, that the ultimate effect of Bcr-Abl signalling via the PI3K/AKT pathway is pathological interference with the post-transcriptional regulation of essential genes.

Other pathways downstream of Bcr-Abl

Similar approaches have been described in pre-clinical studies for inhibitors of other pathways downstream of Bcr-Abl (reviewed in Tipping and Melo). These include an inhibitor of Janus kinase 2 (Jak-2), AG490, and the cyclin-dependent kinase inhibitor, flavopiridol. Both compounds were shown to synergize with imatinib, in in vitro studies, indicating that they may enhance its antileukaemic activity.

Conclusion

A successful molecular therapy for CML must ‘switch off’ or antagonize the oncogenic signal generated by the uncontrolled tyrosine kinase activity of Bcr-Abl. Because the Bcr-Abl oncoprotein is the only molecule that is unique to CML cells, it represents the ideal target for therapeutic intervention. Novel pharmacological agents continue to be developed and the rapid progress being made in this field is well illustrated by the example of the pyrido-[2,3-d]pyrimidine compounds. Moreover, the efficacy of existing treatments can be improved if a drug combination results in synergy. Concerted efforts are underway to identify compounds that undergo synergistic interactions when paired with each other.

References

FLT3 tyrosine kinase as a target in acute leukemias

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Over the last decade, there have been tremendous advances in the understanding of the events that cause human leukemias. More than 300 different chromosome translocations or other mutations have been identified, and are often associated with specific types of acute or chronic leukemias. Although there are many oncogenes associated with leukemias, they tend to fall into two large groups: those that primarily alter differentiation of normal hematopoietic stem cells, and those that promote viability and proliferation. By analyzing these mutated genes in animal models, a theme has emerged indicating that the development of acute leukemias generally involves cooperativity between at least one oncogene that blocks differentiation and at least one that enhances proliferation and viability. Currently, the latter category of oncogenes is of particular interest in the development of novel therapeutics, because many are activated tyrosine kinases or other signaling proteins such as p21RAS, which are amenable to inhibition by small molecule drugs. For example, BCR/ABL is associated with the immune system, as well as a reduction in myeloid progenitor cells. Targeted disruption of the FL gene in mice is associated with significant impairment of the immune response. Targeted disruption of the FL gene in mice is associated with significant impairment of the immune response. Targeted disruption of the FL gene in mice is associated with significant impairment of the immune response. Targeted disruption of the FL gene in mice is associated with significant impairment of the immune response. Targeted disruption of the FL gene in mice is associated with significant impairment of the immune response. Targeted disruption of the FL gene in mice is associated with significant impairment of the immune response. Targeted disruption of the FL gene in mice is associated with significant impairment of the immune response. Targeted disruption of the FL gene in mice is associated with significant impairment of the immune response. Targeted disruption of the FL gene in mice is associ...
FLT3, several studies have demonstrated biallelic mutations in FLT3, as well as patients in whom the residual wild-type allele is lost.

An additional group of AML patients contains mutations in the so-called activation loop of FLT3. The activation loop is a general component of tyrosine kinases and when the kinase is in the ‘inactive’ state, it functions to block access of ATP and substrate to the kinase domain. After activation, which would normally be through ligand binding in the case of RTKIII family members, a specific tyrosine residue within the loop is typically phosphorylated, causing the loop to adopt an ‘activated’ configuration allowing access to the kinase. D835 substitutions have been reported in 7% of AML, 3% of MDS, and 3% of ALL patients. D835Y is the most common substitution, but other substitutions included D835V, D835H, D835E, and D835N. Recently, mutations in other sites have also been reported. For example, we recently found N841 point mutations in three of 65 patients without FLT3-ITD mutations. Taken together, these data indicate that approximately 30–35% of AML patients have acquired mutations in FLT3 comprised of either FLT3-ITD mutations (24%) or FLT3-activating loop mutations (7–10%). FLT3 is thus the single most commonly mutated gene in AML.

Recently, the crystal structures of the FLT3 JM and kinase domains have been solved, revealing that the JM domain interacts with the kinase domain when the receptor is in an inactivated configuration. JM domain mutations in FLT3 may cause the JM domain to fall away from the kinase domain, facilitating kinase activation, transphosphorylation, and initiation of signaling. The available evidence indicates that either length mutations in the juxtamembrane domain, or activating loop mutations result in constitutive activation of the FLT3 kinase. Several groups have documented constitutive activation of FLT3-ITD, and activation of downstream targets known to be activated by the native FLT3 in response to FL, including the STAT5 and RAS/MAPK pathways. FLT3-ITD confers factor-independent growth to hematopoietic cell lines that are dependent on IL-3 for growth. Retroviral transduction of FLT3-ITD mutations into primary murine bone marrow cells results in a myeloproliferative phenotype in a bone marrow transplant (BMT) assay. Similar data have been obtained demonstrating that the D835 substitution mutations in FLT3 result in constitutive kinase activation. Thus, both JM length repeat and activating loop mutations result in constitutive activation of the FLT3 kinase and expression of FLT3-activating mutations in primary hematopoietic cells results in a myeloproliferative phenotype indicating below, that additional mutations are required for development of AML.

The majority of retrospective data indicate that FLT3 mutations are an independent variable that confer a poor prognosis in AML, particularly in older patients. In a large series of 854 AML patients treated on the United Kingdom Medical Research Council AML 10 and 12 Trials, 231/854 (27%) of patients were FLT3-ITD positive. FLT3-ITD correlated with higher leukocyte and blast counts, with decreased remission induction rates ($P=0.005$), and decreased disease-free survival (DFS), event-free survival (EFS) and overall survival (OS).

There has been intense focus on the development of FLT3 inhibitors because of the high frequency and poor prognosis of AML patients with mutant FLT3. Proof of principle for this strategy has been reported using cell culture and murine models of leukemia mediated by FLT3-ITD. There are several promising compounds that have recently been reported that are currently in Phase I or II clinical trials, including CEP-701, CT53518, SU5614, SU5416, SU11248, and PKC412. Each of these orally available compounds are potent FLT3 inhibitors. PKC412 will be discussed in more detail, as an example of this type of therapy.

FLT3 is a promising molecular target for therapy of AML. However, enthusiasm should be tempered by several considerations. First, it is likely that, as for CML in blast crisis, AML associated with activating mutations in FLT3 will have additional mutations that may not be sensitive to FLT3 inhibition. One might therefore predict a response rate to FLT3 inhibition in AML comparable to that of STI571 in CML blast crisis of approximately 30%. It is likely that additional agents will be required for effective therapy of AML.

Another open question for FLT3 is whether this mutation is present in all AML blasts in a patient or in a subclone. Nakano et al. followed FLT3 mutational status in 28 patients at diagnosis through relapse. Internal tandem duplications of the FLT3 gene (FLT3/ITD) were present at diagnosis and relapse in five patients, lost at relapse in one patient, and detected only at relapse in six patients. Similar results have been reported for patients with Asp835 mutations. Shih et al. analyzed Asp835 mutations in 120 patients at diagnosis and at relapse. Eight of the 13 patients with the mutation at diagnosis, lost the mutation at relapse, while five retained the original clone. Another six patients acquired the mutation only at relapse. These studies suggest that FLT3 mutations are frequently acquired as the underlying AML clone evolves.

Only a fraction of patients with AML have activating mutations of FLT3. It is not yet known what effect FLT3 inhibition might have on AML associated with overexpression of the wild-type FLT3 allele. None of the inhibitors currently under investigation is truly specific for FLT3. The toxicities of these drugs will need to be carefully evaluated and may be due to inhibition of other targets not involved in the pathogenesis of AML. However, as has been suggested for STI571, the additional targets may in some cases prove beneficial. Thus, while a number of different inhibitors may be found to effectively inhibit FLT3 in vivo, therapeutic efficacy may vary considerably depending on the other targets. Lastly, we can expect resistance to develop to FLT3 inhibitors, as has been observed with STI571 therapy of CML blast crisis. In some cases, resistance may be due to point mutations near the ATP-binding site. It will be important to anticipate this problem, and to characterize molecular mechanisms of resistance to each inhibitor.
References


RBC CONGENITAL ANEMIA

Congenital dyserythropoietic anemias

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Congenital dyserythropoietic anemias (CDAs) constitute a heterogeneous group of rare inherited disorders, characterized by moderate to severe anemia due to a markedly ineffective erythropoiesis with an erythroblastic bone-marrow and specific erythroid morphological anomalies.1 Three main types have been described: although CDA1 and CDAII are rare diseases, reported cases exceed several hundreds of individuals. CDAIII concerns mostly three large families, one in Sweden, one in the US, and one in Argentina. Other types, most of which are still ill-defined, have been reported as individual cases or isolated families. Most CDAs are associated with mild or moderate peripheral hemolysis. Biochemical as well as clinical signs such as icterus, cholelithiasis and iron overload stem from both the destruction of hemoglobinized erythroblasts in the bone marrow and peripheral hemolysis. Splenomegaly is a common feature that can lead to diagnosis. In spite of recent genetic progress, the mechanisms leading to the premature death of erythroid cells is still unknown. A better insight should shed light on disturbed and normal erythropoiesis and improve patients management.

Congenital dyserythropoietic anemia type I

CDA type 1, an autosomal recessive disease, is characterized by a chronic macrocytic anemia. Blood smears can show, beside macrocytosis, elliptocytes, occasional dacryocytes and rare figures of fragmentation. Bone marrow smears exhibit a megaloblastoid erythroid hyperplasia and the presence of some nuclear chromatin strands joining pairs of nearly completely separated erythroblasts (Figure 1). This important morphological sign has to be cautiously searched for as it can concern only 1% of erythroblasts or less. It has to be distinguished from cytoplasmic strands that are devoid of any diagnostic value. About 5% of erythroblasts are binucleated and a few cells may be trinucleated or tetranucleated. Specific ultrastructural abnormalities have led to an undefeatable diagnosis criterion: the heterochromatin of about 60% of intermediate and late erythroblasts has a spongy appearance with extensive disruption of the nuclear membranes leading to drive cytoplasm and cytoplasmic organelles into the nucleus.1 In these patients, red cell deformability, as assessed by osmotic gradient ektacytometry, is normal in our experience. However, SDS-PAGE electrophoresis of erythrocyte membrane proteins reveals a small but significant reduction of protein 4.1, thought to be a secondary phenomenon. Yet, the decrease is always mild when compared to gels obtained from patients with hereditary elliptocytosis stemming primarily from a lack of protein 4.1.

Associated nonspecific congenital abnormalities are often present (in 8/12 patients in our experience) including mostly short stature, bone malformations predominantly of hands and feet, and deafness in some families.1,2 Age at diagnosis is highly variable from birth to adulthood, according to the phenotypic expression of the disease and to local diagnosis availability.

Neonatal manifestations can include anemia, jaundice and/or splenomegaly; these features are reported in at least 55% of patients in retrospective studies, even though the diagnosis can be delayed. Most patients belonging to an isolate of Bedouin families living in the Negev Desert, in the southern part of Israel, where the disease prevalence has been shown to be prominent, have been transfused during the first months of life.3–5 Recent advances in fetal medicine have led to rescue a severely anemic fetus by in utero transfusion and diagnosis of CDA1 was achieved after birth. In that particular case, the patient was severely affected with regular transfusion needs until appropriate therapy.6 It is most likely that some hydrops fetalis resulting in fetal death are due to undiagnosed CDA1. In addition, attention has been drawn on the potential association of neonatal persistent pulmonary hypertension and CDA1, probably by the bias of anemia-related left ventricular failure leading to high pulmonary artery pressure.7

The common hematological form of moderate intensity is usually discovered during childhood, adolescence or early adulthood (mean age at diagnosis in our
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Gene, could be identified. The gene, has 28 exons

Individuals.6,9 This fascinating connection between
ill-defined threshold, which probably varies with

Treatment is discontinued or prescribed under a yet

Delayed, when patients are treatment-dependent and will relapse

Spleen enlargement and iron overload. However,

In reticulocyte and hemoglobin values, and a decrease in

Hepatitis C has resulted, in all published cases, in a rise

Starting with the usual dose prescribed for chronic

Regular subcutaneous injections (twice or thrice weekly)

Conditions which can transiently enhance the severity of

Anemia or disrupt its tolerance.1 The coincidental

Finding of a rise in hemoglobin value in an adult patient

In early childhood or in case of pregnancy or of other

Transfused several times during their life, predominantly

Assessment of iron stores is mandatory, leading to iron

In the absence of numerous transfusions. A regular

Chelation as soon as necessary. Most patients have been

Experience is 6 years) when facing a moderate macro-
cytic anemia with hemoglobin values ranging around

90 g/l, or a splenomegaly of unknown origin, or the early

Onset of a cholelithiasis with biological evidence of increased hemolysis. The evolution of CDA1 is doomed

By iron overload that can occur with aging, even in

The absence of numerous transfusions. A regular

Assessment of iron stores is mandatory, leading to iron

Chelation as soon as necessary. Most patients have been

Transfused several times during their life, predominantly

In early childhood or in case of pregnancy or of other

Conditions which can transiently enhance the severity of

Anemia or disrupt its tolerance.1 The coincidental

Finding of a rise in hemoglobin value in an adult patient

Affected both by CDA1 and blood-borne hepatitis C has

Undoubtedly led to demonstrate the effectiveness of interferon-α in promoting erythropoiesis in CDA1.8

Regular subcutaneous injections (twice or thrice weekly)

Starting with the usual dose prescribed for chronic

Hepatitis C has resulted, in all published cases, in a rise

In reticulocyte and hemoglobin values, and a decrease in

Spleen enlargement and iron overload. However,

Patients are treatment-dependent and will relapse

After a delay of several weeks or months, when

Treatment is discontinued or prescribed under a yet

Ill-defined threshold, which probably varies with

Individuals.6,9 This fascinating connection between

Immunology and erythropoiesis has not generated

Intense curiosity and the mechanism of action of the

Restoration of effective erythropoiesis by interferon-α

Remains a matter of speculation. In any case

Morphological abnormalities of erythroid progenitors are

Persistent under treatment, although probably decreased

But precise quantitative assessment is uneasy) and one has to keep in mind the potential
deleterious effects of long-term interferon-α treatment.

A genomewide search, conducted with the use of the

DNA of 25 Bedouins affected by the disease and

Belonging to four large consanguineous families has

Led to localize the gene on chromosome 15q15–15.3,

Within a 7cM area9 and, some years later, the candidate
gene, could be identified. The gene, has 28 exons

Spanning 15 kb of genomic DNA and encodes a protein

Of 1226 amino acids, called codanin-1.11 A total of 12

different mutations in nine families have been identified. Affected

Patients may be homozygotes or compound

Heterozygotes. In two cases only one missense mutation

Has been evidenced and the other mutation was not

Identified. The mutations reported are diverse with no
clear genotype/phenotype correlation. The diversity of

The severity of the disease among homozygous Bedouins

Patients with CDA1 suggests that clinical expression can

Be modulated by modifier genes. The gene appears to be

Ubiquitously expressed and it has been suggested that
codanin-1 might be involved in nuclear integrity

By connecting the nuclear membrane and the micro-
tubules.

Congenital dyserythropoietic anemia type II

CDA II is an autosomal recessive disease. A noticeable

Number of cases have been described in Western Europe,

Mainly Italy and North Africa. A high number of

Affected individuals originate from south Italy, but a

Founder effect could not be definitely demonstrated.12

Anemia is of variable severity, jaundice and splenome-
galy are usually present. The age at presentation

Ranges in the International European Registry including

98 patients, from 1 month to 25 years (mean 5.2 years).

About 25% of affected individuals are anemic
during the neonatal period and 15% require one or

Several transfusions. However, the transfusion

Needs usually decrease during the first years of life

And only 5% will require transfusions during

Adulthood.13

Anemia is usually normocytic with a reticulocyte
count ranging from 50 to 150 G/l. In our experience,

Study of red cell deformability by osmotic gradient
ektacytometry yields a decrease in maximum deform-
ability index and a curve similar, although less

Pronounced, to those of patients affected with heredi-
tary spherocytosis. In many patients, spherocytes can be

Observed on blood smears and cell counters can detect a

Noticeable percentage of dense cells. For long, the gold

Standard for diagnosis has been the Ham test and the
disease was called HEMPAS (hemolytic erythroblastic
anemia multinuclearity associated with a positive
acidified-serum test).

However, it has been shown that CDAII is one of the

Congenital disorders involving N-glycosylation of pro-
teins14 and in our experience all patients exhibit defective
glycosylation of band-3 (AE1) on polyacrylamide gel
electrophoresis in the presence of sodium dodecylsulfate

(SDS-PAGE ) of red cell proteins (Figure 2). This

Reliable test is now a gold standard for the diagnosis.15

On bone marrow smears erythroid hyperplasia with bi-
or multinuclearity (about 10–30% of erythroblasts are

Binucleated) is the classical clue for the diagnosis.

( Figure 3). Electron microscopy shows a more or less

discontinuous double membrane running parallel to the

erthroblast cell membrane.1 A genomewide linkage

Analysis has mapped the yet unidentified gene to

Figure 1
chromosome 20q11.2.\textsuperscript{16} Long-term evolution allowed to observe, even in moderate forms without repeated transfusion, that splenomegaly, cholelithiasis and iron-overload are all secondary consequences of ineffective erythropoiesis and peripheral hemolysis.\textsuperscript{15} Coheritance with Gilbert’s syndrome can enhance hyperbilirubinemia and icterus.\textsuperscript{17} Interferon-\alpha is ineffective and there is no medical treatment for CDAII. Splenectomy can alleviate or suppress transfusion needs but does not prevent iron overload.\textsuperscript{15} Iron overload has to be managed on due time by appropriate regular chelation. In severe forms, geno-identical bone marrow (or cord blood) transplantation has to be considered.\textsuperscript{18}

Congenital anemia type III is the rarest form of well-defined dyserythropoietic anemias.\textsuperscript{1} The mode of inheritance is clearly autosomal dominant in the largest family reported where 37 cases descend from a couple born in Northern Sweden in the late 19th century.\textsuperscript{19} Two other families have been reported, one in USA and one in Argentina. Only sporadic cases have been reported in other countries. Anemia is mild or moderate with macrocytosis and basophilic stippling of the red cells. Surprisingly, the hemolysis associated with dyserythropoiesis appears to be intravascular with spontaneous hemosiderinuria and patients seem to be devoid of the risk of iron-overload. Light microscopy of bone marrow shows erythroid hyperplasia with bi- or multinucleated erythroblasts. Giant multinucleated erythroblasts with up to 12 nuclei of variable size are typical.\textsuperscript{19} With electron microscopy, various non specific dysplastic features are found. A propension for lymphoproliferative disorders such as monoclonal gammopathy, myeloma, or lymphoma has been suggested. Visual disturbance with macular degeneration and angiod streaks has been reported in several members of the Swedish family.\textsuperscript{20} The causative gene, yet unidentified, maps to an 11 cM interval within 15q21–q25.\textsuperscript{21}

Other forms of CDA are rare or still ill-defined. Occasional associations with other hematological disorders such as thalassemia trait or mild hereditary spherocytosis have sometimes to be taken into account. Group IV includes congenital severe anemia with megaloblastoid erythroid hyperplasia, but devoid of specific erythroid morphological anomalies. Inheritance is suggested to be recessive as there is a parental consanguinity in the only family reported. In CDA group V erythroid hyperplasia is grossly normoblastic without peripheral hemolysis, suggesting a destruction of erythroblasts of normal appearance or of early immature reticulocytes, before bone-marrow egress.\textsuperscript{1}

Attention has recently been drawn on the association of chronic recurrent multifocal osteomyelitis, an autosomal syndrome whose gene has been located on chromosome 18q21.3–18q22\textsuperscript{22} and a microcytic congenital dyserythropoietic anemia in consanguineous Arab families.\textsuperscript{23}

Dyserythropoiesis of unknown mechanism can be the initial manifestation of FAS receptor mutations resulting in auto-immunity and lymphoproliferation.\textsuperscript{24} Auto-immune hemolytic anemia is often associated but can occur only after a noticeable delay following the first manifestation of dyserythropoiesis. Deficiency of erythroid CD44 together with a unique blood cell phenotype has also been reported and is associated with dyserythropoiesis and peripheral hemolysis.\textsuperscript{25} Mitochondrial congenital disorders can lead to apparently isolated congenital dyserythropoiesis with in most cases vacuoles in erythroid precursor cells and ring sideroblasts.\textsuperscript{24} Specific assessment of respiratory chain disorders and/or molecular biology can identify the disease before other apparently unrelated degenerative manifestations are evidenced with time.\textsuperscript{26} Finally, acquired disorders of erythropoiesis such as myelodysplasia or syndactyly have also to be cautiously ruled out.\textsuperscript{27}
References


Vaso-occlusion in sickle cell anemia: role of interactions between blood cells and endothelium

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Introduction

Sickle cell anemia (SCA) is one of the most common inherited blood disorder. It has been defined as a molecular disease more than 50 years ago and since then has given rise to an enormous amount of investigations.¹ SCA is usually characterized by three major features, namely hemolytic anemia, painful vaso-occlusive crises (VOC) and susceptibility to infections. Other potentially life-threatening complications include splenic sequestration, acute chest syndrome, and stroke. Finally, evolution during adulthood, now commonly observed, is complicated by chronic organ damage. Despite a unique mutation in the beta-globin gene and a common basic mechanism based upon the polymerization of deoxyhemoglobin S (Hb-S) and vaso-occlusion by sickled red blood cells, its phenotypic expression is highly variable, and the occurrence of the major complications is unpredictable. Most particularly, events triggering vaso-occlusion still remain largely unknown. In the recent years, the discovery of an abnormal adhesion of sickle red blood cells to the vascular endothelium has provided important new insights. It is clear now that all the circulating cells as well as the endothelium are part of the phenomenon and that cell activation and inflammatory processes play a major role in the pathogenesis of the disease.

Basic pathophysiological mechanism of SCA

Understanding the pathophysiology of the disease has been a continuous challenge due to the progressive identification of the various mechanisms involved.¹ The disease results from the single base A→T mutation in the triplet encoding the sixth residue of the beta-globin chain, resulting in a valine for glutamic acid substitution and the abnormal Hb-S with an altered polarity. The hydrophobic valine tends to establish hydrophobic interactions with an adjacent hemoglobin molecule and reduced oxygen pressure leads to its polymerization in paracrystalline polymers. Subsequent formation of intracellular fibers results in the loss of deformability and possible distortion of the cells, the next step being an obstruction of the postcapillary microveinules by these rigid cells. This basic mechanism was first proposed in the early 1970 and thought to infer directly the pathophysiology.

Damage of the red cell membrane is a secondary effect, leading to dehydration and thus potentiating polymerization itself. Cell dehydration is associated with shedding of microvesicles and subsequent increase of the intracellular concentration of hemoglobin (MCHC). MCHC is also the result of a loss of K⁺ and a gain of Na⁺. Two channels, the Ca²⁺-activated K⁺ channel and the K–Cl cotransport, are the major determinants of this ion leakage.² Moreover, the unstable nature of the mutant hemoglobin causes it to denature and to produce oxidants. Different membrane or cytoskeleton proteins can be damaged in the process, and a shorter survival of the cell is a cumulative result.

Although this initial simple scheme is intellectually satisfactory, it cannot fully explain the pathophysiological events that lead to vaso-occlusion since it has been shown that sickling takes place only after a delay time, that is normally longer than the time required in the circulation to reoxygenate the blood.³ Thus, in the steady state of the disease, polymerization should be reversed before reaching fiber formation, precluding the occurrence of VOC. In the same time, it is clear that any event that would reduce the blood flow can maintain the red blood cells at low oxygen pressure for a longer time and thus could the triggering event initiating the vicious circle of vaso-occlusion.

The sickle red blood cell is an adhesive cell

The possible implication of adhesion events in slowing down blood flow and initiating VOC has been suggested since the 1980’s.⁴,⁵ It was confirmed by many experimental data during the following decade. For long though, different experimental approaches (static versus flow conditions) and different systems (various origins of the endothelial cells) were used, making a synthetic view difficult. The identification of the molecular partners involved both on the red blood cells and on the endothelial cells provided the final demonstration of the reality of this abnormal phenomenon in SCA. The group led by RP Hebbel, although not the
Figure 1 Schematic representation of the major systems involved in sickle red blood cells adhesion to the endothelium. Stress reticulocytes express proteins that are not normally present in mature circulating cells. VLA-4 interacts with VCAM-1 at the surface of an activated endothelial cell. CD36 interacts with another CD36 molecule on endothelial cells through thrombospondin (TSP) bridging. In large vessels, high molecular weight von Willebrand factor (HMW vWF) bridges a GP Ib/IIa-like molecule on the reticulocyte to a GP Ib-like molecule on the endothelium. Other systems involve the interaction between the BCAM/Lu antigen on the RBC and the subendothelial laminin as well as IgGs and clustered charges at the RBC surface.

It came as a surprise to realize that adhesion is actually mostly the fact of very young cells that emerge prematurely from the bone marrow as a consequence of the anemic stress, rather than of the older, dehydrated and more rigid cells. These stress reticulocytes still express proteins used for their docking in the marrow, and normally no longer found at the cell surface of circulating mature red cells (Figure 1). Among them, VLA-4 (very late antigen-4), also called integrin alpha4beta1, on the red cell, directly recognizes VCAM-1 (vascular cell adhesion molecule-1) as its partner on the activated endothelial cell.7 Actually, VLA-4 is the only known ligand of VCAM-1. CD36, also called GPIV (platelet glycoprotein IV), expressed on stress reticulocytes, was shown to bind another CD36 molecule at the endothelium surface through a thrombospondin bridge.8 These two high-affinity interactions under flow conditions are probably the most important ones. They are responsible for stress reticulocyte adhesion to the endothelium in postcapillary venules, the major site of vaso-occlusion in SCA. Some other proteins are also involved. For instance, partnership was documented between B-CAM/Lu on the red cell and the subendothelial laminin,9,10 pointing out the potential implication of endothelial damage in the pathogenesis of SCA. High molecular weight multimers of the von Willebrand factor (vWF) are probably involved as well.11 Adhesion of aged red blood cells to the endothelium may result from alterations of the cell membrane including negative charges clustering and IgG binding, but it is unlikely that this phenomenon plays a major role in initiating VOC. Still it is possible that all these adhesive processes act in a cooperative manner, or differentially depending upon the circumstances or the vascular territory. CD36, for instance, is expressed only on the endothelium of the microcirculation; on the contrary, vWF seems to play a role in large vessels. Thus, vaso-occlusion in the microcirculation seems to result from a two-step mechanism in which young cells, namely stress reticulocytes, first adhere strongly to an activated endothelium, thereby reducing blood flow and leading to the secondary low-affinity binding and trapping of sickled red blood cells.

All the circulating blood cells are involved as well as plasma proteins

In this multifactorial adhesion process, plasma proteins are involved. IgGs bind to the altered sickle red blood cells; thrombospondin forms the bridge between two CD36 molecules on the stress reticulocyte and on the endothelial cell, respectively. The implication of the von Willebrand factor has been mentioned above. In addition, other proteins of the clotting system are probably involved, including fibrinogen and fibronectin.

A role has also to be given to the other blood cells, including polymorphonuclear neutrophils, monocytes, and platelets.12-14 All these cells are activated in SCA. Activated platelets secrete thrombospondin, the role of which has been mentioned in the CD36-mediated adhesion of stress reticulocytes to the endothelium. In this context it is also interesting to mention that membrane alterations on the sickle red blood cell induce phospholipid changes and the appearance of a procoagulant activity. All these factors are responsible of a procoagulant status in patients with SCA that may contribute to the vaso-occlusive process.

A high neutrophil count is a good index of morbidity and even mortality in SCA. It is interesting to note that it rapidly decreases under hydroxyurea therapy the only drug to date that has been shown efficient in SCA, and that it is the parameter that is the best correlated with the observed clinical benefit. Activated neutrophils also adhere to the endothelium by interacting with selectins and ICAM-1. It is thus possible that increased neutrophil activation and adhesion to the endothelium in SCA participate to a decreased blood flow in the microcirculation and to the triggering of VOC.

A proadhesion endothelium is an activated endothelium

It is interesting to realize that VCAM-1, one of the major participant to the adhesion of stress reticulocytes to the endothelium is expressed at the cell surface only when the endothelium has been activated by pro-inflammatory cytokines or viruses. Actually, more and more data accumulate to suggest a major role of inflammation in the pathophysiology of SCA and the triggering of vaso-occlusion. IgG-mediated adhesion of altered sickle cells is also increased when the endothelium has been activated by the herpes simplex type 1 virus (HSV1). Indeed, HSV1 activation induces the
expression of the Fc receptor at the cell surface. Activated endothelial cells also secrete thrombospondin and thus might also contribute to the high thrombospondin plasma level observed in SCA patients, particularly during VOC.

The role of endothelial activation in SCA has initially been hypothesized from in vitro data. However, the more recent observation of activated circulating endothelial cells in SCA patients¹⁵ not only demonstrates endothelium damage but also now provides the opportunity to study the endothelium activation status in the various phases of the disease. Activated circulating endothelial cells express tissue factor and thus contribute to the procoagulant status observed in SCA patients.

The endothelium also plays a crucial role in the control of the vascular tone by producing both endothelin-1 (Et-1), a potent vasoconstrictive peptide, and nitric oxide (NO), a powerful vasodilator. Clearly, vasoconstriction is another factor that may reduce blood flow and facilitate vaso-occlusion. Indeed, elevated levels of plasma Et-1 have been reported in SCA. Similarly, shear-stress-induced production of NO is increased, but plasma Et-1 have been reported in SCA. Similarly, shear-stress-induced production of NO is increased, but this is a futile production since the vessel does no longer respond to NO. The mechanism of this loss of shear-stress adaptation of vessel diameter in SCA patients remains obscure. Endothelial cell-produced NO is stoichiometrically scavenged by hemoglobin, this reaction being 1 000 times more rapid with a cell-free lyzant than with hemoglobin compartmentalized within erythrocytes. It has been hypothesized that chronic hemolysis could induce a permanent decompartmentalization and a dysregulation of the balance between NO production and its scavenging.¹⁶ The plasma of SCD patients has been shown to contain free ferrous hemoglobin and to consume increased quantities of NO, even in the steady state with a further increase during crises, and major systemic effects on the NO bioavailability. The presence of hemoglobin in plasma also correlates with an increased level of soluble VCAM-1, demonstrating that scavenging of NO may also interfere with the adhesion process.

**Therapeutic consequences**

It is clear now that cell adhesion to the endothelium and the endothelium itself play a major role in triggering vaso-occlusion in SCA. This opens new directions for the development of innovative therapeutic approaches in this disease for which to date only one drug, namely as mentioned above, hydroxyurea, has shown its clinical efficacy. It is also to be mentioned that the long-term effects of a life-long treatment with this S-phase-blocking agent are unknown. One could then imagine to block specific cell-cell interactions by using antibodies or RGD peptides for the interactions implicating integrins. Could it be possible also to modulate endothelium activation by using NF-κB inhibitors? Could NO therapy, or pharmacological modification of NO production be of any therapeutic values? These are open questions, some of which are already under investigation.

In this context, it is interesting to note that it has been shown recently that hydroxyurea might interfere with cell adhesion to the endothelium and abnormal regulation of the vascular tone in SCA. Hydroxyurea was initially administered to SCA patients with the hope of reinducing fetal hemoglobin (Hb-F) production, thereby diluting Hb-S within the cell and preventing deoxy-Hb-S polymerization. However, there is no correlation between the Hb-F response that is delayed and highly variable from patient to patient and the rapid and almost constant clinical benefit, at least in children.¹⁷ Hydroxyurea has been shown to decrease VLA-4 and to a lesser extent CD36 on the reticulocytes of patients treated with the drug.¹⁸ It has been shown also to modulate the expression of adhesion molecules by human endothelial cells in culture and to downregulate ET-1 production.¹⁹ Finally, hydroxyurea corrects the abnormal activation of neutrophils in SCA patients.²⁰ Thus, hydroxyurea seems to be a multitarget drug with pleiotropic effects. Its effects on the processes described above, in correlation with its proven clinical efficacy, are encouraging in exploring ways to pharmacologically interfere with them.

**Conclusions**

Of course, SCA results from the production of the abnormal Hb-S; of course, in fine, polymerization of deoxy-Hb-S remains the central pathophysiological process that leads to hemolytic anemia and vaso-occlusion in SCA. Stress reticulocytes are found in other inherited hemolytic anemias, such as pyruvate kinase deficiency, without resulting in vaso-occlusion. Still, the recent data reported above have shed a new light on the events that contribute in the initiation of vaso-occlusion in SCA. Of high significance is the fact that some of the molecular interactions leading to cell adhesion to the endothelium may be prompted by intercurrent pathologies. During VOC, the observed changes do not affect the red blood cells but the endothelium, plasma and the other circulating cells. Clearly, cell activation in general, often in an inflammatory context should now be considered as a major determinant in the pathophysiology of vaso-occlusion in SCA.

**References**

3. Hofrichter J, Ross PD, Eaton WA. Kinetics and mechanism of deoxyhemoglobin S gelation: a new approach to...
12 Hofstra TC, Kalra VK, Meiselman HJ, Coates TD. Sickle erythrocytes adhere to polymorphonuclear neutrophils and activate the neutrophil respiratory burst. *Blood* 1996; 87: 4440–4447.
Molecular pathology of thalassemia intermedia

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Heterozygous thalassemia is characterized by a mild degree of anemia and pronounced microcytosis. There is no jaundice and the spleen is not usually palpable. Typically, Hb A2 is increased and Hb F is either normal or slightly increased. Homozygous or compound heterozygous forms of β-thalassemia on the other hand, if not transfused, are associated with severe life-threatening anemia, marked splenomegaly, icterus, growth retardation and gross skeletal deformities. Since 1940, it has become clear to clinicians that there is an intermediate severe form of thalassemia.¹ Thus, the term thalassemia intermedia was introduced to describe thalassemic patients with a hemoglobin level persistently below 90 g/l, splenomegaly, and a varying degree of hemolysis. It rapidly became evident that thalassemia intermedia has an extraordinarily diverse clinical spectrum with hemoglobin values varying between 60 and 90 g/l, some patients presenting skeletal deformities and growth retardation with a phenotype close to that of thalassemia major. However what clearly distinguishes these patients from those with Cooley’s disease is the fact that the anemia is not life threatening and consequently not transfusion dependent. This clinical heterogeneity of thalassemia intermedia anticipated its complex molecular pathology. In this chapter, we will review the molecular pathology of thalassemia intermedia in the light of four cases studied recently in our hematology clinic.

Case 1

This infant of Portuguese origin has been anemic almost from birth, and first consulted us at the age of 10 years. Clinically, he presented a growth but not mental retardation. He was pale and icteric and there was marked splenomegaly in the left abdomen. Hb was 85 g/l, MCV 54.5 fl, MCHC 311 g/l, WBC 10.8 × 10⁹/l with normal differential, platelets 164 × 10⁹/l. Serum iron was 21.8 μmol/l, serum ferritin 96 μg/l. IEF of hemoglobin revealed no abnormal Hb. Hb A2 and Hb F measured by HPLC were 6.6 and 9.5% respectively. Bilirubin and LDH were increased while haptoglobin was decreased. Direct Coomb’s test was negative. DNA analysis of the alpha globin cluster by Southern blotting excluded deletional alpha thalassemia as well as alpha globin gene triplication/quadruplication. Diagnosis of thalassemia intermedia was established. Family studies showed that the father was a beta thalassemia carrier (Hb 129 g/l, MCV 63 fl, Hb A2 5%, Hb F 0.4%). Sequencing of his beta globin gene revealed the IVS I nt 1 G→A mutation creating β⁰ thalassemia. The mother, however, had a normal hemogram: Hb 146 g/l, MCV 86.6 fl, MCHC 337 g/l. Hb A2 was at the upper limit (3.6%) and Hb F slightly increased (1.6%). Keeping in mind beta thalassemia silent carrier as a possible diagnosis, the whole beta globin gene, the promoter region, and the 5'UTR and 3'UTR were sequenced. Results showed the presence of the relatively common mutation of the distal CACCC box at position −101 (C→T). Sequencing of the patient’s DNA revealed both paternal and maternal mutations (data not shown).

The identification of silent beta thalassemia alleles resulted from further analysis of one – at first sight hematologically normal – parent of thalassemia intermedia patients. These mutations are seen in the promoters, the 5' and 3' UTR, and in the IVS 2 region.²–⁴ Table 1 summarizes the mutations identified to date (April 2004). Generally, there is no anemia or microcytosis in carriers. However, as in our case, a slight increase of Hb A2 and Hb F may be seen. The combination of these mutations with β⁰ or severe β⁺ thalassemia alleles results in a clinical phenotype of thalassemia intermedia. A mild thalassemia intermedia phenotype may also be seen in patients homozygous for silent β-thalassemia alleles, or equally may result from the association of mild β-thalassemia mutations found at the same region of the beta globin as the β-silent ones.⁵ This group includes a number of mutations involving the poly(A) addition site⁶ (see Table 1).

The mechanisms by which these silent and mild beta thalassemia mutations decrease expression of β-globin gene are not always clear. Recent experimental data concerning mutations within 5'UTR indicate that they decrease mRNA levels by disrupting the function of the downstream promoter region,⁷,⁸ that is, the transcription initiator element, the NF-E2/AP-1 site and the E-box. The mechanism of action of +22 G→A is particularly interesting as this mutation creates a new ATG site 28 nucleotides before the normal initiation codon. It is possible that +22 ATG is utilized partially for translation and this leads to the mild β-thalassemia effect. Recently, a novel silent β-thassemia mutation was described in the −6 Kozak sequence (β⁷+45 G→C). In
vitro transcription/translation experiments demonstrated that this mutation decreased the translation efficiency of the beta globin chain by about 30%. This slight impairment is consistent with the observed clinical phenotype.

The mutations that occur in the distal and proximal CACCC boxes seem to downregulate globin-gene transcription by decreasing the binding of transcription factors. The termination codon +6 C→G mutation is common in Greece and was found to lead to a 20–34% reduction in mRNA. The precise mechanism of such an effect is not known.

To conclude about this group of mutations leading to beta thalassemia intermedia, we must add that counseling for couples where one partner has beta thalassemia minor is difficult because the hematologically normal partner may be a silent beta thalassemia carrier. Sequencing of the promoter region as well as the 5′ and 3′ UTR is thus mandatory.

Case 2

The patient, a young woman of mixed Brazilian-Portuguese origin, was initially referred to our service because of chronic fatigue. Clinical and laboratory examination revealed chronic hemolytic anemia and splenomegaly: Hb was 84 g/l, MCV 65 fl, MCHC 316 g/l, reticulocytes 462 × 10^9/l. Examination of the peripheral blood showed considerable anisopoikilocytosis with numerous erythrocytes showing basophilic stippling. Direct human antiglobulin test was negative. Hb A2 and Hb F were 3.6 and 5.1% respectively. Bone marrow showed marked erythroid hyperplasia; supravital staining with brilliant cresyl blue revealed large inclusions in the cytoplasm of about 3% erythroblasts. Sequencing of the whole beta globin gene, including promoter regions and the 5′ and 3′ UTR, showed a C→T mutation at codon 39 present in the heterozygous state. This mutation leads to a phenotype of β^0-thalassemia. Analysis of the α-globin clusters by Southern blotting showed the presence of pathologic fragments specific for the anti-4.2 α-globin gene quadruplication. Electron microscopy confirmed the presence of large electron-dense inclusions in erythroblasts from the bone marrow aspirate (Figure 1). Immunofluorescence staining of bone marrow cells showed that the Hb α-chain, but not the Hb β-chain, was specifically expressed in erythroblasts. Alpha/beta mRNA quantification experiments confirmed that the extra α-genes were expressed (Figure 2).

This case illustrates an additional mechanism leading to thalassemia intermedia: association of β-thalassemia
with a chromosome carrying more than the usual number of alpha-globin genes, very often triplicated, rarely quadruplicated z-globin-gene arrangement. In this form of thalassemia, the severity of anemia results more from the hemolysis due to alpha chain excess than from an important deficit of Hb A production. Thalassemia intermedia resulting from this interaction (triplicated/quadruplicated z-globin gene and heterozygous severe b-thalassemia) is thus characterized by: (a) pronounced abnormalities of erythrocytes in peripheral blood smears; (b) splenomegaly; (c) microcytic anemia of varying degrees with hemolytic features; (d) Hb A2 levels comparable to those seen in beta-thalassemia trait; (e) high level of Hb F but less than 10% (this feature is important for differential diagnosis between thalassemia intermedia secondary to double heterozygous b0/b+ [mild form] or b0/b+/-thalassemia silent state where Hb F is more than 10%). The differential diagnosis of this form of thalassemia intermedia (association of b-thalassemia with z-globin gene triplication/quadruplication) is made with that of thalassemia intermedia secondary to dominant beta thalassemias (see the next section).

Case 3

A 32-year-old Swiss female was diagnosed with chronic hemolytic anemia/thalassemia intermedia on the basis of Hb 9.5 g/dl, MCV 72 fl, reticulocytes 2.7%, total bilirubin 32 mol/l, HbA2 4.9%, HbF 3.8%. The spleen was enlarged (17 cm on ultrasound). Peripheral blood smear showed marked anisocytosis, poikilocytosis and numerous erythrocytes with basophilic stippling. Bone marrow was hypercellular with a marked increase of erythroid precursors (M:E ratio 1:1:2). Upon brilliant cresyl blue staining, numerous erythroblasts with large inclusion bodies were identified (data not shown).

Figure 3 shows sequencing data for the patient. From this, the presence of an undescribed 2bp deletion (−GA) at position 131/132 can be clearly observed, with the presence of double bands from the point of the mutation onwards as is characteristic of insertion or deletion cases. As a result, CD131 becomes CAA rather than CAG with subsequent frameshift, which theoretically should produce a transcript with nonsense codon at position 138, generating a truncated peptide of 137 aminoacids with an altered C-terminus from position 131 onwards. No abnormal protein was found upon isoelectric focusing of hemoglobin, HPLC or ionization mass spectrometry of globin chains. However, small quantities of protein may still be present, but further studies such as antibody binding or more advanced mass spectrometry would be needed to give a more definite answer.

This case shows that some b-thalassemia mutations are inherited in a dominant manner, that is, individuals carrying a single allele of this type, with no other associated Hb pathology, present a more severe hematological picture, including moderately severe anemia and splenomegaly. This phenotype has been included in the thalassemia intermedia group and was originally called ‘inclusion body b-thalassemia’. Where b-thalassemia intermedia is produced by simple heterozygous alleles, the physiopathology of the disease is thought to be somewhat different from that previously described (see cases 1 and 2). Most of the mutations of this type reported so far have occurred on exon-III of the b-globin gene; in some of these cases large intraerythroblastic inclusions have been seen. About 20 different b-globin exon III mutants have been identified so far, and interestingly not all of them are associated with b-thalassemia intermedia. This raises some important questions regarding the mechanisms leading to this form of the disease. Recent evidence suggests that mRNA and protein stability probably play an important role in determining the phenotype of the pathology.

Mutations such as the 121 (+A) and the 106 (+G) produce a less severe phenotype that seems to be more like a b-thalassemia trait. These two mutants together with the 131/132 mutant have a +1 frameshift and lead to the production of a 137 amino-acid truncated chain.
On the other hand, +2 frameshift mutations such as the 109 (−G),\(^{23}\) 123 (−A),\(^{16}\) 124 (−A)\(^{22}\) and 114 (−CT+G),\(^{15}\) produce an elongated fragment of 157 AA. All of these mutants have a more severe phenotype (higher percent of reticuloctyes, more severe anemia, and therapeutic splenectomy in older patients) which may be related to the elongated peptide itself, even though in the case of the CD114 there is very little mutant transcript available for translation. Moreover, an exon II mutation at position 94 (Hb Agnana)\(^{23}\) in which a +TG insertion is present also leads to the 157 AA peptide, and was observed to produce a severe type thalassemia intermedia. Another interesting mutation which was first described in 1973\(^{24}\) results from a complex rearrangement in which there are two deletions of 4bp and 11bp separated by a 5bp insertion. For this mutant variant, the β-globin chain would be expected to be of 153 AA and similar in its carboxyl end to the +2 frameshift peptides. Thalassemia in patients with this mutation is again of the severe type, similar to Hb Agnana and other mutants with an elongated chain (CD114, CD109, CD123, CD124), reinforcing the hypothesis that the nature of the peptide is a crucial factor in the molecular pathology of the disease.

Case 4

A 7-year-old patient of Burmese origin suffers from severe but transfusion-independent congenital microcytic anemia: Hb 54 g/l; MCV 70 fl; reticulocytes 96 g/l. He presented a growth retardation (weight 13.5kg; height 107cm at 6 years and 8 months of age), as well as hepatomegaly and splenomegaly (2cm below the costal margin). He had thalassemic facies and muscular atrophy. Total bilirubin was 30 µmol/l; ferritin 725 µg/l.

Bone age was found to be 3.5 years and a chest X-ray revealed cardiomegaly (cardiothoracic index 72%). L. Bone age was found to be 3.5 years and a chest X-ray revealed cardiomegaly (cardiothoracic index 72%). Hematological analysis shows that he expresses 2.1% Hb A2 and 97% HbF. Red blood cell morphology is shown in Figure 4a. HbF was found to be heterocellularly distributed among the RBCs (Figure 4b).

Gene mapping of the α-cluster showed the presence of four alpha globin genes (αz/αz) on chromosome 16. Gene mapping of the β-globin gene cluster showed an absence of deletions. Several common deletion types of δβ-thalassemia (Sicilian, Corfu, Laotian and Thai) were excluded using PCR analysis following a previously described method.\(^{25}\) The patient’s haplotype pattern was found to be similar to haplotype 3, termed ‘Senegal’ type (− + − + + + + −). Direct sequencing of the total β-globin gene revealed the presence of the IVS-1 nucleotide 1 G→T mutation in a homozygous state. This mutation completely abolishes normal splicing and provides the phenotype of β\(^{-}\)-thalassemia.\(^{26}\) Sequencing of the promoter regions of the duplicated γ-globin genes revealed the presence of the previously described common C→T substitution at position −158 of the Gγ-globin gene in a homozygous state. No other known mutations were found on the Gγ and Aγ promoter sequences. Sequence variations on the LCR 5’ HS-2 segment were analyzed to determine the presence of associated polymorphisms. The AT repetitive region at position −10623 to −10570 from the cap site of the e-gene read as follows: 9(AT)−A−2(CA)−2(TA)−CGT−10(AT), which is also characteristic of haplotype 3. Moreover, a C→T substitution was also identified in a homozygous state at position −10488, which has not been previously described. Similar AT repeats, (AT) × Ty have been localized in the 5’ flanking region of the beta globin gene and associated with regulation of gene expression on the beta globin gene cluster by binding to a nuclear protein BP1 known to act as a transcription silencer.

Direct sequencing of PCR amplified fragment was performed to characterize this β-globin promoter region from position −555 to −520bp to the cap site. The patient was found to present the common (AT)\(_n\)-T, configuration.

This young patient, homozygous for the β\(-\)-thalassemia IVS1-1 G→T mutation, was diagnosed with thalassemia intermedia because of the concomitant association of hereditary persistence of fetal hemoglobin (HPFH), heterocellular distribution type. Studies at the molecular level, in which mRNA ratios were measured, showed that high Hb F was mainly composed of G gamma. Studying the mechanisms underlying HPFH, we found that the patient had (a) the −158 C→T substitution (homozygous state) 5’ to the Gγ-gene (this mutation is known to increase HbF production, especially in the presence of extreme erythropoietic stress such as in our case of homozygote β\(-\)-thalassemia); (b) the haplotype 3/Senegal that is reported to have Hb F levels significantly higher than other haplotypes;\(^{27}\) (c) the typical purine-pyrimidine repetitive region of (AT)\(_9\)−N\(_{12}\)−(AT)\(_{10}\), previously observed in a β\(-\) chromosome with haplotype 3 and also associated with increased Hb F expression.\(^{28}\)
This case illustrates that certain forms of heterocellular HPFH may have the capacity to considerably ameliorate homozygous β^+ thalassemia, transforming the expected thalassemia major to thalassemia intermedia. The molecular basis of such cases is not fully known. Some family studies indicate that, in some patients at least, the determinant for HPFH is not linked to the β-globin gene cluster but to other chromosomes, for example a link to chromosome Xp22.2–p22.3 or chromosome 6q22.2–q23.1 is strongly suspected.^{29,30} Although there is enough evidence to implicate these interactions as an important cause of thalassemia intermedia, a full understanding of how the increased Hb F production is mediated will have to await the isolation of the genes involved.

Conclusions

In the light of four clinical cases of thalassemia intermedia, we have reviewed the main molecular interactions that can produce this condition. The described molecular pathology explains almost 88% of the cases of thalassemia intermedia, the association of 1 or 2 mild beta thalassemia alleles being the most frequent molecular mechanism (it accounts for more than 37% of cases), followed by dominantly inherited β-thalassemia (10%) and association of β-thalassemia with alpha globin triplication/quadruplication (12%). However, in approximately 12% of cases no known modulating factor and/or uncharacterized β-thalassemia alleles are found.^{5} Such cases represent an opportunity to discover new mechanisms leading to old diseases such as thalassemia intermedia. For example, Badens et al recently reported a case of thalassemia intermedia where the patient is constitutionally heterozygous for a β-thalassemia mutation inherited from his father. The authors beautifully demonstrated that, after conception, a second hit (deletion of the normal maternal allele in one of the early precursors of the hemopoietic lineage) led to somatic reduction, hemizygosity, and mosaicism of blood cells that have either one or no functional beta globin gene. This novel mechanism accounts for the thalassemia intermedia of the reported patient.^{31}

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References

ted globin chain \( \beta ^{Makabe}. \) Brit J Haematol 1990; 75: 393–399.


18 Hopmeier P, Krugluger W, Gu LH, Smetanina NS, Huisman THJ. A newly discovered frameshift at codons 120–121 (\(+A\)) of the \( \beta \) gene is not associated with a dominant form of \( \beta \)-thalassemia. Blood 1996; 87: 5393–5394.


22 \( \text{\c{C}u\text{"u}r{"u}k} \) MA, Molchanova TP, Postnikov YV, Pobedimskaya DD, Liang R, Baysal E *et al.* thalassemia alleles and unstable hemoglobin types among Russian pediatric patients. Am J Hematol 1994; 46: 329–332.

23 Ristaldi MS, Pirastu M, Murru S. A spontaneous mutation produced a novel elongated \( \beta ^{0} \) globin chain structural variant (Hb Agnana) with a thalassemia-like phenotype. Blood 1990; 75: 1378–1379.


28 Adekile AD, Dimovski AJ, Oner C, Lanclos KD, Huisman THJ. Haplotype-specific sequence variations in the locus control region (5' hypersensitive sites 2, 3, 4) of \( \beta S \) chromosomes. Hemoglobin 1993; 17: 474–478.

29 Dover GI, Smith KD, Chang YC, Purvis S, Mays A, Meyers DA *et al.* Fetal hemoglobin levels in sickle cell disease and normal individuals are partially controlled by an X-linked gene located at Xp22.2. Blood 1992; 80: 816–824.


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