Emergence of the haematopoietic system in the human embryo and foetus

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The first haematopoietic cells are observed in the third week of human development in the extraembryonic yolk sac. Recent observations have indicated that intraembryonic haematopoièse occurs first at one month when numerous clustered CD34+ Lin- haematopoietic cells have been identified in the ventral aspect of the aorta and vitelline artery. These emerging progenitors express transcription factors and growth factor receptors known to be acting at the earliest stages of haematopoièse, and display high proliferative potential in culture. Converging results obtained in animal embryos suggest that haematopoietic stem cells derived from the para-aortic mesoderm – in which presumptive endothelium and blood-forming activity could be detected as early as 3 weeks in the human embryo by differential expression of the CD34 and Flk-1/KDR genes – play an essential role in the foundation of definitive haematopoièse. Aorta-associated CD34+ cells also represent a unique localised accumulation of primitive haematopoietic stem cells worthy of in-depth molecular characterisation. Differential screening of a cDNA library has already revealed the expression of novel genes in this population, one of which appears to be involved in the development of both haematopoietic and endothelial cells. Active blood formation is observed in the liver and bone marrow by the end of the first trimester. Inception of haematopoièse occurs earlier in the liver, where CD34+ cells are detected as early as 30 days, than in the marrow, where haematopoietic cells are not observed before week 11.

Current interest in early human blood cell ontogeny may be partly related to the growing use of foetal stem cells for transplantation at postnatal stages, and to emerging cell and/or gene therapies of the blood system in utero, which justify a thorough characterisation of embryonic and foetal human haematopoièse. In addition, the prenatal haematopoietic system is characterised by an outstandingly high rate of progenitor cell expansion, migration and differentiation and hence can be seen as a privileged model to identify novel factors involved in these processes. In this setting, the recent identification in animal but also in human embryos of unique intraembryonic sites of haematopoietic stem cell emergence and proliferation could be of particular interest.

We shall briefly review here the successive steps of human haematopoietic development, emphasising the recent progresses made in our understanding of the origin and identity of human embryonic and fetal stem cells.

Primary haematopoièse in the human embryo and foetus

As is the case in other mammals, human haematopoièse starts outside the embryo, in the yolk sac, then proceeds transiently in the liver before getting stabilised until adult life in the bone marrow. Only T lymphocytes are produced in the same tissue at embryonic, foetal and postnatal stages.

The yolk sac

It is at about 18.5 days of development (early head process) that primitive haematopoietic cells appear inside forming blood vessels in the intermediate mesodermal layer of the human yolk sac wall. Studies on human haematopoietic cell emergence at these early stages are scarce, but our own observations suggest that the sequence described in animal models also applies to the human yolk sac: mesoderm-derived clusters of primitive haematopoietic stem cells – the blood islands – develop in close association with the endothelium of emerging blood vessels, possibly from a common ancestor cell or haemangioblast. The coexpression of the CD34 surface molecule by haematopoietic precursor cells and endothelial cells can be traced back to these initial stages, which may support the hypothesis of their common origin. Migliaccio et al. described several generations of clonogenic progenitors in the human yolk sac from 4.5 weeks of development, including pluripotential (CFU-GEMM), granulomonocytic (CFU-GM) and erythroblastic progenitors (BFU-E and CFU-E). The human yolk sac starts regressing at about 45-50 days post-ovulation and virtually all clonogenic progenitors have disappeared from that tissue by week six.

The liver

The liver emerges during the 4th week of development when the hepatic bud, an endodermal out-
growth of the foregut, invades the adjacent mesodermal septum transversum. These two tissues contribute hepatocyte cords and vascular sinuses, respectively. We have detected CD45+CD34+ haematopoietic cells from day 23 of development in the liver anlage while the first CD34+ haematopoietic progenitors could be recognised on day 30.1 In vitro colony-forming cells, i.e. BFU-E, CFU-GM and, slightly later, CFU-E have been indeed detected at 4.5-5 weeks in the liver rudiment, where their frequency then increases dramatically, paralleling their sharp decline in the yolk sac.3 At the end of the first trimester, and onwards, more primitive progenitors - CFU-GEMM and HPP-CFC - have also been detected in the liver. Earlier studies, confirmed by more recent immunohistochemical approaches, have documented the extensive erythro-myeloid haematopoiesis that takes place extravascularly in the human embryonic and foetal liver, and have stressed the prominence of erythropoiesis therein (reviewed in ref. #4). Other myeloid cells present in the haematopoietic liver are granulocytes, macrophages and rare megakaryocytes. B-lymphopoiesis has been traced in the liver from about 9 weeks of gestation by detection of surface IgM+ cells.

The bone marrow

A cartilaginous presumptive skeleton is present in the 6-8-week human embryo. Bone rudiments are then surrounded by a dense network of CD34+ capillaries, by CD68+ monocytes and by osteoblast precursors which all invade the diaphyseal cartilage at 8.5-9 weeks. Incoming macrophages rapidly digest the cartilage, leaving only intact small islets of chondrocytes that soon become surrounded by osteoblasts, from which ossification proceeds in a typically endochondral manner. In-between ossifying trabeculae, large vascular sinuses develop leading to the completion, at about 10 weeks, of bone marrow cavities.3 Marrow haematopoiesis starts during the 11th week of development in specialised mesodermal structures or primary logettes, constituted by a loose network of mesenchymal cells supported by dense fibrillar material and surrounding a central artery, inside which CD15+ granulocytes appear first, closely followed by erythroid cells. Haematopoiesis then develops dramatically in rapidly enlarging logettes which by week 15 are densely packed with cells of the erythroid and granulocytic series.3

Haematopoietic stem cell emergence in early human development

As mentioned above, the emergence of the CD34 cell surface antigen on ontogeny seems to be contemporary with the earliest commitment of mesodermal cells in the 3-week yolk sac to haematopoiesis and vasculogenesis. CD34 expression is then consistently detected on the surface of haematogenous cells in the liver and bone marrow.

Concepts on the filiation of the stem cells that found definitive haematopoesis have changed in the past few years with the demonstration that, in animals, these emerge inside the embryo, and not in the yolk sac as previously believed. In mice and birds the original blood-forming territory develops intrinsically in the para-aortic splanchnopleural mesoderm constituting the presumptive aorta- gonad-mesonephros (AGM) region of the embryo and contributes, at pre-stage stages, multilineage haematopoietic progenitors and eventually long-term reconstituting true haematopoietic stem cells. The existence in the human embryo of an equivalent site of haematopoietic stem cell generation has been suggested by the identification, at 4-6 weeks of development, of numerous clustered CD34+ haematopoietic cells on the ventral endothelium of the aorta and vitelline artery.1,6 These cells express surface antigens that typify early blood cell progenitors, being CD45+, CD34+, CD31+, CD43+, CD44+, CD164+; but display no CD38 or lineage-specific markers. In situ hybridisation on embryo sections and screening of cDNA libraries prepared from these sorted aorta-adherent progenitors have also revealed that they express genes known to be associated with the early steps of haematopoietic development, such as Tal1/SCL, c-myb, GATA-2, GATA-3, fik-1/VEGFR2 and c-kit.1 When directly assayed in methylcellulose, human intraembryonic aorta-associated CD34+ cells exhibited negligible clonogenic potential. In contrast, following a 4-10-day co-culture on murine bone marrow stromal cells (M5-5 cell line), they generated about six times more progenitors, which yielded large multilineage colonies in methylcellulose, than the liver rudiment.6 Of note, the para-aortic splanchnopleura - but no other intraembryonic tissue - exhibited dramatic haematopoietic potential in culture as early as day 23 of development, i.e. several days before CD34+ stem cells can actually be identified on the aortic wall. This result, as well as provocative semi-thin section histology pictures, suggest that haematopoietic stem cells emerge from mesoderm in that territory, and not merely migrate there from another location.6

Differential screening of a cDNA library built from sorted embryonic aorta-associated CD34+ cells with probes prepared from embryonic liver and foetal bone marrow CD34+ stem cells is in progress. Several differentially expressed genes have already been found, one of which encodes a serine-threonine kinase which, interestingly, is co-expressed in all developing endothelial and haematopoietic stem cells.1 This argues for the existence of haemangioblasts, i.e. common progenitors for vascular and blood cells.

We have reported that KDR, the human homologue of VEGFR2/Flik1, is strongly expressed in the human embryo by endothelial cells but barely detectable in the first haematopoietic stem cells arising in the wall of the aorta.2 Conversely, the CD34 protein is detected from early stages at the surface of both cell types.
We took advantage of this differential expression pattern to trace the emergence of putative human haemangioblasts and their segregation into endothelial and haematopoietic lineages. A population of KDR+ CD34– mesoderm cells emerges in early-somatic human embryos, by the beginning of the 4th week of gestation. During blood vessel formation these KDR+ CD34– cells gradually co-express increasing levels of CD34. Simultaneously, in the yolk sac, the sole haematopoietic tissue at that stage, most haematopoietic progenitors exhibit a KDR– CD34+ phenotype. Remarkably, as development proceeds, a KDR+ CD34– compartment persists in the splanchnopleura until just prior to the emergence of aorta-associated haematopoietic cell clusters. This cell compartment may include the putative haemangioblastic precursor of human haematopoietic and endothelial lineages.8

Conclusions

The localised accumulations of haematopoietic stem cells observed along intra-embryonic artery walls and in the yolk sac in the third-fifth weeks of gestation probably reflect phases of progenitor cell emergence and amplification that do not occur any later in development. These unique, transient haematogenous territories are presently being actively studied: deciphering the molecular control of mesoderm commitment towards blood cell lineages is of prime interest for developmental biologists, while haematologists suspect that novel factors are to be identified at these early stages that could be used to manipulate the survival/renewal/proliferation of adult haematopoietic cells. Experiments to that end are based on in vitro cell or organ culture of the mouse or human yolk sac, para-aortic splanchnopleura and derived AGM tissues, the ability of which to drive the expansion and differentiation of co-cultured stem cells is tested in homospecific or xenogeneic combinations. Much emphasis is also being put on subtractive cloning of novel genes whose function can be tested in vitro and in living models of overexpression and inactivation such as mouse ES cells, transgenic mice and zebrafish.

A better understanding of the microenvironment of these different haematopoietic tissues is of theoretical and practical interest in order to unravel similarities with or differences from adult bone marrow stroma in terms of critical cells and mediators;6 progress in this domain should be obtained via the generation of immortalised stromal cell lines.

References

Characterisation and biology of normal human haematopoietic stem cells

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Haematopoiesis is a life-long process responsible for replenishing both haematopoietic progenitor cells and mature blood cells from a pool of pluripotent, long-term reconstituting haemopoietic stem cells (HSC). The daily turnover in a normal adult of approximately $10^{12}$ blood cells is tightly regulated, involving, in part, a complex interaction between soluble and membrane-bound stimulatory and inhibitory cytokines and their corresponding receptors. The molecular cloning of these haemopoietic growth factors and their receptors has been instrumental in the partial delineation of the pathways that lead from a single HSC to the various terminally differentiated cells in the haemopoietic system.

The HSC compartment is distributed through the separated bone marrow (BM) locations and HSC are known to traverse in low numbers via the peripheral blood (PB) between these compartments. The HSC compartment is heterogeneous in various aspects, possibly as a result of different mitotic histories of the individual HSC caused by stochastic mechanisms that regulate cycling activity in a predominantly quiescent HSC population. In analogy to the situation in the mouse, the human HSC compartment probably represents a hierarchy of primitive cells on the basis of decreasing ability to generate new HSC, decreasing pluripotentiality and proliferative potential, and increasing turnover rate. In a transplant setting this heterogeneity may be reflected in the different time periods that individual HSC clones contribute to long-term reconstitution of a conditioned host, irrespectively of whether this reconstitution includes all haemopoietic lineages, and whether these lineages can be detected at the same moment in time.\(^1\)

HSC assays

HSC cannot be detected in bone marrow smears because they occur with low frequencies and have no unique features that allows their enumeration. Depending on the HSC assay used, HSC frequencies in normal BM are estimated to be between $1 \times 10^4$ to $1 \times 10^6$ cells. The HSC compartment is typically characterised by its ability to self-replicate and to generate life-long blood cells of a multiplicity of lineages. In the laboratory, these properties are often projected on individual HSC and the assays used are tuned to reveal one or more of their abilities. From physical sorting of various haemopoietic grafts it has become apparent that HSC assays should at least test for multilineage cell production over extended periods of time (i.e. months rather than weeks), in vitro but preferentially in vivo. Preferentially such assays should be able to allow HSC frequency analysis as well as assessment of the proliferative ability of (individual) HSC subsets. Assays that allow such a read-out include (a) analysis of long-term stroma-supported haemopoiesis and generation of progenitors (long-term culture, LTC) and frequency analysis using miniaturised LTC’s in limiting dilution (cobblestone area forming cell or CAFC assay; LTC initiating cell or LTC-IC assay);\(^2\,3\) (b) assessment of human multi-lineage haemopoiesis in the bone marrow of immunodeficient mice, e.g. in NOD/SCID mice (Scid repopulating cell or SRC assay),\(^4\) or (c) in sheep, where the human cells are infused during the pre-immune foetal stage (human-foetal sheep model).\(^5\) The LTC, which is performed in flask cultures, gives an insight into the capacity of a graft to produce progenitor cells while it does not reveal how many HSC and progenitors contribute to it. The latter issue is highly relevant when differences are anticipated in the progenitor cell generating capacity of HSC e.g. on the basis of disease, chemotherapy or in vitro methods for physical or chemical purging or selection. As frequency analysis requires a limiting dilution set-up of the assay it will be evident that animal assays are unfit to enumerate HSC on a routine basis, but nevertheless, probably provide the investigator with a more relevant estimation of the total graft ability than do in vitro assays. Typical drawbacks of all these assays are that they take a long time to complete and are often tedious to quantify.

Extensive studies using physically sorted murine BM have allowed regression analysis on the applicability of the murine CAFC assay as an in vitro equivalent of assays for a series of HSC subsets in vivo. These studies have indicated that, both in vivo and in vitro, early-developing, short-lived clones are initiated by the transient repopulating spleen colony-forming cells (CFU-S day-12), whereas later developing and more permanent clones are descendants of more primitive, long-term repopulating HSC, that induce stable chi-
maerism for more than a year. The CAFC assay, therefore, seems fit to quantify HSC subsets with different repopulation potentials.

In order to facilitate more rapid enumeration of HSC and progenitor cells other methods have been and are being investigated, including colony formation and phenotypic analysis. Although stem cells may form (multi-lineage) colonies in vitro, they are greatly outnumbered by the more mature progenitor cells which therefore renders colony formation unsuitable for HSC enumeration in a graft. Determination of the replating efficiency of individual colonies may give more information on the proliferative ability of colony-forming cells, but, again requires a large time expenditure.

Data from many laboratory and clinical investigations indicate that almost all lymphohemopoietic stem cells and all their progenitor cells are contained in approximately 1% of human BM mononuclear cells that express the surface marker CD34. Because stem cells are a rare cell type in the CD34+ cell population, investigators have subdivided the CD34+ cell population in order to enrich stem cells further. The CD34+/ CD38- cell subset comprises less than 10% of human CD34+ adult BM cells (equivalent to < 0.1% of mononuclear cells), lacks lineage (i.e. it is Lin-) antigens, contains cells with in vitro replating and long-term haemopoietic culture capacity, and is predicted to be highly enriched for in vivo repopulating stem cells. BM-derived CD34+/CD38- cells also contain SRC and generate long-term, multilineage human haemopoiesis in the human-foetal sheep transplantation model, and human cells harvested from chimaeric foetal sheep BM have been shown to engraft secondary recipients successfully, providing evidence for the long-term repopulating potential of AC133+ cells.

Another interesting glycoprotein antigen is AC133, which is selectively expressed on CD34bright HSC and progenitor cells derived from human foetal liver and BM, and PB. AC133-selected cells, which also include some CD34- cells, engraft successfully in a foetal sheep transplantation model, and human cells harvested from chimaeric foetal sheep BM have been shown to engraft secondary recipients successfully, providing evidence for the long-term repopulating potential of AC133+ cells.

It should be noted that enumeration of HSC using phenotypic analysis may, or may not, generate similar data as those from in vitro or in vivo functional assays. Firstly, it would require great skill, superb probes and instrumentation to allow rare event detection for enumeration of HSC in unseparated grafts using the presently available technology. Thus, although we may find that the bulk of HSC activity is contained in a CD34+/CD38- fraction, not every CD34+/CD38- is necessarily a HSC. Moreover, some HSC that do not meet these criteria may be overlooked as was demonstrated by the recent finding that perhaps the most potent, long-term repopulating HSC do not express the CD34+ antigen but are Lin CD34-. A second issue with phenotypic analysis is that the markers that we use may be promiscuous, or may not relate to function following manipulation of HSC. Ex vivo HSC manipulation protocols, e.g. for expansion of HSC from umbilical cord blood cells (UCB), somatic gene transfer or tumour cell purging, include either stimulation with a variety of cytokines, or use of selection procedures, or both. It has been frequently observed that large numbers of CD34+ cells and progenitors can be generated ex vivo, however, without a substantial maintenance or increase of HSC as tested by functional assays (e.g. SRC, LTC-IC, CAFC). We have recently found over 102-fold expansion of the CD34+/CD38- cells in 11 week cultures of human umbilical cord blood CD34+ cells, whereas LTC-IC/CAFC were only modestly (1-100-fold) expanded and although SRC were initially expanded, no SRC activity was detected at 11 weeks of culture. While the freshly uncultured CD34+/CD38- cells were Lin+, it could be shown that most of the cultured and CD34+/CD38- cells, although still containing all CAFC activity, expressed lineage markers in that fraction. These data show that one should be extremely careful in extrapolating a function-phenotype relationship as defined in fresh specimen to other circumstances.

**HSC quiescence, chemosensitivity and radiosensitivity**

Virtually none of the stem cells from the mobilised peripheral blood (MPB) and BM is cycling. Indeed, LTC-IC activity is higher in CD34+ cells isolated in G0 than in those residing in G1, G2 phase HSC show longer persistence of CD34 expression in suspension culture than do G1 phase HSC, and maintain in vitro haemopoiesis for longer periods. The deep quiescence of most HSC explains why they are refractory to a single treatment with cycle-specific drugs (e.g. 5-fluouracil, cytosine arabinoside, hydroxyurea, vincristine), whereas repeated chemotherapy may lead to HSC activation and their recruitment into a drug-sensitive state. In contrast, some alkylating drugs, e.g. busulphan, preferentially kill the most primitive CAFC (week 6) while sparing most of the transiently repopulating CAFC (week 2). M ultiple rounds of chemotherapy may, therefore, not only result in decreased numbers of HSC in the BM and PB, but may also lead to loss of the ability of the individual HSC to produce progeny.

Ample evidence in the murine model has shown that the most primitive, long-term repopulating HSC and CAFC-week 6 have the lowest sensitivity for ionising radiation (gamma, X-ray and fission neutrons) and display unexpected high sub-lethal damage (SLD) repair. In contrast, transiently repopulating HSC, CAFC-week 2 and cells that form spleen colonies in irradiated recipients (CFU-S) are highly sensitive and lack SLD repair.

The regulated localisation, conservation, commitment and terminal differentiation of undifferentiated
HSC is believed to occur in niches or local area networks in BM stromal microenvironments. This results in preservation of the stem cell pool while permitting controlled cell proliferation and differentiation. The exact nature of such niches is only slowly emerging from many experiments. It is clear that complex interactions between stromal cells and haemopoietic stem and progenitor cells involve cell adhesion molecules, and extracellular matrix molecules that may bind and present elaborated cytokines and chemokines. Stromal cells display membrane bound cytokines (e.g. stem cell factor) and their receptors (e.g. c-kit), and specific heparan sulphate proteoglycans containing high 6-O-sulphation on the glucosamine residues. These interactions lead to specific docking of haemopoietic cells where they co-localise with regulatory molecules in an as yet insufficiently characterised context. Specific niches might exist that induce conservation and maintenance of primitive progenitors and other niches that promote proliferation and differentiation, depending on the specific cytokines and matrix components present within.

In vitro, primitive HSC require combined stimulation by multiple cytokines for growth, but some cytokines selectively promote viability rather than growth when acting individually. These cytokines, e.g. Interleukin-3 (IL-3), the ligands for c-kit (KL) andflt3 (FL), but especially Tpo, exert direct and selective viability-promoting effects on a small fraction of CD34+CD38–, but not CD34+CD38+, human bone marrow progenitor cells at the single-cell level. Tpo mRNA is expressed in many stromal cell cultures, while there is variable expression of KL and FL.

Mobilisation and homing of HSC

Although in the steady state low HSC numbers can be detected in the PB, numerous agents, varying from lipopolysaccharides to specific cytokines, are able to increase the numbers of HSC in the blood dramatically. The probable underlying drive of the haemo- poietic system is (a threatening) depletion of the body's HSC reserve due to infection, blood loss or treatment with antineoplastic agents. It is clinically useful that repeated infusions of some cytokines (e.g. G-CSF), in combination or not with chemotherapy, mobilise large numbers of quiescent HSC into the blood that can be harvested by leukapheresis and used for autologous or allogeneic transplantation. This method is an advantage for the stem cell donors who would otherwise have to undergo anaesthesia and repeated bone marrow punctures.

Following their transplantation, the HSC have to find the BM niches that guarantee their life-long regulated preservation and outgrowth according to the body's demands. Homing of transplanted HSCs in the recipient BM cavity is thus a critical step in the establishment of long term haemopoiesis after BM T, as only the cells that home to the marrow in a murine model are capable of reconstituting lethally irradiated secondary hosts long-term. Quiescent HSC, especially those in G0, have been demonstrated to show more effective seeding in the BM than do G1 or cycling HSC. What is more, using PKH26-labelled murine cells that were enriched for either transient or long-term in vivo repopulation suggestive evidence was found that even at 48 hours after transplant long-term repopulating cells are still quiescent in the bone marrow (BM). Short-term ex vivo cycle progression of HSC, in the absence of cell division, appears to reduce the seeding efficiency and long-term engraftment capacity of both human and murine HSC.

From murine studies it appears that only about 1 of 4 infused HSC homes to the total BM of a conditioned recipient, although other studies have suggested a 100 percent seeding efficiency. The homing of human CAFC in the NOD/SCID model is extremely low and less than 1 percent of infused human BM-derived HSC can be recovered 24 hrs after injection from the total recipient's BM (Van Hennik et al., unpublished results).

A wide variety of adhesion molecules and other ligands that mediate cell-to-matrix and cell-to-cell interactions have been implicated in HSC adherence to vascular endothelium in the BM and subsequent transmigration of haemopoietic progenitor cells across it. Specific HSC homing is probably a complex multistep process of rolling, crawling and nesting of the HSCs. There is evidence that a family of selectins (L, P and E) can mediate initial tethering, rolling and subsequent adhesion of HSC to endothelial cells. In fact, all of the different classes of adhesion molecules appear to play roles in anchoring HSCs within the BM or the promotion of differentiation. Intercellular adhesion molecule (ICAM-1) in the Ig family, very late antigen 4 (VLA-4), an integrin, L-selectin and CD44 are examples of such important molecules. Another important consideration is the functional activity of these adhesion receptors. The integrins can be activated by different cytokines, including GM-CSF, IL-3 and KL. The chemokine stromal cell-derived factor-1 (SDF-1), too, was found to be critical for bone marrow engraftment. SDF-1 binds to its receptor CXCR4, which is expressed on many cell types including some CD34+CD38– cells. SDF-1 attracts CD34+CXCR4+ HSCs and its important role in homing is illustrated by the absence of haemopoiesis in the bone marrow of mice that lack SDF-1 or do not express CXCR4. Recently, Peled et al. demonstrated that KL and IL-6 induce CXCR4 expression on human CD34+ cells. CXCR4 expression potentiates migration to SDF-1 and engraftment in primary and secondary transplanted NOD/SCID mice. Moreover, anti CXCR4 antibody completely abrogated stem cell engraftment in this model.

The use of HSC in clinics

Primitive HSC and progenitors from BM, MPB and recently also UCB are targets for high-dose chemotherapy or radiotherapy. With the discovery of the
currently known cytokines and chemokines and the improved definition of culture ingredients, liquid cultures of unmanipulated or physically sorted human BM, MPB, UBC and foetal liver have been and will be increasingly used in an attempt to expand repopulating HSC, purge malignant cells and permit somatic gene therapy. Other potential clinical applications of these ex vivo graft manipulations include T-cell depletion for allogeneic HSC cell transplantation, adoptive immunotherapy via T-lymphocytes that are grown and educated in culture, and haemopoietic support for haemopoietically compromised patients. Still more extensive study is required in these areas to overcome issues such as loss of repopulating stem cells due to manipulation, and inefficient gene transfer and expression in human HSC and their progeny.

References

New approaches for analysis of gene regulation in normal and leukaemic haematolymphopoietic progenitor/stem cells

While recent studies provided insight into gene regulation in normal and leukemic HPCs/HSCs, new approaches and model systems may be required to further our understanding in diverse key areas. Specifically, (i) HPCs have been stringently purified and extensively characterised, but isolation and phenotyping of HSCs is still unsatisfactory; (ii) novel methodology is required for analysis of gene regulation at single HPC/HSC level; (iii) delineation of negative regulatory mechanisms for HPCs/HSCs is still unsatisfactory: particularly, the role of apoptotic mechanisms in normal haematopoiesis is uncertain; (iv) finally, while leukaemogenetic models have been established in immortalised cell lines and transgenic animals, an in vivo system for leukaemic transformation of normal HPCs/HSCs is still unavailable. This report reviews recent advances addressing these aspects.

The KDR receptor allows isolation and characterisation of normal HSCs.

The haematolymphopoietic hierarchy is defined by functional assays. Pluripotent HSCs, endowed with extensive self-renewal capacity, are assayed in vivo on the basis of their capacity to repopulate the haematolymphopoietic system, i.e., to xenograft irradiated NOD-SCID mice and pre-immune sheep foetuses. The major hurdle in HSC studies has been the lack of a specific positive marker, comparable to CD34 for early haematopoietic precursors: this hampered purification, characterisation and utilisation of this extremely rare cell population. Indeed, HSCs are usually enriched in the CD34+CD38- fraction, which comprises only 0.1-0.2% repopulating HSCs in adult bone marrow (BM) and cord blood (CB). While HSC identification is still elusive, recent observations have suggested a role for vascular endothelial growth factor receptor 2 (VEGFR2, KDR in humans and flk-1 in mice) in murine embryonic haematoangiogenesis. Targeted gene disruption studies indicate that flk-1 is required for initiation of primitive/definitive haematolymphopoiesis and vasculogenesis; this suggests a role for flk1 in the generation of haemoangioblasts, i.e., hypothetical stem cells bipotent for haematolymphopoietic and endothelial lineages.

We observed that in human post-natal haematopoietic tissues [CB, BM, normal or mobilised peripheral blood (PB, MPB)] CD34+ cells comprise 0.1-0.5% of haematolymphopoietic tissues [CB, BM, normal or mobilised peripheral blood (PB, MPB)].CD34+KDR+ cells is 22% in BM by NOD-SCID mice and foetal sheep xenografts and pre-immune sheep foetuses.

CD34+KDR+ cells are enriched and highly expressed in the CD34+KDR- fraction. Based on limited dilution analysis, the HSC frequency in CD34+KDR+ cells is 22% in BM by NOD-SCID mouse assay and 25-42% in PB, CB by 12-wk LTC assay.

The latter enrichment values rises to 53-63% in LTC supplemented with VEGF, thus suggesting a functional role for the VEGF/KDR system in HSCs: the purification index rises yet further to >95% in the CD34+KDR+ cell fraction. Conversely, oligo-unipotent HPCs with no self-renewal capacity are restricted to and highly purified in the CD34+KDR- cell fraction. Based on limiting dilution analysis, the HSC frequency in CD34+KDR- cells is 22% in BM by NOD-SCID mouse assay and 25-42% in PB, CB by 12-wk LTC assay.

These results indicate that KDR is a functional marker defining pluripotent repopulating HSCs and distinguishing them from oligo-unipotent HPCs: these findings pave the way to characterisation and functional manipulation of HSCs/HSC subsets, as well as innovative approaches for HSC clinical utilisation.

Unicellular-unilineage erythropoietic cultures: molecular analysis of regulatory gene expression at sibling cell level

In vitro studies on haematopoietic control mechanisms have been hampered by the heterogeneity of the analysed cell populations, i.e., lack of lineage specificity and developmental stage homogeneity. We developed unicellular culture systems for unilineage differ-
Apoptosis revealed that several immature erythroblasts undergo regulation of erythropoiesis. Possible involvement of Fas and Fas ligand (FasL) in the thyroid cell production. We therefore studied the possible consequences of a decreased requirement for erythropoietin, which might deliver negative signals to neighbouring cells, as a consequence of an increased requirement for erythropoietin.

Apoptotic role of fas/fas ligand system

In the culture system reported here (i) the GF stimulus induces CB HPCs to proliferate and differentiate/mature exclusively along the erythroid lineage, (ii) this erythropoietic wave is characterized by <4% apoptotic cells, (iii) asymmetric divisions are virtually absent, i.e., nonapoptotic HPCs with no erythropoietic potential are forced into apoptosis; (iv) the system is cell division controlled, i.e., the number of divisions performed by each cell is monitored. Single-cell reverse transcriptase (RT)-PCR analysis was applied to this culture system to investigate gene expression of diverse receptors, markers of differentiation and transcription factors (EKLF, GATA-1, GATA-2, NF-E2, PU.1, SCL/Tal1) at discrete stages of erythropoietic development. Freshly isolated CD34+ cells expressed CD34, c-kit, PU.1 and GATA-2 but did not express CD36, erythropoietin receptor (EpOR), SCL/Tal1, EKLF, NF-E2 or GATA-1 and glycoporphin A (GPA). In early to intermediate stages of erythroid differentiation, we monitored the induction of CD36, Tal1, EKLF, NF-E2 and GATA-1, which preceded expression of EpOR. In late stages of erythroid maturation, GPA was upregulated, while CD34, c-kit, PU.1 and GATA-2 were barely or not detected.

In addition, competitive single-cell RT-PCR was used to assay CD34 mRNA transcripts in sibling CD34+CD38− cells differentiating in unilineage erythroid cultures: this analysis allowed quantification of the gradual downmodulation of CD34 mRNA from HPCs through their differentiating erythroid progeny. It is concluded that this novel culture system, coupled with controlled single-cell RT-PCR analysis, may eliminate the ambiguities intrinsic to molecular studies on heterogeneous populations of haematopoietic progenitors/precursors growing in culture, particularly in the initial stages of development.

Apoptotic role of fas/fas ligand system in the regulation of erythropoiesis

In the BM, erythropoiesis occurs in discrete anatomical units, the erythroblastic islands, consisting of one or two macrophages surrounded by one or more rings of erythroblasts at different maturation stages. The inner erythroblastic layers contain immature cells, whereas the more mature cells are at the periphery of the island. This spatial association of mature and immature erythroblasts may play an important role in erythropoiesis as homocellular cell-cell interaction seems to be required for erythroid cell growth and maturation. We speculated that maturating erythroblasts might deliver negative signals to neighbouring cells, as a consequence of a decreased requirement for erythroid cell production. We therefore studied the possible involvement of Fas and Fas ligand (FasL) in the regulation of erythropoiesis.

Immunohistochemistry of normal BM specimens revealed that several immature erythroblasts undergo apoptosis in vivo. Analysis of BM erythroblasts and purified HPCs undergoing unilineage erythroid differentiation4 showed that Fas is rapidly upregulated in early erythroblasts and expressed at high levels through terminal maturation. However, Fas crosslinking was effective only in less mature erythroblasts, particularly at basophilic level, where it induced apoptosis cross-linking by high levels of Epo. In contrast, FasL was selectively induced in late differentiating Fas-insensitive erythroblasts, mostly at the orthochromatic stage. FasL is functional in mature erythroblasts, as it was able to kill Fas-sensitive lymphoblast targets in a Fas-dependent manner. Consequently, FasL-bearing mature erythroblasts displayed a Fas-based cytotoxicity against immature erythroblasts which was abrogated by high levels of Epo.

These findings suggest the existence of a negative regulatory feed-back between mature and immature erythroid cells, whereby the former cell population might exert a cytotoxic effect on the latter one in the erythroblastic island. Hypothetically, this negative feedback operates at low Epo levels to moderate the erythropoietic rate; however, it is gradually inhibited at increasing Epo concentrations coupled with enhanced erythocyte production. Thus, the interaction of Fas and FasL may represent a major apoptotic mechanism for erythropoiesis, contributing to the regulation of red blood cell homeostasis.

A novel in vitro leukaemogenic model: PML-RARα expression in normal HPCs dictates the leukaemic phenotype

While the role of fusion proteins in acute myeloid leukaemia (AML) is well recognized, the leukaemic target cell and the cellular mechanisms generating the AML phenotype are largely unknown. To address this issue we have established a novel in vitro leukaemogenic model: highly purified human HPCs/HSCs are transduced with retroviral vectors carrying cDNAs of the fusion protein and the green fluorescent protein, purified to homogeneity and induced into multi- or unilineage differentiation by specific GF combinations.

Expression of PML/RARα fusion protein in human HPCs/HSCs dictates the acute promyelocytic leukaemia (APL) phenotype, largely via previously reported effects: (i) rapid induction of HPC/HSC differentiation to the promyelocytic stage: this is followed by maturation arrest, which is abolished by retinoic acid; (ii) reprogramming of HPC commitment to preferential granulopoietic differentiation, respectively of the HGF stimulus: transduction of single sibling HPCs formally demonstrated this effect; (iii) HPC protection from apoptosis induced by HGF deprivation. A PML/RARα mutated in the co-repressor N-CoR/histone deacetylase binding region7 lost these biological effects, showing that PML/RARα alters the early haematopoietic programme via N-CoR-dependent target gene repression mechanisms.
These observations identify the cellular mechanism underlying development of the APL phenotype, showing that the fusion protein directly dictates the specific lineage and differentiation stage of leukaemic cells.

Altogether, this model system allows us to analyse the proliferation/differentiation potential of transduced primary HPCs/HSCs starting from the earliest phases after oncogene transfer through differentiation/maturation along each haematopoietic pathway. The investigative potential of this approach is shown by the fact that it allowed reproduction, in primary HPC culture, of the PML/RARα effects detected so far in cell lines (i.e., maturation block, protection from apoptosis, sensitisation to RA differentiative stimulus), while unveiling novel biological actions of the fusion protein (i.e., HPC reprogramming and rapid differentiation to the promyelocytic stage). We believe that this model system will have a wide impact, in that it represents a novel experimental tool for studies aimed at recapitulating in vitro the genetic events leading to haematopoietic neoplasias.

References

Vaccination as a treatment for cancer is an attractive option, particularly in the setting of a low level of residual disease. However, the task of activating a defeated immune system to recognise and destroy persistent tumour cells is formidable. Haematological tumours are a major challenge since those cells have survived the full power of the immune system, possibly by inducing tolerance. Three developments, all arising from molecular biological technology, may allow us to overcome the anticipated obstacles. First, there has been a large expansion in our knowledge of candidate tumour antigens; second, we have a greater understanding of immune mechanisms; third, vaccine vehicles are being developed for delivery of tumour antigens via pathways able to activate the most effective immune attack.

Our focus is on tumours of B lymphocytes, which include a broad range of diseases, with various current treatment options. Low grade tumours are responsive to chemotherapy, but are likely to relapse and are usually incurable. Even tumours such as diffuse large cell lymphoma, which may be cured by standard chemotherapy, prove lethal in 60% of cases, and plasma cell tumours such as multiple myeloma have a poor outlook with current treatment. However, patients with B-cell tumours can often achieve remission, in some cases with transplant support, offering an opportunity to intervene with an immunotherapeutic approach. For vaccines it is of course necessary that remission allows recovery of immune capacity.

A widening range of potential tumour antigens is being identified on B-cell tumours, including viral antigens, mutated proto-oncogene products, oncofoetal antigens, sequences arising from chromosomal translocation events, mucins and idiotypes. Candidate antigens can be placed into various categories depending on how they are expressed by the tumour cell. One group of antigens is expressed as glycoproteins at the cell surface; a second group is presented as peptides bound to MHC class I or II molecules; and a third group consists of secreted antigens. Each category of tumour antigen will require an appropriate immune effector mechanism for successful attack, and we need to tailor our vaccines with this in mind.

We have investigated idiotypic determinants, which are expressed by the immunoglobulin of neoplastic B lymphocytes as defined clonal markers, with the private idiotypic determinants being tumour-specific. They arise from the processes of genetic recombination and somatic mutation which occur during normal B-cell maturation, and are largely preserved following neoplastic transformation. Idiotypic Ig can be a surface protein, as in most lymphomas, or a secreted protein, as in multiple myeloma. Development of vaccines which can specifically suppress both these categories of B-cell tumour would have relevance for other tumour antigens found in these cellular sites. Idiotypic protein vaccines have been found to induce protective immunity against B-cell lymphoma in several mouse models, largely mediated by anti-idiotypic antibody, and a small clinical trial in patients with low grade lymphoma is showing promising results. However, idiotypic protein is difficult to prepare on an individual patient basis, and antibody is not likely to be useful if there is a significant level of secreted protein. Both these problems could be solved by turning to DNA vaccines which are simple to construct, and which are known to activate T cell responses.

DNA vaccines

DNA plasmid vaccines consist of a backbone of bacterial DNA containing a cDNA sequence encoding the potential antigen. Transcription is usually driven by the powerful CMV promoter, and stimulation of the immune system occurs due to unmethylated CpG dinucleotide repeats within the backbone sequence. Vaccination with DNA containing genes from pathogenic organisms has been shown to be effective in inducing all arms of the immune response, and in protecting against infection. Injection is usually in a muscle or skin site, and a gene gun has been used to deliver DNA to intradermal sites. There is evidence for direct transfection of antigen-presenting cells (APCs) in skin, and possibly in muscle. However, delivery of antigen from a muscle site is probably mainly by secretion and uptake by APCs, or by cross-priming. Clinical trials of DNA vaccines against infectious diseases are in progress, with immune responses being induced.

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DNA vaccines against B-cell tumours

To construct a DNA vector for idiotypic vaccination, it is necessary to identify the VH and VL genes used to encode tumour Ig, and this can be achieved by PCR/cloning methods. We have chosen to assemble genes as single chain Fv (scFv), but it is also possible to construct vectors to express whole Ig, containing either human or mouse constant region sequences. It soon became clear that DNA vaccines containing scFv alone, or as homologous Ig, could not activate a significant anti-idiotypic immune response. The presence of human constant region sequences improved the antibody response to attached mouse V-region sequences, and inclusion of cytokine sequences or peptides could also increase responses. However, we obtained a dramatic improvement in anti-idiotypic responses by fusing a gene encoding a fragment derived from tetanus toxin to the 3' end of the scFv. Attachment of this non-toxic fragment (fragment C[FrC]) activated anti-idiotypic responses against a range of human scFv sequences in mice, where scFv alone had failed. Importantly, the scFv appeared to fold effectively in the scFv-FrC fusion protein. Promotion of immunity probably results from both increased recognition by APCs, and by a massive increase in T-cell help. This help is provided by T cells specific for FrC and, since gene fusion is an absolute requirement, this acts via a classical hapten-carrier mechanism.

To assess the generality of the approach for other tumour antigens, carcinoembryonic antigen (CEA), we replaced the scFv with a different tumour-associated antigen, also expressed at the cell surface. A similar promotional effect of fusion was seen with this antigen, with CEA sequence alone inducing poor antibody responses, whereas the CEA-FrC fusion gene induced very high levels of anti-CEA antibodies. This suggests that the same fusion format may be useful for many surface-expressed tumour antigens.

Figure 1. Induction of protective immunity against B-cell tumours by DNAscFv-FrC vaccines. Mice were vaccinated with DNAscFv-FrC or DNAscFv derived from either: A, a mouse lymphoma (A31); or B, a mouse myeloma (ST33), at days 0, 21 and 42. Anti-idiotypic antibody levels against the tumour Ig were measured, and mice were then challenged with tumour. In each case, anti-idiotypic antibodies and protection were induced by the DNAscFv-FrC fusion vaccine but not significantly by the DNAscFv alone.
DNA vaccines induce protective immunity against tumour

A DNA vaccine containing scFv-FrC fusion genes derived from a mouse lymphoma, A31, was injected into syngeneic mice and was found to induce anti-idiotypic antibody. Importantly, this vaccine protected mice against challenge with a malignant lymphoma (Figure 1). It appeared, therefore, that this design was effective as a vaccine against a cell surface tumour antigen. We then tested the same vaccine design against a mouse myeloma, 5T33. This myeloma is one of a series which show characteristics similar to human myeloma, including osteolytic lesions. The neoplastic plasma cells are completely surface Ig-negative, but secrete monoclonal Ig. Interestingly, the scFv-FrC fusion gene also induced protective immunity against this tumour (Figure 1). Although the vaccine-induced high levels of anti-idiotypic antibody, it was unlikely that antibody could mediate protection. This was confirmed by the fact that vaccination with idiotype protein/CFA, which induced comparable levels of anti-idiotypic antibody, completely failed to protect against tumour. We were unable to define a motif in the V-gene sequences suitable for binding to MHC class I, and it is therefore likely, but not yet proven, that CD4+ T cells are involved in protection.

Clinical trials of scFv-FrC DNA vaccine

A phase 1 clinical trial of idiotypic DNA vaccines containing only scFv has been carried out in 7 patients with end-stage low grade follicular centre lymphoma (FCL) largely to assess any toxicity. No ill effects were observed, and we have now been allowed to proceed with a second trial using DNA scFv-FrC fusion genes. The patients have FCL in first remission. We shall investigate escalating doses of DNA, and will measure antibodies against both tumour-derived scFv, and against FrC. This will provide information on the ability of patients to respond to both a tumour antigen and an exogenous pathogen-derived antigen, both delivered by DNA.

Summary

DNA vaccines against cancer have to activate an inadequate or damaged immune system in order to attack residual cancer cells. Although the potential problem of tolerance may be overcome by transplantation, provision of high levels of T-cell help is likely to be an important factor in stimulating effective immune pathways. The fusion gene approach appears to provide the required help, and offers a rational design for raising both antibody and T-cell mediated attack against lymphoma and myeloma, which express idiotypic antigen at the cell surface or as a secreted protein respectively. Intriguingly, preliminary data indicate that the fusion gene approach promotes antibody responses against a different cell surface tumour antigen. CEA. Strategies for using DNA vaccines to induce attack on processed peptides bound to MHC class I molecules are also being developed. We hope and anticipate that all categories of tumour antigen may be susceptible to this powerful new technology. The critical clinical requirement, however, will be to treat the presenting tumour with maintenance or restoration of immune capacity. We await results of the preliminary clinical trials with great interest.

References

Monoclonal antibodies in the treatment of non-Hodgkin's lymphoma patients

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During the last ten years, significant progress has been made in the treatment of non-Hodgkin's lymphoma (NHL) patients. The refinement of the classification of the lymphomas and the incorporation of prognostic indices in decision making have allowed identification of groups of patients who may be cured and others that need further research to find the correct therapeutic options. Therapeutic improvements came from the introduction of G-CSF allowing higher dose of curative drugs with less risk of severe infections, the development of high-dose therapy with autotransplantation, and the addition of interferon to multidrug chemotherapy regimens. Through these options, around half of the patients with de novo NHL may expect to be alive ten years later. However, the difficulty of overcoming tumour cell resistance to standard drugs, the toxicity of the newly developed regimens, and the ageing population of patients challenge us to develop less toxic but more effective treatments.

The idea of developing monoclonal antibodies (MoAb) against cancer cells, particularly lymphoma cells, appeared more than 20 years ago with the description of the different antigens found on cell membranes. Since the first attempt reported in 1980, the exponential increase in the progress in molecular biology and protein engineering has recently culminated in the approval, by drug agencies, of rituximab and, in a near future, of radioimmunoconjugates.

Antibodies mediate cell death through different mechanisms because of its own difference from the normal cells and increase the toxicity of the treatment. Unconjugated antibodies must remain on the cell surface to allow the Fc portion of the antibody to activate immunologic mechanisms. Antigen density and MoAb binding affinity may influence the cytotoxic efficacy for unconjugated antibodies but radioimmunoconjugates emit particles with enough energy to kill adjacent cells, cells with a low antigen density or non-antigen-bearing cells. However, they may kill vital normal cells and increase the toxicity of the treatment.

The MoAb must reach tumour cells in every parts of the organism and at all sites of the disease. This may be a problem in large tumours that are poorly vascularised. The presence of circulating antigens may be a problem leading to rapid clearance of the MoAb. The MoAb must not be eliminated through immunologic mechanisms because of its own difference from the host. When xenophobic Ab are used, rapid appearance of human anti-mouse antibodies (HAMA) may alter the pharmacokinetics of the MoAb, particularly during the re-treatment phases, and then further decrease their activity. Genetic engineering has allowed the humanisation of antibodies and the creation of chimeraic proteins with a small antigen-binding mouse part and a large human constant Fc region.
These chimaeric MoAbs have substantially lower immunogenicity and, thus, prolonged half-life. They also have an improved ability to mediate complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC), which increases their potency.

The MoAb may kill the lymphoma cells through a variety of different mechanisms. Radioimmunoconjugates or immunotoxins kill cells through the emission of particles or the internalisation of the toxin. Unconjugated MoAb may trigger CDC or ADCC. They may have direct cytotoxic effects on tumour cells, either on blocking the binding of an endogenous ligand, which deprives the cell of a critical survival signal, or by mimicking it, which triggers growth arrest. These functions may potentiate the effect of chemotherapy.

A large variety of antigens may potentially be chosen as targets. While the early trials focused on Ig idiotype, the CD20 antigen is probably the ideal target for B-cell lymphomas. It is not expressed on stem cells or precursor B-cells but is found on normal mature B-cells and malignant B-cells, with the exception of plasma cells and myeloma cells. It is usually present on all cells of the tumour clone. It is expressed in high density on all B-cell lymphoma but not chronic lymphocytic leukaemia cells. This antigen is stable in the membrane of B-cell, does not have any known variant, is not shed, and does not modulate or internalise in response to antibody binding. While its biologic function is not fully known, it appears to function as a calcium channel and it either forms a membrane pore or controls a pore that is involved in calcium transfer during the cell cycle. The majority of the molecule is within the membrane or inside the cell and there is a small loop of 40 amino acids outside the cell. All anti-CD20 appear to bind to the same section of this external loop except L26, which binds to an intracellular epitope of the molecule.

Unconjugated MoAbs

Unconjugated MoAbs constitute the simplest application of targeted MoAb treatment. Table 1 lists the different antigens that have been used. The first trials used MoAbs directed against the idiotype of the surface Ig of lymphoma cells which certainly represents a unique tumour-specific antigen. Most of these trials were conducted by Levy at Stanford.4 In different trials, anti-idiotype antibodies produced responses in 50% to 70% of the patients. Although the median duration of these responses was only 6 months, some patients with complete response had long remissions. However, patients relapsed with idiotype-negative cells. The presence of circulating shed idiotype and the formation of HAMA further limited the efficacy of unconjugated MoAbs. This, associated with the constraint of making anti-idiotype specific for each patient, precluded further development of this therapy.

Subsequently, investigators used pan-B antigens, such as CD20, and humanised antibodies. Rituximab (MabThera®) is the most extensively studied unconjugated MoAb to date. This chimeric antibody consists of the murine variable regions from the 2B8 MoAb grafted onto a human IgG1 constant region. In a phase I trial, the dose limiting toxicity was not reached which attests to a low side-effect profile of the drug.5,6 Most phase II trials used the dose of 375 mg/m² once a week for 4 weeks.7,8 These trials accrued predominantly patients with indolent follicular lymphoma (FL), refractory to standard chemotherapy regimens. In more than 200 patients, the response rate was around 50%, with 6% complete responses, and responses were observed in different subgroups with adverse prognostic features, such as previous autotransplant or bulky tumour. Many patients had no detectable residual tumour cells in blood or bone marrow, even as detected by PCR analysis for the t(14;18) translocation (molecular remission). The median time to response was about 2 months, with many patients showing progressive responses for several months. This may be correlated with the long half-life of the antibody, some patients having residual levels detectable 6 months after the last infusion. Median time to progression in responding patients was longer than 12 months. Interestingly, patients who progressed after a first response could be retreated and 50% of them responded. Several successive responses were observed in some patients. Because of these results drug agencies approved the indication of rituximab for relapsing FL patients.

Most adverse events were minor and associated with the first infusion. They consisted primarily of fever, chills, mild nausea, mild fatigue, or malaise. Rarely, patients developed more serious reactions, including hypotension, bronchospasm, or sensation of throat swelling. These symptoms were usually managed by temporarily slowing or stopping the antibody infusion. The most serious reactions were observed in patients with peripheral blood involvement or large tumours. These reactions could be prevented by stopping the

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infusion once mild adverse reactions occur. Myelo-suppression was rare. As expected, the normal B-lym-phocytes rapidly declined and recovered over 6 to 9 months. However, there was no increase in infection rate and no occurrence of opportunistic infections, probably because Ig levels and T-cells remained normal. HAMA was observed in less than 1% of the patients and HACA (anti-chimaeric antibody) was not observed.

Subsequently, rituximab was used in patients with more aggressive B-cell lymphoma, mantle cell lymphoma (MCL) and diffuse large B-cell lymphoma (DLCL). In a phase II trial, patients with relapsing disease showed a 32% response rate. In another phase II trial, mantle cell lymphoma patients, in first line therapy or relapsing, showed a 40% and 30% response rate, respectively. Most of the responses in these studies were incomplete, with less than 10% being complete responses, and median time-to-progression was less than 12 months. These results indicate that rituximab has an anti-tumour activity in nearly all B-cell lymphomas. Currently, it is being tested in chronic lymphocytic leukaemia, post-transplant lymphopro liferative diseases, and multiple myeloma. Although plasma cells do not express the CD20 antigen, there is some indication that the clonogenic precursors may.

Rituximab has been used in conjunction with chemotherapy in FL and DLCL patients, mostly with the CHOP regimen. In a small study of 40 patients a response rate of 95% was reached with 55% complete responses. At time of publication, 74% of the responding patients had not progressed with a median follow-up of 29 months. Seven of the 8 patients with bcl-2-rearrangement converted to PCR negativity after completion of the treatment. The adverse events were not different from those expected from the CHOP regimen or rituximab treatment. No specific toxicity was observed with the combination of rituximab and CHOP. Currently, it is being tested in chronic lymphocytic leukaemia, post-transplant lymphoproliferative diseases, and multiple myeloma. Although plasma cells do not express the CD20 antigen, there is some indication that the clonogenic precursors may.

Studies are only beginning or have been short lived with other MoAb and no or few further developments are expected with them.

**Immunotoxins**

An alternative approach to increase the activity of MoAb is the development of an immunotoxin, a construct conjugating the antibody to cytotoxic plant or bacterial toxic proteins. The commonly used toxins, ricin and diphtheria toxin, are highly potent natural products that disrupt protein synthesis. Unlike unconjugated MoAb, immunotoxins must be internalised after antigen binding to allow the toxin access to the cytosol. Although the conjugation to MoAb confers some target specificity, the toxin continues to mediate non-specific toxicity to normal tissues. Deglycosylated ricin A-chain has been used to eliminate such non-specific toxicity.

The vast majority of the immunotoxin trials have been phase I studies designed to determine the maximum tolerated dose (Table 2). These trials have shown that therapeutic serum levels may be achieved with tolerable toxicity. A relatively uniform toxicity has been observed with vascular leak syndrome, hepatotoxicity, and myalgia. A strong immunologic response against the construct or the toxin was observed and re-treatment was not feasible in most patients. The different trials have shown a low response rate of 10% to 25% partial responses without durable efficacy. The future of this therapy will depend on decreasing toxicity, decreasing immune response against the construct, and on increasing the anti-tumour activity.

**Radiolabelled antibodies**

These consist of a radionucleotide, usually 131iodine (131I) or 90yttrium (90Y) emitting particles, coupled to a MoAb (Table 3). These compounds may selectively deliver ionizing radiation to tumour cells. These agents seem to possess several advantages over other antibody constructs: they do not rely on recruitment of patients’ immune effector mechanisms and the particles are capable of killing cells from a distance of several cell diameters permitting the killing of antigen-negative tumour cells. Most studies of radio-

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<th>Antibodies</th>
<th>Toxins</th>
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targeted to HLA-DR, CD20, CD21, or CD22 anti-MoAb administered to the patients. Although $^{131}$I and $^{90}$Y infusion of non-radiolabelled (cold) MoAb is often used to maximise the doses distributed to normal tissues, a first dose may interfere with the distribution of radiation. To minimise tumour burden, particularly in the spleen, can make the doses to normal tissues. A first dose to ensure that the radiation doses delivered to normal tissues. Both compounds require an onsite radiopharmacy and dosimetry calculations that may also limit their application.

Radiolabelled MoAb therapy is associated with myelosuppression, toxicity not found with cold MoAb, although a considerable inter-patient variability has been observed. With low dose radiation therapy neutropenia and thrombocytopenia are observed 3 to 4 weeks after the infusion and may persist for 16 weeks but with higher doses these haematological effects are more rapid, profound, and prolonged. Extensive bone marrow involvement can lead to a greater binding and a larger radiation dose delivered to the normal haematopoietic cells. In addition the same adverse effects as those which are observed with cold MoAb treatment exist and the use of iodine conjugates can cause hypothyroidism. Murine MoAb both emit beta-particles, $^{90}$Y emits higher energy particles, which have a deeper tissue penetration, and $^{131}$I also emits radiation and has a longer half-life. These last characteristics may present safety concerns limiting its use to large centres with strict radiation isolation. Both compounds require an onsite radiopharmacy and dosimetry calculations that may also limit their application.

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The trials with non-myeloablative doses have used $^{131}$I targeted to HLA-DR, CD20, CD21, or CD22 antigens and have achieved responses in 5% to 80% of treated patients. Preliminary studies with the $^{131}$I-labelled Lym1 antibody in refractory patients yielded a 50% response rate. The $^{131}$I-labelled anti-CD20 antibody (Bexxar®) produced durable CR in patients with recurrent FL. Patients received an unlabelled dose of the antibody followed by a trace-imaging dose labelled with $^{131}$I. One or two weeks later, the patient received an additional dose of unlabelled antibody followed by a therapeutic dose of $^{131}$I calculated to produce 75 cGy to the whole body. A 60% response rate was observed, with 27% CR, and a median duration of CR longer than 1 year. In de novo patients, a higher response rate was reported. Longer follow-up in more patients is necessary to determine whether this approach will result in long-term disease control. In phase I-II trials, $^{90}$Y-labelled 2B8 antibody administered after a rituximab infusion yielded a 82% response rate. Larger numbers of patients are needed to determine this antibody’s place.

Even greater response rates have been reported in studies that used myeloablative doses of $^{[13]}$-labelled anti-CD20 antibody. Patients received a therapeutic infusion of the antibody, were isolated, and 10-12 days later were given their cryopreserved stem cells. Toxicity of this approach included severe infections and cardiomyopathy. However, 70% to 80% of the CR patients remained disease-free 18 months later. Longer follow-up and randomised study will determine whether this approach confers survival advantage over conventional high-dose regimens.

Conclusions

In conclusion, impressive responses have been documented with MoAb therapy, even if their role in the treatment of lymphoma remains to be determined. Although the unconjugated MoAb, and particularly rituximab, have showed activity as single agents, they may have a greater role in combination with chemotherapy or as maintenance in responding patients. Their ability to purge blood and bone marrow to an undetectable level of lymphoma cells may allow re-infusion of minimally contaminated haematopoietic stem cell harvests after high dose therapy. The combination of these MoAb with a radionuclide may result in greater efficacy but a greater toxicity, particularly myelosuppression. The definitive use of these MoAb cannot be recommended before the results of current prospective studies are available.

References

Antisense oligonucleotides for haematological malignancies
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LRF Molecular Haematology Unit, Institute of Child Health, London UK

The growth, differentiation, appearance and function of all living cells is dictated by proteins. The code for each protein is stored in the form of double helix DNA within the nucleus of every cell. Expression of such a gene is performed by transcribing the base sequence of the DNA into single stranded messenger RNA (mRNA) which is able to move from the nucleus to the cytoplasmic compartment of the cell where it is translated into a protein. Many disease processes are characterised by inappropriate or excessive expression of normal or chimaeric proteins as a consequence of altered DNA transcription. Current drugs predominantly function by altering protein expression within the target cell, however, the process is far from efficient and often lacks specificity. It is an attractive proposition to use novel approaches to block transcription or translation of individual genes in order to lower disease-causing proteins. An emerging and powerful approach is the use of antisense oligonucleotides (ASO) consisting of short sequences of synthetic single stranded DNA or RNA (around 12 to 20 bases) complementary to aberrantly expressed genes. ASO were reported to inhibit gene expression as far back as 1978 with the inhibition of the Rous sarcoma virus. Understanding of their kinetics and mode of action at this time was, however, poor. Their design permits Watson-Crick base pairing with a particular mRNA, blocking its translation either sterically or by the action of ribonuclease H (RNAse H) enzyme to cleave the ASO-mRNA duplex. Some antisense oligonucleotide modifications inhibit gene expression by the formation of triplex DNA, inserting a stretch of nucleotides into the major groove of the nuclear double helix which forms side-to-side hydrogen bonds (Hoogsteen base pairs) with one of the strands. The resultant triple helix is unable to transcribe RNA. While triplex formation has the aesthetic advantage of blocking protein production at source the triplex technology is considerably more complex and less advanced. Antisense oligonucleotides have a number of inherent problems. These include nuclease degradation, cellular delivery, effective sequence specificity, formulation and in vivo pharmacokinetics. Greater understanding of all these areas has lead to improved ASO molecules and the prospect of effective molecular therapy.

Antisense oligonucleotide design
Optimising choice of target
The ability of naturally occurring RNA to anneal is a crucial process for living cells. However, the ability to design artificial antisense oligonucleotides has been hampered by the frequent inability of the ASO to anneal successfully to the mRNA. Efficient RNA-ASO annealing involves the interaction between highly structured RNA elements. Empirical trials of a large selection of oligonucleotides for an mRNA sequence has tended to be the method for effective ASO selection, with many ineffective molecules being discarded. A lack of understanding of the structure of RNA and the rules governing the annealing properties underlies this failure. Two approaches have been effective in improving ASO design. The first is an empirical approach, essentially generating a large number of synthetic oligonucleotides to cover all the possible selections and hybridising these against the target mRNA. This may be performed by generating gridded arrays of ASO logically covering all computations for a mRNA onto a glass plate and then hybridising the labelled message against the grid. The ASO that can access the RNA structure and anneal gives a positive signal. Identification of a good ASO molecule is readily provided but the technique is limited to examining a maximum of 400 bases of RNA for each grid. In most cases this is quite adequate. A greater length of gene specific RNA can be examined by hybridising the labelled RNA against random or semirandom libraries (pools of single stranded ASO) of oligonucleotides, the latter having been shown to be more effective. The library approach is more prone to some false negative results but does not require the polymer technology required to attach the oligonucleotides to the glass for the gridded arrays. Both are extremely effective at identifying accessible RNA sites for ASO. The second ASO selection approach is based on the computer supported structural design of RNA. The secondary structure of the target RNA may be predicted by the programme mfold 2.0 and the structural parameters recorded. Favourable ASO structures are searched for by examining for a maximal number of external bases and components and a minimised loop degree. Simplistically this means an ASO that complements an mRNA region with a weak secondary structure will.
effectively compete with the RNA structure, displace the mRNA-mRNA double stranded binding and anneal to exert its antisense effect. Such a theoretically based selection approach has improved ASO identification considerably although again some erroneous predictions still occur. It is becoming clear that some mRNA sites are inaccessible to ASO due to their structure. These may include chimaeric junctional points from chromosomal translocations (as found in some cancers) limiting their use against tumour specific mRNAs. The site of optimal ASO sequences remains equivocal on occasions, however, some logical themes are evolving.

Protection against nuclease degradation
Antisense technology has in the past suffered from poor quality control for ASO manufacture and before modifications are considered, an emphasis on good quality manufacture is essential. Breakdown of unmodified ASO (PO) by circulating enzymes (nucleases) leads to rapid removal of the effective agent before it has a chance to enter the cell. Chemically modifying the oligonucleotide backbone will confer resistance to nuclease degradation and considerably extend its active half life from minutes to days. The first generation of ASO (providing a negative charge to the molecule) were modified by changing the oxygen backbone to a sulphur atom and were termed phosphorothioates (PS). An alternative substitution with the methyl group (creating methylphosphonates [MP]) is as effective for nuclease protection, is relatively non-toxic to cells, but is poorly water soluble and RNAse H activity with outer MP combines a central core of seven PS molecules (to retain communication, to form peptide nucleic acid (PNA) oligonucleotides when injected intravenously (IV), intraperitoneally (IP), or subcutaneously (SC). The kinetics are similar with all three routes of administration. There is some knowledge about the pharmacokinetics of in vivo ASO delivery shows an altered tissue distribution, predominantly to the lung and liver, which may limit their application. The possibility of conjugating the ASO to a polymeric drug delivery system has a greater potential for improved oligonucleotide delivery in vivo and may also permit differential tissue targeting to give greater organ specificity. Optimisation of an intracellular delivery system remains an area for considerably more research if antisense therapy is to be improved further.

Mechanism of action
There are a number of mechanisms of ASO effect. Direct competition with aminoacyl tRNA for sites on the mRNA molecule with subsequent blockade of ribosomal reading represents one, while the formation of the mRNA-DNA duplex with ionic ASO (i.e. PS or Gapmers) and subsequent activation of the enzyme RNAse H leading to duplex breakdown is another. It is apparent that several mechanisms are responsible for ASO mediated inhibition of gene expression and it is important to demonstrate a decrease in the amount of protein produced by the gene targeted.

Pharmacokinetics of in vivo therapy
There have been several recent reports of the in vivo efficacy of antisense molecules targeting oncogenes in hamatological malignancy using the severe combined immunodeficient (SCID) mouse as a model. There is some knowledge about the pharmacokinetics of PS oligonucleotides when injected intravenously (IV), intraperitoneally (IP), or subcutaneously (SC). The kinetics are similar with all three routes of administration. PS molecules are strongly protein bound. Although 30% of the dose is excreted in the urine within 24hrs, there are detectable levels in most tissues for up to 48hrs, with only 15-50% degradation. Plasma clearance by both routes is biphasic with an initial half life of 15-25 minutes and a second half life of...
20 to 40 hours. The current human studies have administered the ASO by either a two hour infusion (IV) or by continuous SC infusion over a two week period. It appears that both of these routes of administration lead to a maximum tolerated dose of phosphorothioate oligonucleotide dose in the region of 6 mg per kilogram per day. Above this dose reversible falls in the platelet count (thrombocytopenia) are observed and require therapy to be stopped. Platelet recovery is readily reversible. The pharmacokinetics of the second and third generations of ASO are not yet clarified.

How ASO should be assessed

Antisense research must be interpreted with adequate reference to control parameters. These include essential controls such as sense control (the complementary sequence to the antisense sequence), non-sense or scrambled control and mismatched control (the same oligomer but for one or two mismatches in the central section). Most published antisense experiments have utilised a minimum of two controls and this should be regarded as a basic requirement when designing antisense work.

Antisense experiments are targeted towards a particular oncogene and should therefore be able to downregulate the translation of its mRNA, producing a decrease in its protein production. If this phenomenon cannot be demonstrated then the suspicion of non-specific effects must be raised.

Non-specific effects due to chemical composition of oligonucleotides

Oligonucleotides are polyanions and can have physiological roles such as those of naturally occurring polyanions including heparin and dermatan sulphate. Base composition of the oligonucleotide such as the presence of four contiguous guanosine residues can result in it having an antiproliferative role which may mimic an antisense effect if not adequately controlled for. Other non-specific effects induced by oligomers, with palindromic sequences of six or more bases, mimic an antisense effect if not adequately controlled for. Other non-specific effects include induction of $\alpha$- and $\gamma$-interferon production, which may have effects on enhancing natural killer cell activity in vivo tumour models such as in the SCID mouse. These examples represent a fraction of the number of potential interactions between oligonucleotides and cellular proteins, and may give an explanation for some of the non-sequence-specific effects seen with control oligomers.

Application of antisense oligonucleotides for human disease

There are currently a number of first generation phosphorothioate ASO that are being investigated in the human setting. These include the fields of cancer (lymphoma), cardiac disease (coronary vein grafting), chronic inflammatory conditions (bowel disease) and infectious disorders (CMV retinitis). All conditions are associated with overexpression of a specific gene or the presence of a foreign infectious agent.

**ASO for haematological malignancies**

In the field of cancer, chromosomal translocations often lead to deregulation of proto-oncogenes, or to the formation of fusion genes, making them potential targets for manipulation by ASO in an attempt to downregulate oncogene expression and, it is to be hoped, to have an anti-tumour effect. The systemic use of ASO also has its attractions where it might be hoped to have a specific anti-tumour effect while avoiding the many non-specific toxicities caused by chemotherapeutic substances. In B-cell lymphomas the t(14;18) and t(8;14) translocation involving the Bcl-2 and MYC genes respectively have been targeted for antisense therapy. The currently most advanced human Phase I/II study has been in B-cell lymphomas with high Bcl-2 expression. The Bcl-2 oncogene has been implicated in the oncogenicity of a wide variety of malignancies. Bcl-2 protein directly prolongs cellular survival by blocking chemotherapy-induced programmed cell death (apoptosis) making it an attractive target for antisense therapy. Antisense, control sense and nonsense oligonucleotides to the open reading frame of Bcl-2 were evaluated using a lymphoma cell line with high Bcl-2 expression and gave specific downregulation of Bcl-2 protein with subsequent induction of apoptosis. An in vivo lymphoma model was set up with the same cell line. Anti-tumour effect in vivo was demonstrated with a two week infusion of Bcl-2 antisense (G3139, Genta USA) at 100 µg daily achieving a plasma level of approximately 0.1 µM. Currently a human Phase I trial of this Bcl-2 antisense is being completed and shows it to be well tolerated with promising evidence of efficacy. It is envisaged that the ASO may be used in a clinical setting to overcome Bcl-2 mediated drug resistance by combining Bcl-2 ASO to prime the patient before chemotherapy, followed by the conventional chemotherapy. Such an experimental approach has been taken using G3139 and chemotherapy in a SCID mouse melanoma model and is now being translated into a human trial for both melanomas and lymphomas. Significant tumour regression is observed. Alternatively it may prove to be of benefit as a form of maintenance therapy being given over a longer period. Caution will, however, be needed to ensure that long term toxicities are not induced. Similar Phase I studies are now underway for other solid tumours using a Raf isoform antisense as an inhibitor of MAP kinases (ISIS 5132, ISIS Pharmaceuticals, Carlsbad CA) and Protein Kinase C-α (ISIS 3521).

**ASO for other fields of medicine**

The potential for ASO in the fields of medicine is vast. The field of myocardial infarction and cardiac vein grafting have both been investigated with favourable results. Antisense to cdk2 kinase mRNA prevents arterial intimal thickening after vein grafting...
and may be a useful addition to cardiac surgery to prevent coronary artery restenosis and could be useful in preventing progression of atherosclerosis. In the field of inflammatory bowel disease an ICAM-1 antisense (a protein mediating inflammation) has entered a Phase I study (ISIS 2302) and has been reported to have significantly reduced the use of steroids in the ASO treated group. It is envisaged that other systemic inflammatory conditions such as rheumatoid arthritis or systemic auto immune diseases could benefit from such an ASO approach. Recently the downregulation of Bcl-xI an anti-apoptosis gene by ASO in the intima of vascular lesions has resulted in regression, suggesting that targeting apoptosis by ASO may play a major role as a novel therapy for vascular disease, a major cause of death in the Western world.

The other area of considerable interest and one in which there are currently very few effective pharmaceutical agents is that of antiviral therapy. Initial attempts to eradicate HIV have not shown much success, possibly due to a failure to identify the correct gene to target. However, an ASO to the mRNA for the IE2 gene of cytomegalovirus (CMV) (ISIS 2922) administered directly into the eye for CMV infection of the retina, which causes blindness particularly in immunocompromised patients, has been successful. This compound is now completing the licencing procedure for clinical use as the first ASO to come onto the commercial market. This is a major advance for antiviral therapy and illustrates the versatility of ASO. Similar approaches could also be taken for bacterial infections.

Future perspectives

The potential ability of ASO to downregulate the expression of disease-causing genes, with minimal toxicity it has been demonstrated and is now opening up a whole new approach to the management of some human diseases. In many respects antisense therapy has been the forerunner of gene therapy as many of us understand it and have permitted us to show that some genes truly play a significant role in human disease. ASO give us the ability to switch off inappropriately expressed genes and modify the target cells’ biological behaviour. Vector-mediated gene therapy will in the future allow replacement of genes that are lacking in the cell, that is to say switch on genes. ASOs represent the first generation of a new class of pharmacological agents based on a biological understanding of the diseased cell. Other synthetically derived molecules such as ribozymes and DNAzymes are also capable of silencing genes but still have a long way to go before they reach the clinic. Oligonucleotides are currently the most advanced in the human therapeutic setting. Total replacement of conventional therapy is not going to occur but we will be able to use gene modification to enhance current pharmacological agents and reduce their toxicity. Combinations of antisense molecules may further enhance their efficacy and alterations of the antisense chemistry may improve their tolerance and usefulness. This is a whole new area of research and as such requires much evaluation if it is to be applied optimally. Although in vitro efficacy may be established, it is highly desirable to test the hypothesis in vivo animal models. With care novel therapies based on the biology of the malignant cell may be determined on a scientific basis and may help improve the treatment of patients with disease.

References

Dendritic cell maturation and generation of immune responses

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Dendritic cell maturation

Dendritic cells (DC) represent a system of professional antigen presenting cells (APC). Their function is to capture incoming antigens and present them in secondary lymphoid organs to naive T cells in order to generate immune responses. DC precursors constitutively migrate at low rate from blood into non-inflamed tissues, but the rate of migration can be dramatically increased if there is an inflammation. Here the DC, which are in their so-called immature state, encounter pathogens and undergo a complex change in their properties that is collectively called maturation.

Evidence for DC maturation has been gained initially from in vivo observations where immature DC have been shown to migrate from peripheral tissues to secondary lymphoid organs while acquiring T cell stimulatory capacity. The molecular details and the kinetics of the maturation process as well as the signals that trigger it have been defined using an in vitro system of monocyte-derived DC. Human peripheral blood monocytes cultured in GM-CSF and IL-4 develop without dividing into immature DC that can respond to various stimuli by undergoing the maturation process. This process involves many aspects of cell physiology, such as changes in the rate of endocytosis, MHC biosynthesis, expression of co-stimulatory molecules and cytokines, as well as molecules involved in adhesion and migration (Figure 1). We will discuss the properties of immature DC, the signals that induce maturation, and the consequence of maturation for the generation of the immune response.

Mechanisms of antigen capture by immature DC

Immature DC have a high and constitutive level of macropinocytosis that allows them to take up large volumes of fluid and concentrate the macromolecules in the endocytic compartment. This capacity is dependent on the developmentally-regulated expression of aquaporins and amiloride-sensitive sodium channels, which endow these cells with a very high capacity to transport water and ions across the membranes leading to concentration of macromolecules. Immature DC also express high levels of the mannose receptor, a pattern-recognition molecule that allows efficient uptake of mannosylated and fucosylated antigens, as well as the Fcg receptor, CD32, that allows capture of immune complexes. Immature DC are consequently extremely efficient antigen presenting cells (APC) for soluble antigens. They can present tetanus toxoid (TT), which is taken up by fluid phase at concentrations of ~10^{-10} M, and are therefore as efficient as TT-specific B cells that take up this antigen via specific mIg. The efficiency of presentation can be further boosted (~100 fold) by targeting the antigen to CD32 or to the mannose receptor. This can be achieved by complexing the antigen with antibodies or by mannosylation.

Stimuli that induce dendritic cell maturation

The availability of cultured immature DC has been instrumental in the identification of stimuli that induce DC maturation. These include inflammatory cytokines (TNF-α and IL-1) and bacterial and viral products (LPS and double-stranded RNA). These stimuli are likely be met by DC in peripheral tissues and are important to the initiation of the maturation process. Once maturing DC have reached the secondary lymphoid organs, they interact with activated T cells that can deliver further stimulatory signals via CD40L and TRANCE, resulting in induction of cytokine production and increased survival. DC maturation can be dissected into several distinct sub-programmes that involve changes in endocytosis, MHC biosynthesis, expression of co-stimulatory molecules, cytokine production and migration (see Figure 1).

Antigen presentation on MHC class II molecules

In DC the maturation stimuli optimise antigen presentation on class II molecules. In immature DC, newly synthesised class II molecules are loaded efficiently with antigenic peptides and transported to the cell surface. From the cell surface, class II molecules are continuously internalised and recycled back to the surface. Thus, in immature DC, antigenic peptides can be loaded on both newly synthesised and recycling molecules. The class II recycling compartment is particularly prominent in these cells and allows rapid loading of T cell epitopes that are different from those loaded into newly synthesised molecules. Maturation induced by LPS or TNF-α results in DC...
in an increase of ~3-4 fold in the rate of class II synthesis, which is sustained for at least 24 hours, while at subsequent time points the synthesis is shut off. The transient boost of class II biosynthesis provides the maturing DC with a large number of molecules that can be loaded with antigenic peptides. At the same time the maturation process leads to a progressive decrease of endocytosis which results in a dramatic increase in the life expectancy of class II molecules. Indeed, in immature DC, class II molecules have a relatively short half-life of ~10 hours, which shifts to more than a 100 hours in mature DC. As a consequence of these co-ordinated changes in class II biosynthesis and stability, DC, soon after induction of maturation, assemble a large number of peptide MHC complexes that are retained in a stable form for long periods of time in the absence of further class II synthesis. This mechanism optimizes the presentation of infectious antigens, i.e., antigens, derived from pathogens, which stimulate DC directly (for instance via LPS), or indirectly (for instance via production of TNF-α).

Antigen presentation on class I molecules

A particular challenge for an APC is to present a cytopathic virus. To do this the cell must be susceptible to infection in order to produce viral protein, but at the same time, it must be able to resist the cytopathic effect of the virus. In some cases, for instance infection with influenza virus, DC are able to achieve this difficult task. Immature DC are susceptible to infection with influenza virus and synthesize large amounts of viral proteins. But, soon after infection, they become resistant to the cytopathic effect of the virus. The dsRNA of the influenza virus stimulates DC maturation, which results in upregulation of synthesis of MHC class I molecules necessary for efficient presentation of viral antigens. As part of the maturation programme, DC produce type I IFN which upregulates MxA, a protein that instates resistance to the cytopathic effect of the virus. By this autocrine mechanism of stimulation, DC rapidly resist the cytopathic effect of the virus and efficiently present viral antigens by continuously loading antigenic peptides on newly synthesized class I molecules. Unlike that which is observed for class II molecules, the biosynthesis of class I molecules is sustained for days while the half-life does not change significantly, remaining relatively short (~10-20 hours). This half-life may reflect an intrinsically greater instability of class I as compared to class II molecules in living cells.

Immature DC are also particularly efficient in capturing apoptotic or necrotic cells. In this case, the antigens present in the phagocytosed cells can be processed and presented on both class II and class I molecules. This mechanism (defined as crosspriming) may be important for presentation of tumour antigens and, in general, of antigens which are carried exclusively by non-professional APC.

T cell stimulation and polarisation

Immature DC are very poor stimulators for naive T cells since they do not express co-stimulatory molecules. On the other hand, all maturation stimuli induce marked upregulation of B7.1 and B7.2 and consequently mature DC acquire a marked capacity to
depending on the type of cytokines produced. The capacity to polarise T cells towards Th1 or Th2, while all mature DC have comparable capacity to trigger the Th2 response to influenza virus. Altogether, it appears that lack of requirement for help in the generation of a CTL response explains the strong stimulation induced by pathogens. The nature of the maturation stimuli determines cytokine production and consequently T cell polarisation.

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Therapeutic approaches based on DC maturation

The better understanding of the mechanism of DC maturation is going to have an impact on several therapeutic areas. First, antigens can be targeted more efficiently to dendritic cells by mannosylation, opsonisation and special formulation (with apoptotic bodies or exosomes being particularly interesting options). These mechanisms may also be used to eliminate DC when required. Second, adjuvants to be used for vaccination may be designed in a more rational way to prime naive T cells. Although most aspects of DC maturation (such as the downregulation of endocytosis, increased MHC biosynthesis and increased co-stimulation) are induced to a comparable extent by all maturation stimuli, for cytokine production the nature of the stimulus makes a difference (see Table 1). In general, pathogens or helper T cells provide a much more powerful maturation stimulus for cytokine production than TNF-α or IL-1, and push DC to a higher T cell stimulatory state. For instance, IL-12 production is triggered selectively by CD40L and/or LPS, but not by TNF-α. The strong stimulation by CD40L explains the requirement for T cell help in the induction of cytotoxic responses to cellular antigens such as some minor histocompatibility antigens. On the other hand, the strong stimulation induced by pathogens explains the lack of requirement for help in the generation of a CTL response to influenza virus. Altogether, it appears that while all mature DC have comparable capacity to trigger T cell proliferation, they differ remarkably in their capacity to polarise T cells towards Th1 or Th2, depending on the type of cytokines produced.

Table 1. The nature of the maturation stimuli determines cytokine production and consequently T cell polarisation.

References

Most solid tissue cells including tumours express MHC class I-molecules, but lack co-stimulatory molecules important for appropriate cytotoxic T lymphocyte (CTL) activation. Therefore, optimal CTL induction requires processing and presentation of peripheral antigens by professional antigen presenting cells (APC). This cross-presentation of antigens provides the immune system with a mechanism by which it can detect and respond to antigens expressed in non-lymphoid tissues. Antigen-specific CTL responses can be induced regardless of the haplotype of the immunising cell. For induction of many MHC class I-restricted tumour-specific immune responses, cross-presentation of antigens that have been captured by professional APC plays a dominant role. Cross-presentation of antigens by professional APC is the pivotal mechanism of CTL-priming even when cells are transfected with the co-stimulatory molecule B7, or when plasmid DNA, inoculated in muscle tissue, is used as the immunogen. A role for bone marrow (BM) derived cells (most likely dendritic cells (DC) able to present MHC class I restricted antigens in a TAP-dependent fashion) appears to be important in the induction of tumour-specific CTL responses by cross-priming.

These observations thus indicate that exogenous antigen-uptake by professional APC, that process and present these antigens to CTL precursors, is the dominant mechanism by which specific CTL responses are primed with the exception of CTL responses induced by directly infected APC. Viruses and other microorganisms can also directly intrude into professional APC with or without concomitant induction of the APC activation programme required for CTL induction. Cross-presentation of antigens by professional APC is important for efficient CTL-priming, but the same mechanism might also be important for tolerance of autoreactive CTL. In studies with transgenic mice expressing a membrane-bound form of ovalbumin (OVA) in pancreatic islets, BM-derived APC cross-presented OVA to adoptively transferred OVA-specific CTL in lymph nodes draining the site of OVA expression. After an initial phase of proliferation, OVA-specific CTL were ultimately deleted from the periphery. This deletion only required antigen recognition on a BM-derived APC. Thus, cross-presentation of antigens can also lead to tolerisation of CTL. These studies provide a mechanism by which potentially auto-reactive CTL can be deleted from the periphery when the antigen recognised by these CTL is expressed outside the recirculation pathway of naive T cells, but contrasts with the activation of OVA-specific CTL by cross-priming APC that occurs when OVA-expressing cells are used for immunisation. The discrepancy between these observations can be explained by lack (tolerance) or presence (priming) of antigen-specific CD4+ Th cells. Indeed in the OVA-transgenic mouse system, injection of T cell receptor transgenic OVA-specific CD4+ Th cells prevents the deletion of OVA-specific CTL and favours the induction of autoimmunity. These observations thus indicate that provision of help by CD4+ Th cells is an important factor in the prevention of peripheral CTL-tolerance as well as for the induction of CTL-immunity.

The nature of this help was unknown until recently. Th cells must recognise antigen on the same APC that presents the CTL-epitope. This clarifies the requirement for epitope linkage between Th cell- and CTL epitopes important for induction of CTL responses, and was previously explained by a proximity requirement for the efficient delivery by Th cells of soluble factors, such as IL-2. Alternatively cognate interactions between Th cell and APC convert the APC to a state that is capable of priming naive CTL, as now reported by 3 groups, including ours. This model could explain our observation that the inability of female B6.129pm12 mice (which harbour a mutated MHC class II molecule resulting in a minimal H-Y-specific helper response) to reject male skin-grafts is overcome by immunisation with (in vitro) activated DC. Importantly, by communicating with the cross-priming APC, a Th cell can assist in CTL priming without requiring the simultaneous interaction of a (rare) antigen-specific CTL precursor, the presence of which cannot be noticed by the Th cell (murine CTL are class II-negative) in the immediate vicinity of the same APC. As a result, the activity of only a few antigen-specific Th cells can in this way be amplified, since one Th cell can activate a number of APC which can then prime an array of antigen-specific CTL.

We were alerted to this scheme of cellular interac-
tions by studying the role of CD40-CD40Ligand interaction in the delivery of help for CTL-priming. This interaction is important in the activation of professional APC. CD40 is a member of the tumour necrosis factor gene family and is expressed on several cell types, including DC, B cells, macrophages, endothelial cells and proximal tubular epithelial cells in the kidney. CD40Ligand (CD40L) is expressed shortly following TCR triggering on CD4+ T cells. We demonstrated that CD40 signalling can replace CD4+ T cells in the priming of helper dependent CD8+ CTL responses. Vaccination of B6 mice (H-2b) with completely allogenic tumour cells of BALB/c origin (H-2d), transformed by the human adenovirus type 5 early region 1 (Ad5E1), leads to the induction of a strong H-2Db restricted CTL-response directed against an AdE1B-encoded CTL-epitope. Since these tumour cells themselves cannot present the E1-derived CTL epitope to E1B-specific CTL (they lack H-2Dd), the CTL must be primed by non-tumour cells that have processed and presented the E1-peptide to E1-specific CTL (cross-priming). In vivo depletion studies revealed that cross-priming of E1B-specific CTL is strictly Th cell dependent, as mice depleted for CD4+ Th cells prior to immunisation with BALB/c Ad5E1 cells no longer mount an E1B-specific CTL response. Administration of a CD40 activating monoclonal antibody (mAb) to either CD4-depleted or B6 I-Ab knock-out mice, lacking functional MHC class II-restricted CD4+ T cells in the periphery, resulted in efficient restoration of E1B-specific CTL responses. Moreover, blockade of CD40L by in vivo administration of a mAb that blocks CD40L on the CD4+ T cells, resulted in a profound inhibition of CTL-priming that was overcome by CD40 triggering. B cells are neither required as APC for cross-priming of Ad5E1B-specific CTL nor essential for the CD40-mediated restoration of cross-priming in the absence of CD4+ cells. Similarly CD40- or CD40L-deficient mice are unable to generate OVA-specific CTL responses after vaccination with OVA-expressing cells, showing that expression of both CD40 and CD40L is indeed required for cross-priming of CTL. Moreover, mice devoid of CD4+ Th cells, are not able to mount OVA-specific CTL after vaccination with OVA expressing cells, unless immunisation is performed together with injection of a mAb that triggers CD40. Taken together, these results show that CTL can be primed in the absence of Th-derived cytokines, and demonstrate the crucial importance of CD40-CD40L interaction. The contribution of CD4+ Th cells involves the activation of professional (non B-cell) APC via CD40-CD40L interactions. Indeed, help for priming of H-Y specific CTL can be bypassed by activation of DC through CD40. Female MHC class II knock-out mice are not able to mount an H-Y-response when injected with male DC, but do respond when CD40-modulated DC are used for vaccination. These results show that the Th cell and CTL do not need to meet simultaneously at the surface of the antigen-presenting DC to enable CTL-priming. Moreover these results indicate that DC that have received an activation signal through CD40-CD40L interactions can prime naive CTL, whereas unmodulated DC that have not been in contact with antigen-specific, CD40L-expressing Th cells are unable to do so.

References

The role of chemokines and chemokine receptors in T cell priming and Th1/Th2-mediated responses

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Chemokines and chemokine receptors
The immune system is made of mobile cells and its function is dependent on the capacity of these cells to migrate to the right place at the right time. T cell priming occurs in lymphoid tissues and requires encounters between naive T cells and antigen-loaded dendritic cells (DC) that have captured antigens at peripheral sites. In contrast, effector responses such as delayed type hypersensitivity (DTH) or allergic reactions occur in peripheral tissues following interaction of Th1 or Th2 cells with effector leukocytes. Leukocyte migration is controlled at the level of expression of selectins, chemokine receptors and integrins that co-operate in an ordered fashion in the process of extravasation and migration within the tissues. Recent evidence indicates that chemokines and chemokine receptors provide a flexible code to determine the selective migration of T cells, DC, and other leukocytes involved in the immune response.

Chemokines can be operationally divided into two categories. The first is represented by those which are produced constitutively in bone marrow, thymus and secondary lymphoid organs and which are denoted constitutive or immune as they control leukocyte traffic under physiological conditions (Table 1). The second is represented by inflammatory chemokines, that are induced or upregulated by inflammatory stimuli and are involved in the recruitment of effector leukocytes at site of tissue injury (Table 2). While there may certainly be exceptions to this rule, it represents a useful paradigm to approach the complexity of chemokine regulation.

Chemokine receptors can be specific for a single chemokine or can recognise several. The expression of these receptors is highly specific for a particular cell type and determines the cell’s capacity to migrate to particular districts where the cognate ligands are produced (Tables 1 and 2). An emerging principle is that in DC, B and T lymphocytes chemokine receptor expression is developmentally regulated. In addition, upon activation, chemokine receptor expression is rapidly modified by transcriptional or post-translational mechanisms, resulting in the acquisition of novel migratory capacity. We will give a few examples of how changes in chemokine receptor expression may be instrumental in inducing selective migration of cells during the immune response.

Chemokines in antigen presentation
The induction of T cell responses requires migration of DC from tissues, where they sample antigens, to the T cell areas of lymph nodes, where they stimulate naive T cells. Immature DC and monocytes express receptors for inflammatory chemokines and consequently are attracted to inflamed tissues where the cognate ligands are produced. Here the DC will take up antigens and will be stimulated to mature by pathogens or inflammatory cytokines. Maturating DC are themselves an extremely abundant source of inflammatory chemokines and therefore contribute to attracting immature DC as well as effector leukocytes. The high level of inflammatory chemokines produced leads to a prompt downregulation of the cognate receptors on maturing DC. At the same time, receptors for constitutive chemokines (especially CCR7) are upregulated at the transcriptional level. CCR7 allows maturing DC to enter the afferent lymph attracted by SLC which is bound on lymphatic endothelial cells, and subsequently to localise in the T cell areas of lymph nodes, following a gradient of ELR, another CCR7 ligand which is produced by resident mature DC. Thus, in their life-cycle DC first express receptors for inflammatory chemokines, then produce inflammatory chemokines, then upregulate receptors for constitutive chemokines, and finally, after a further time lag, start synthesising constitutive chemokines. This time-dependent expression of receptors and ligands allows DC to tightly regulate their migratory capacity in an autonomous fashion, which is consistent with their role as sentinels of the immune system (Figure 1).

Chemokines in T cell priming
Naive T cells continuously recirculate from the blood into secondary lymphoid organs such as lymph nodes, where they scan DC for antigens and re-enter the blood stream through the efferent lymph. Naive T cells express L-selectin and CCR7 which allow them to interact with endothelial venules (where the ligands for these receptors, CD34 and SLC are expressed on endothelial cells). Following extravasation the cells enter the T cell areas, where ELC is pro-
duced by resident mature DC (Figure 1). The importance of CCR7 and its ligands in orchestrating the T-DC encounter is exemplified by the fact that mice lacking SLC or CCR7 are incapable of mounting primary T cell responses6 (and M. Lipp, personal communication).

Chemokines in T-B collaboration

Naive B cells also home to the lymph node where they localise to the B cell areas. Resting B cells express CXCR5 which is essential for their localisation to the B cell follicles7 where the cognate ligand BCA-1 is produced by as yet undefined cell types. Activation of B lymphocytes to antibody production requires the encounter of two extremely rare cells, the antigen-specific T and B cells. It is therefore not surprising that this interaction needs to be tightly regulated. Following stimulation with antigen, the B cells upregulate CCR7 and are therefore attracted to the T cell areas where ELC is produced. Conversely, some T cells that are activated by encounter with antigen on DC upregulate CXCR5 and thus migrate towards the B cell areas, guided by BCA-1. In this way, T and B cells move out of their own areas and migrate towards each other, meeting at a T and B boundary where they can interact in an antigen-specific fashion (Figure 1).

Chemokines in Th1/Th2-mediated responses

Following priming in the lymph node, T cells acquire effector function, i.e. the capacity to produce cytokines following antigenic stimulation. Th1 cells produce IFN-γ and are involved in responses against intracellular pathogens and DTH reactions, while Th2 cells produce IL-4 and IL-5 and are involved in responses against extracellular parasites and generate allergic reactions. Together with the acquisition of the cytokine producing capacity, effector T cells acquire new homing potential. They lose CCR7 and acquire receptors for inflammatory chemokines depending on the type of polarisation (Figure 2). CCR3, a receptor for eotaxin, which is also expressed by basophils and eosinophils, is selectively expressed by Th2 cells.8,9 The expression of CCR3 allows these three cell types to co-localise together at sites of allergic reactions. This may play a pathogenetic role because the Th2-derived cytokines IL-4 and IL-5 function as activation and survival factors for basophils and eosinophils. CXCR3, a receptor for Mig and IP-10, two chemokines induced by IFN-γ, is expressed at higher levels on Th1 than on Th2. This receptor may be important to localise Th1 cells to sites of DTH reactions. CCR5 is preferentially, but not exclusively expressed on Th1 and its expression is upregulated by IL-2. CCR4 is expressed on Th2 cells but also on non-polarised T cells that are incapable of producing IL-4. Finally, CCR1 and CCR2 are

Table 1.

<table>
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<th>Constitutive/lymphoid chemokines</th>
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Abbreviations: Mo, monocytes; MF, macrophages; mDC, mature DC; act., activated; T, T lymphocytes; B, B lymphocytes; MDC, macrophage derived chemokine; TARC, thymus and activation regulated chemokine; ELC, EBI1 ligand chemokine; SLC, secondary lymphoid tissue chemokine; SDF-1, stromal cell derived factor 1; BCA-1, B cell attracting chemokine 1.

Table 2.

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</table>

Abbreviations: Mo, monocytes; MF, macrophages; iDC, immature DC; Eo, eosinophils; Ba, basophils; N, neutrophils; T, T lymphocytes; B, B lymphocytes; MIP-1, macrophage inflammatory protein 1; RANTES, regulated on activation of normal T cell expressed and secreted; MCP, monocyte chemotactic protein; Eot, eotaxin; LARC, liver and activation regulated chemokine; GCP-2, granulocyte chemotactic protein 2; GRO, growth related oncogene; ENA, epithelial cell derived neutrophil attractant; IP-10, interferon inducible protein 10; Mig, monokine induced by IFN-γ; I-TAC, interferon-inducible T cell alpha chemoattractant.
Selective expression of chemokines and chemokine receptors orchestrates the afferent and effector phases of the immune response. The critical migratory steps and cellular interactions in the immune response are highlighted, together with some of the molecules involved. (A) Monocytes and immature DC are recruited by inflammatory chemokines to sites of antigen challenge. (B) Pathogens induce DC maturation resulting in a switch in chemokine receptor expression. (C) DC enter lymphatic vessels and are drained to the lymph node where they home to the T cell areas. (D) Naive T and B cells home to the T and B cell areas of the lymph node. (E) T cells interact with DCs and are activated. (F) Effector T and B cells migrate to B cell areas where they stimulate antigen-specific B cells. (G) and (H) Th1 or Th2 together with effector cells migrate to peripheral sites of DTH or allergic reactions.

Developmental and activation induced control of chemokine receptor expression in T cells.
expressed on both Th1 and Th2 cells. Interestingly the expression of chemokine receptors on effector T cells can be rapidly shifted by activation signals (Figure 2). Following TCR triggering effector T cells downregulate receptors for inflammatory chemokines and transiently upregulate CCR7 as well as other receptors for constitutive chemokines. This rapid switch occurs at the transcriptional level and results in novel homing capacities. The transient CCR7 upregulation may allow T cells that have been activated in the tissues to migrate back to the lymph nodes via the afferent lymphatics, or alternatively, to localise within the chronically inflamed tissues to areas where ELC and SLC are produced.

Conclusions
We have just started to appreciate the complexity of the chemokine system and its regulation. This system provides a very flexible mechanism for regulating migration of different cells as well as their encounters. Chemokine receptors can be useful markers of cell populations and their selective blockade may represent a promising therapeutic strategy to interfere with the afferent or efferent limb of the immune response.

References
Autoimmune thrombophilic syndromes

MIKE GREAVES
Department of Medicine and Therapeutics, University of Aberdeen, Scotland

The role of autoantibodies in thrombosis has been acknowledged for many years, but the range of thrombotic disorders in which an autoimmune pathogenesis is suspected continues to expand (Table 1). In the 1960s the disorder which subsequently became known as the antiphospholipid syndrome was first described. Paradoxically, although this condition has been subjected to extensive investigation, the pathogenetic mechanisms causing thrombosis are less well defined than those in more recently described autoimmune thrombotic disorders, such as heparin-induced thrombocytopenia with thrombosis.

Antiphospholipid antibodies and thrombosis

Antiphospholipid antibodies are associated with arterial and venous thrombosis, recurrent pregnancy loss and thrombocytopenia. Although the antibodies have not yet been conclusively shown to be causal in thrombosis and miscarriage, they are useful laboratory markers for the antiphospholipid syndrome (APS). The identification of the syndrome is clinically important because of the risk of recurrent thrombosis and the need for antithrombotic therapy in many cases. Diagnosis and treatment of APS are significant challenges, however, due to the protean clinical manifestations and associations, limitations of currently available laboratory tests for antiphospholipid antibodies and the lack of clear evidence-based guidance on optimal management.

Antiphospholipid syndrome

Autoantibodies with apparent specificity for negatively charged phospholipids have long been recognised. The terms lupus anticoagulant (LA) and anticardiolipin (ACL) have been used to describe these antibodies. LA is an immunoglobulin which acts as a coagulation inhibitor but which does not recognise a specific coagulation factor. It slows the rate of thrombin generation, and therefore clot formation in vitro, through interference in the interactions which require phospholipid. It is therefore detected in coagulation assays. The criteria for LA positivity are the prolongation of a phospholipid-dependent coagulation test, with evidence of an inhibitor demonstrated by incomplete correction of the clotting time on mixing with normal plasma and confirmation of phospholipid-dependence of the antibody. In contrast, ACL is usually detected by enzyme-linked immunosorbent assay, in which the anionic phospholipid cardiolipin is used to coat wells on plastic microtitre plates. In APS, assays for LA and ACL are complementary.

The antiphospholipid syndrome may be defined as the occurrence of thrombosis and/or recurrent miscarriage in association with laboratory evidence of persistent antiphospholipid antibody, either LA or ACL. Thrombosis may affect arteries or veins. Stroke due to cerebral infarction is a prominent manifestation, but occlusion of visceral and peripheral arteries may also occur. In veins, limb deep vein thrombosis is most common, but involvement of visceral and intracerebral veins, and pulmonary embolism also occur. Thrombocytopenia is an occasional feature. Recurrent miscarriage is a notable manifestation of the syndrome, often occurring in women with no prior thrombotic history.

Where APS occurs in a subject with systemic lupus erythematosus, or less commonly some other disorder, such as systemic sclerosis, rheumatoid arthritis or Behçet’s syndrome, APS is regarded as secondary. In primary APS there is no evidence of other underlying disease.

The nature of antiphospholipid antibodies

Why should antibodies apparently reactive with some phospholipids be associated with a marked thrombotic tendency? A significant advance towards being able to answer this question was the recognition of the dependence of antibody binding upon a plasma protein, apolipoprotein H or β2 glycoprotein I (β2 GPI). It is noteworthy that this protein binds to anionic phospholipids and also possesses weak anticoagulant properties, principally through inhibition of the contact phase of coagulation and of platelet prothrombinase activity. The precise roles of β2 GPI and phospholipid in antibody binding have been disputed. β2 GPI may enhance antibody binding to phospholipid, although antibody binding to β2 GPI immobilised on microtitre plates without the requirement for phospholipid has been noted. One possibility is that β2 GPI bound to a surface, for example cell membrane phospholipid or the plastic of an assay plate, undergoes a conformational change and certain ‘antiphospholipid’ antibodies bind to exposed neoepitopes. An alternative hypothesis is that antibody binding to β2 GPI immobilised on a
surface is not dependent upon expression of neoepitopes but is facilitated by concentration of the protein on the surface, and therefore clustering of antigenic sites, allowing bivalent binding of what are essentially low affinity antibodies. Antibody binding may interfere with essential phospholipid-dependent steps in coagulation, especially assembly of the tenase and prothrombinase complexes on anionic phospholipid. The result is a prolongation of the time to clot formation which is reversible in the presence of excess phospholipid.

Proteins other than \( \beta_2 \) GPI are implicated in antiphospholipid reactivity. Some antiphospholipid antibodies bind to immobilised or phospholipid bound prothrombin. Usually the prothrombin concentration in plasma is normal, consistent with the concept that autoantibody only reacts with surface bound prothrombin. The LA phenomenon can therefore be due to antibodies reactive with prothrombin or with \( \beta_2 \) GPI. Anti-\( \beta_2 \) GPI positive sera are also frequently positive in anticardiolipin assays, whereas LA positive samples which are negative for ACL generally do not exhibit antibody binding to \( \beta_2 \) GPI. Furthermore, some antiphospholipid antibodies appear to bind to phospholipid directly. This is a particular feature of antiphospholipid antibodies associated with syphilis and some other infections.

The concept of heterogeneity amongst autoantibodies in APS is strengthened by the demonstration of antibody reactivity with still other plasma proteins involved in haemostasis. Thus, inhibition of activated protein C and its cofactor protein S by antiphospholipid containing sera has been noted and antiphospholipid antibodies may induce resistance to the anticoagulant effect of activated protein C in vitro. Autoantibodies to thrombomodulin and to phospholipid bound protein C and protein S have also been reported. Other autoantibodies impair the inhibition of thrombin by antithrombin III and still others react with the phospholipid binding protein annexin V.

Cellular reactivity of autoantibodies is also a feature in APS. Whilst \( \beta_2 \) GPI dependent binding of antiphospholipid antibodies to platelets has been demonstrated, other autoantibodies react with the major platelet membrane glycoproteins and are distinct from antiphospholipid antibodies and similar to those responsible for autoimmune thrombocytopenia. Additionally, antiendothelial antibody reactivity has been demonstrated. Although some antiendothelial cell reactivity appears to be \( \beta_2 \) GPI dependent other antiendothelial antibodies in APS are distinct from antiphospholipid antibodies.

In summary, although anti-\( \beta_2 \) GPI is commonly a marker for APS, there appears to be considerable antibody heterogeneity, with reactivity against a range of proteins which bind to phospholipid as well as antigens expressed on cell membranes.

The pathogenesis of thrombosis in APS

The paradoxical association between a marked prothrombotic state and the presence of autoantibodies with in vitro anticoagulant effects has not yet been fully explained. Subjects with APS have evidence of persistent coagulation activation. Vascular occlusion is due to thromboembolism, rather than vasculitis. Some arterial events may also be due to embolisation from sterile vegetations on cardiac valves. Although there are many candidate prothrombotic mechanisms related to the properties of the antibodies listed above, the precise pathogenetic roles of the heterogeneous array of autoantibodies which characterise APS have not been clarified. Additional proposed mechanisms include inhibition of fibrinolysis, promotion of platelet activation and induction of tissue factor on endothelial cells and monocytes. Furthermore, it may be that the antiendothelial antibodies which frequently coexist with antiphospholipid antibodies may be pathogenic, as those found in systemic sclerosis have been shown to induce apoptosis of cultured human umbilical vein endothelial cells. The pathogenesis of thrombosis in APS is almost certainly multifactorial.

A thrombotic pathogenesis of pregnancy failure in APS is suspected. Decidual vasculopathy and placental infarction have been observed but these are not apparent in all cases. Autoantibodies reactive with trophoblast have been reported and some may cause displacement of annexin V from trophoblast and accelerate coagulation. A non-thrombotic pathogenesis, through inhibition of trophoblast proliferation by anti-\( \beta_2 \) GPI, has also been proposed.

Although thrombosis and miscarriage are likely to have an autoimmune basis in many cases of APS, in other instances antiphospholipid antibodies could represent an epiphenomenon, perhaps arising through exposure of neoeptopes on proteins bound to cell membrane anionic phospholipids exposed following cell injury from some other cause.

Problems in diagnosis

Clearly APS is a heterogeneous condition, both in relation to its clinical manifestations and to the associated range of autoantibodies. Whilst the diagnosis of APS can often be made with confidence because of the typical constellation of clinical symptoms and signs and informative laboratory data, these may not be conclusive. Significant diagnostic and prognostic difficulties arise because of the occurrence of antiphospholipid antibodies secondary to infections, in
relation to medications, and in some apparently healthy subjects. The association with infection was first recognised in syphilis and was used to aid diagnosis. Antiphospholipid antibodies also occur in infection with HIV and hepatitis C and in some other infections, including cytomegalovirus. Such antibodies may be transient and are generally not associated with thrombosis. Exceptions occur, however, for example in the syndrome of purpura fulminans which occasionally complicates varicella infection in children in which the presence of LA has been linked to protein S deficiency and extensive thrombosis.

Drug-induced antiphospholipid antibodies may also cause diagnostic confusion.

Because of the heterogeneity of autoantibodies in APS, a comprehensive approach to laboratory investigation is essential. This should include coagulation based tests for LA as well as solid phase assays for ACL, as both tests are positive in only around 50% of cases of undoubted APS. Unfortunately there are major limitations to the laboratory methods available. Detection of LA relies upon prolongation of the clotting times in phospholipid dependent coagulation assays but there are pitfalls relating to the performance of these tests and their interpretation. For example the reagents employed in coagulation assays vary considerably in their sensitivity. Furthermore, heterogeneity of antiphospholipid antibodies leads to variable levels of positivity in the different coagulation assays, antiprothrombin antibodies behaving differently from anti-β2 GPI antibodies.

There are also problems in relation to tests for ACL. Despite the establishment and distribution of calibration materials and the general adoption of units for the expression of quantitative data, consistency between laboratories is poor. Commercial reagents and kits are popular but vary widely in their content. Quantitative data, consistency of performance of these tests and their interpretation. For example the reagents employed in coagulation assays vary considerably in their sensitivity. Furthermore, heterogeneity of antiphospholipid antibodies leads to variable levels of positivity in the different coagulation assays, antiprothrombin antibodies behaving differently from anti-β2 GPI antibodies.

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Heparin-induced thrombocytopenia with thrombosis

Thrombocytopenia is a common consequence of heparin therapy. The more common Type I heparin-induced thrombocytopenia is early in onset, trivial in degree and clinically benign. This contrasts with Type II (heparin-induced thrombocytopenia with thrombosis-HITT), which occurs later (typically after at least 5 days of heparin exposure), is often severe in degree and is frequently associated with extension of, or new, arterial or venous thrombosis. It is an occasional and potentially life-threatening complication of treatment with heparin in prophylactic or therapeutic dosage.

Pathogenesis of HITT

The target antigen for HITT autoantibody is a complex between heparin and platelet factor 4 (PF4). This glycoprotein is released from platelet alpha granules during activation and complexes with heparin, some on the platelet surface. When an IgG autoantibody has developed, it binds to the complex and induces platelet activation through interaction with the platelet Fc-RII receptor. When this receptor is tightly occupied stimulus response coupling occurs, with thromboxane synthesis and platelet release. This leads to platelet consumption in thrombus, with vessel occlusion and thrombocytopenia. Endothelial cell activation may also be involved, perhaps through expression of tissue factor after interaction between autoantibody and heparin-glycosaminoglycan complexes on the endothelial cell plasma membrane.

HITT autoantibodies are predominantly IgG2 and their ability to bind to platelet Fc-gammaRII is deter-
mained in part by a polymorphism in the gene for the receptor (resulting in an Arg-H is substitution at position 131).

Diagnosis and management
The most important diagnostic manoeuvre is the maintenance of a high index of clinical suspicion for the condition. Available tests for the autoantibody are either incompletely sensitive (platelet aggregation and release assays) or not totally specific (immunoassays for heparin-PF4) but assist in clinical assessment. In HIT, heparin therapy must be immediately withdrawn. Where anticoagulant therapy is necessary, the choice usually lies between continuation of coumadin, if oral anticoagulation has been established, and the heparinoid orgaran. Cross-reactivity between HIT autoantibody and orgaran is relatively uncommon. Where absence of cross-reactivity with a low molecular weight heparin preparation can be demonstrated this provides an alternative therapeutic option. More recently lepirudin (a recombinant form of hirudin) has been successfully and safely employed as anticoagulant therapy for thrombosis in subjects with HIT, as well as to cover cardiac surgery and haemodialysis in such individuals. It is a significant addition to the therapeutic armamentarium in HIT.

Thrombotic thrombocytopenic purpura (TTP)
It has very recently been demonstrated that sporadic TTP is an autoimmune disorder in which an IgG autoantibody to plasma von Willebrand factor cleaving protease develops. The enzyme, a metalloprotease, is responsible for the normal processing of secreted vWF through cleavage of the peptide bond between tyrosine 842 and methionine 843 in monomeric subunits, thereby degrading large vWF multimers. Presence of the autoantibody appears to allow the large multimers to circulate and to bind to platelet glycoprotein receptors under high shear conditions and hence to induce platelet aggregation. This accounts for the predominant clinical features-thrombocytopenia and organ dysfunction due to microvascular occlusion by platelet thrombi.

The reason for the transient immune dysregulation in sporadic TTP is unknown, but the above pathogenetic mechanism provides an explanation for the effectiveness of high volume plasma exchange therapy (through removal of large multimers and autoantibody and provision of protease), and for the occasional response to other treatments such as immunoabsorption, immunosuppression and splenectomy.

Other autoimmune thrombophilic states
Recurrent arterial thrombosis and late foetal loss have been attributed to low affinity autoantibodies to vWF which enhance its binding to platelets and lead to platelet activation through antibody interaction with the platelet Fc-γRII receptor. The prevalence of such an autoimmune thrombotic disease is unclear, but the clinical parallels with APS and pathogenetic similarities to HIT are striking.

In a further subject unexplained arterial thrombosis was associated with the presence of an autoantibody against the thrombin anion-binding exosite. The antibody caused prolongation of the plasma thrombin time and inhibited platelet aggregation by thrombin, but also inhibited thrombin-induced synthesis of prostaglandin I2 by cultured endothelial cells and blocked thrombin-thrombomodulin activation of protein C. These last two phenomena suggest that this patient represents a further example of an autoimmune thrombophilic syndrome. It is likely that others will be recognised in the future and that an understanding of the mechanisms involved will lead to more rational therapeutic strategies.

References
selective advantage for those stem cells in which one parental X chromosome is active, relative to the remaining stem cells in which the other parental X chromosome is active. However, whatever the mechanism, the presence of a clonal pattern in granulocytes with polyclonal T cells is clearly not a specific diagnostic marker for the MPD in elderly women.

Despite these limitations some useful information can be gained from clonality studies. In particular, two recent papers have demonstrated that approximately a third of patients with ET have a polyclonal pattern in both granulocytes and T cells.4,5 Although it is not possible to exclude the existence of a subpopulation of clonally derived granulocytes, these data suggest the existence of pathogenetically distinct subsets of patients with ET. It has even been proposed that patients with a polyclonal pattern in both granulocytes and T cells may be at lower risk of thrombotic complications, although this suggestion was based on very small numbers of patients and requires confirmation.

Cytogenetics
Karyotypic abnormalities are rare in ET and no consistent chromosomal changes have been observed. Rare reports of consistent chromosome changes have not been substantiated.

Haemopoietic progenitors
Erythropoietin-independent BFU-E have proved a useful diagnostic test in polycythaemia vera (PV). Similar erythroid colonies are found in ET, although in a smaller proportion of patients.6 Interestingly it has been suggested that many ET patients with erythropoietin-independent BFU-E subsequently develop PV. These results underline the similarities between ET and PV and suggest that erythroid colony assays may be a useful way of distinguishing the two diseases.

The growth of megakaryocyte colonies in the absence of exogenous sources of thrombopoietin has also been investigated. In serum-containing cultures, factor-independent megakaryocyte colonies could be found in the majority of patients with ET and also in a substantial proportion of patients with PV, but not in normal controls or in individuals with reactive thrombocytopaenia.6 However, this assay is difficult to standardise and is not widely available as a diagnostic tool.

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Molecular studies

Haemopoietic progenitors from patients with PV display an abnormal sensitivity to, or a reduced requirement for, several different growth factors. This observation has focused attention on growth factor signalling pathways. In the context of ET this reasoning has been reinforced by the description of two families with inherited thrombocytosis associated with mutations in the thrombopoietin gene.5,6 These mutations resulted in an increased production of thrombopoietin and an increased serum thrombopoietin level. However, no mutation of the thrombopoietin gene has been reported in patients with sporadic ET.

Reduced expression of the thrombopoietin receptor (c-mpl) at both RNA and protein levels has been reported in the platelets of ET patients.9 By contrast, Moliterno et al.10 demonstrated reduced expression in patients with PV and myelofibrosis but not in four patients with ET. The reasons for this discrepancy are not clear, but may involve differences in diagnostic criteria or may reflect pathogenetic heterogeneity. No mutations of the c-mpl gene have been found in ET patients. In the absence of any evidence for mutation of the genes for either thrombopoietin or its receptor in patients with ET, attention is now shifting to intracellular signalling pathways.

Classification and diagnosis

There is an increasing realisation that a number of disease entities may be lumped together under the heading of ET. It may therefore be timely to reconsider how best to classify patients with thrombocytosis (Table 1).

Thrombocytosis is defined as a platelet count greater than 400×10⁹/L. Primary thrombocytosis results from an intrinsic abnormality in megakaryocyte formation. No example of a primary congenital thrombocytosis has yet been described but it seems likely that inherited mutation of the c-mpl receptor or other signalling component may emerge before long. Primary acquired thrombocytosis can result from a number of clonal haematological malignancies including ET and PV. Secondary thrombocytosis is characterised by a normal megakaryocyte compartment responding to an extrinsic stimulus. Inherited thrombopoietin mutations therefore fall into the category of secondary congenital thrombocytosis. Secondary acquired thrombocytosis will include the large number of pathological conditions which are accompanied by increased platelet formation. Finally, idiopathic thrombocytosis is a new term analogous to idiopathic erythrocytosis. What is currently called ET would include patients with idiopathic thrombocytosis. What we need, but currently lack, are good positive diagnostic criteria for ET.

Management

Many patients with ET remain well with minimal treatment. Vascular events provide the main source of morbidity and mortality. The dilemma is that vascular complications, when they arise, can be catastrophic.

Haemorrhage is not common and usually only occurs in the presence of very high platelet counts (greater than 1000×10⁹/L). The major complication is thrombosis, both arterial and venous. Three main factors predispose to thrombosis: increasing age, previous thrombosis and degree of thrombocytosis.11 In addition to its vascular complications, ET can also transform into acute myeloid leukaemia (AML), myelodysplastic syndromes or myelofibrosis. These transformations can all occur in patients who have not received any cytotoxic therapy, but their frequency appears to be influenced by the choice of therapy (see below). When considering the best management for patients with ET it may, therefore, be useful to stratify them into groups with high, intermediate or low risks of vascular events (Table 2).

High risk patients

One of the few clear messages concerning the management of patients with ET is that it is important to lower the platelet count in patients at high risk of vascular events. In 1995 Cortelazzo et al.12 reported a ran-

Table 1. Proposed classification of the thrombocytoses.

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Adapted from Pearson, Diagnosis and classification of erythrocytoses and thrombocytes. Baillière's Clinical Haematology. In press.

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<tr>
<th>Risk category</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>High risk</td>
<td>Age &gt; 60 years, Platelets &gt; 1000×10⁹/L, History of ischaemia, thrombosis or embolic events, Presence of hypertension or diabetes</td>
</tr>
<tr>
<td>Intermediate risk</td>
<td>Age 40-60 years, Absence of high risk features</td>
</tr>
<tr>
<td>Low risk</td>
<td>Age &lt; 40, Absence of high risk features</td>
</tr>
</tbody>
</table>

Table 2. Proposed risk categories for patients with ET.
domised trial comparing hydroxyurea with no platelet lowering drug in high risk patients with ET. Vascular events occurred in 3.6% of patients in the hydroxyurea arm compared with 24% of patients in the control arm over a median follow up of 27 months. Hydroxyurea remains the therapy of choice for most high risk patients. Busulphan is effective but is perceived as having more side effects and as an alkylating agent, it is also more likely to be leukaemogenic. Interferon is also effective, but is limited by cost and also by toxicity, especially in elderly patients.

Anagrelide provides the most exciting potential alternative to hydroxyurea. It acts to lower the platelet count by a novel and poorly understood mechanism. It is not a cytotoxic drug and is, therefore, unlikely to be leukaemogenic, but instead promotes megakaryocyte differentiation. Most strikingly it does not suppress the white cell count or haemoglobin. However, it is a positive inotrope drug and a vasodilator, thus explaining its major side effects of palpitations, headaches and fluid retention. Moreover, anagrelide does not reduce megakaryocyte numbers and the significance of this for the subsequent development of myelofibrosis is unknown. The results of prospective randomised trials comparing hydroxyurea and anagrelide, such as the ongoing MRC Primary Thrombocythaemia trial, should be very informative.

Low risk patients
There is considerable evidence that young patients with ET have a particularly low risk of vascular events. In the absence of any high risk features, such as previous thrombosis or a very high platelet count, it therefore seems reasonable to treat such patients with aspirin alone (75 mg/day). However, it is worth emphasising that the natural history of ET in young patients is obscure, and the incidence of late complications including myelofibrosis remains unknown.

Intermediate risk patients
The management of this group of patients provokes the greatest controversy. Lowering the platelet count exposes patients to the potential side effects of the various platelet-lowering drugs. However, not lowering the platelet count is likely to increase the risk of potentially catastrophic vascular events. Randomised data will be needed to provide rational guidance.

Is hydroxyurea leukaemogenic?
This issue is much discussed but the facts are relatively simple. There is currently no convincing evidence that hydroxyurea increases the incidence of leukaemia when it is used alone to treat ET. Reports of the leukaemogenicity of hydroxyurea are based on anecdotal case reports or on retrospective and therefore systematically biased studies. Retrospectively comparing treated and untreated patients is misleading since the two groups are biologically different.

In a large recently reported study, 251 patients were treated with hydroxyurea alone or in combination with other such cytotoxic agents, and followed up for eight years. The frequency of acute leukaemia or myelodysplastic syndrome was 3.5% in patients receiving hydroxyurea alone and 14% in patients receiving hydroxyurea in combination with other cytotoxic agents. In the absence of any therapy with cytotoxic drugs, the background frequency of AML/MDS in patients with ET is unknown.

What about the use of hydroxyurea in PV? A recent report compared hydroxyurea with pipobroman in the treatment of patients with PV. The risk of leukaemia in patients receiving hydroxyurea was approximately 5% at ten years and therefore comparable to the 3.5% observed in patients with ET. The risk of leukaemia was reported as increasing to 10% over thirteen years but these figures are very unreliable since only a small minority of patients remained under observation at thirteen years. Unlike ET, there are some data on the background incidence of leukaemia in patients not receiving cytotoxic drugs. A 1.5% incidence of leukaemia was reported in patients entered into the phlebotomy-only limb of the PVSG-01 study. However, this figure is misleading for two reasons. First, it was not based on an intention to treat basis. Patients requiring cytoreductive therapy were censored from analysis and the figure of 1.5% therefore relates to a highly selected group of patients. Secondly, the number of patients managed by phlebotomy alone diminished rapidly over time to 10% by the tenth year. Thus the median follow up time is significantly shorter than the other limbs of the PVSG-01 trial.

The jury therefore remains out. Existing data do not demonstrate that hydroxyurea is leukaemogenic when used alone to treat patients with ET or other MPD.

References
5. Harrison CN, Gale RE, Machin SJ, Linch DC. A large proportion of patients with a diagnosis of essential thrombocythaemia do not have a clonal disorder and may be at lower risk of thrombotic complications. Blood 1999; 93:417-24.


Hepatic veno-occlusive disease (VOD) was first described after ingestion of bush tea in Jamaica. Toxic pyrrolizidine alkaloids led to obstruction of small intrahepatic venules.

The most relevant condition associated with VOD today is high dose radiochemotherapy followed by bone marrow or peripheral blood stem cell transplantation. According to our present knowledge, the toxicity of the myeloablative therapy in addition to risk factors and secondary events is responsible for this complication. For transplant patients VOD represents the third leading cause of death and the most severe regimen-related toxicity.

Due to an increasing number of patients treated with high dose chemotherapy and stem cell transplantation the incidence of VOD may rise in the future. On the other hand, the modifications of pre-transplant conditioning regimens (mini transplants) in the allogeneic setting may contribute to a reduction of VOD in this population of patients.

Definition and incidence of VOD

Two definitions of VOD are used: one proposed by McDonald et al. from the Seattle group and the other by Jones et al. from Baltimore.

The first study was performed retrospectively by the Seattle group in 1984 and described an incidence of 22% with fatal outcome in 45%. The definition included two of these four criteria: hyperbilirubinaemia >1.6 mg/dL, painful hepatomegaly and ascites or weight gain before day 30.

In a more recent prospective analysis by the same group the definition was modified and included two of the following events: hyperbilirubinaemia >2 mg/dL, hepatomegaly or right upper quadrant pain of liver origin, and sudden weight gain exceeding 2% of baseline body weight due to fluid retention until day 20 post-transplant. Considering these criteria the incidence of VOD increased to 54% and the mortality rate for patients with VOD before day 100 amounted to 39%.

In this study a widely used classification of VOD was introduced. It was classified as mild (no medication required, complete resolution) in 12% moderate (requirement of sodium restriction, diuretics, analgesics, complete resolution of liver injury) in 26% or severe (adverse effects from liver damage, symptoms and laboratory abnormalities without resolution before day 100 or death, whichever occurred first) in 15% of the patients. The corresponding day 100 fatality rates were 9%, 23% and 98%, respectively.

In another earlier study the Baltimore group defined VOD as follows: hyperbilirubinaemia equal or greater than 2 mg/dL and at least two of the following three criteria: hepatomegaly, ascites, or weight gain of at least 5% before day 21. In this study a VOD rate of 22% and a fatality rate of 45% was reported.

A remarkably lower rate was recently reported by Carreras et al. in an EBMT survey including 1,652 patients. In this largest evaluation to date, VOD was defined as the occurrence of at least two of the following criteria: hyperbilirubinaemia >2 mg/dL, ascites or weight gain (>5% of baseline body weight), and painful hepatomegaly before day 21. A rate of 8.9% after allogeneic and 3.1% after autologous transplants was reported. Whereas in the EBMT trial only 29% of the patients received allogeneic transplants, the vast majority of patients in the Seattle trial were transplanted from allogeneic donors.

When considering several risk factors, in the European study the incidence of VOD for allogeneic transplant recipients with a Karnofsky performance score <90% who received high dose chemotherapy, was 25%. This rate is comparable to the incidence found in the Seattle and Baltimore analyses.

These differences in the VOD rate point to the importance of the definition used, the composition of the population studied in terms of autologous or allogeneic transplants, and pre-existing risk factors.

Pathophysiology

The pathophysiology of VOD is incompletely understood. Endothelial cell injury due to the toxicity of the conditioning regimen, however, is believed to be the first step in the pathogenesis. The observation, that TNFα is elevated in patients with VOD, that TNFα exerts procoagulant and hypofibrinolytic effects and that plasminogen activator inhibitor 1 (PAI-1), the main inhibitor of the fibrinolytic system,
is remarkably elevated in VOD patients led us to propose a two step model of the pathogenesis of VOD. The initial conditioning-induced endothelial cell damage is followed by a second step resulting in Kupffer cell activation and cytokine release, which induces the release of PAI-1 from endothelial cells thereby promoting hypofibrinolysis. This second event may be the translocation of bacteria through the damaged gastrointestinal mucosa leading to endotoxaemia. Furthermore, the possible relevance of a depletion of glutathione, which can be provoked by radiochemotherapy and may promote hepatocyte or endothelial cell damage, is presently being investigated. Deleve et al. have demonstrated that radiochemotherapy-induced alterations in glutathione levels leading to increased vulnerability of the endothelium are linked to endothelial cell damage.

Risk factors for the development of VOD

McDonald described elevated pre-transplant transaminase levels, high dose chemotherapy and persisting fever during conditioning as independent indicators of severe VOD. Amphoter cin was associated with an increased risk of severe VOD and vancomycin and acyclovir with an increased risk of a fatal outcome. Jones confirmed a pre-existing enzyme elevation (aspartate aminotransferase, AST) as a risk factor by multivariate analysis, whereas the conditioning regimen and type of graft did not influence the VOD incidence. In 1998, Hägglund described pre-transplant nor ethisterone treatment, bilirubin >26 µmol/L before BMT, one HLA-mismatch, previous abdominal irradiation and conditioning with busulphan as risk factors for VOD in a multivariate analysis.

In the large EBMT survey, Carreras et al. described elevated levels of AST before transplant, allogeneic BMT, high dose cytoreductive therapy, Karnofsky performance score <90% and prior abdominal irradiation as independent variables associated with an increased risk of VOD. In this analysis VOD was classified as being mild, moderate and severe in 8%, 64.4% and 27.6%, respectively.

Patients with pre-existing hepatitis are at a high risk of developing VOD. To predict the course of VOD after conditioning, Bearman et al. developed a calculation model which was found to be highly specific and moderately sensitive.

Clinical features and differential diagnosis

Patients typically present with an increase of bilirubin, hepatomegaly, right upper quadrant pain, ascites and otherwise unexplained weight gain. Whereas sudden weight gain and hepatomegaly are early signs occurring as soon as day 0, hyperbilirubinemia (median day 6), peripheral oedema (day 7) and ascites (day 12) follow at later stages with remarkable interindividual differences. Although VOD is a complication of the early post-transplant phase, manifestations at later stages can occur.

Differential diagnosis includes other toxic liver injury, mostly due to the conditioning regimen, cyclosporine, antibiotics, methotrexate or other medication in the early phase after transplantation. Hepatic graft-versus-host disease (GvHD) is another important differential diagnosis. It is rarely associated with ascites. In the majority of cases GvHD of the liver manifests later than VOD and is often associated with skin or gut symptoms. Nevertheless, there is a notable overlap between VOD and hepatic GvHD symptoms and signs.

Fungal and viral infections or cholangitis in the course of sepsis must be considered, although liver damage in hepatitis B and C is mediated by a functioning immune system. In addition, cardiac, renal and pancreatic diseases may cause abnormal liver function and/or ascites and must be distinguished from VOD.

Making the diagnosis - histological, laboratory and technical investigations

VOD can not be adequately diagnosed by clinical criteria alone, but is most reliably confirmed by biopsy. Characteristic histological findings have been reported by Shulman. Severe VOD is associated with the histological findings of venous occlusion, zone 3 hepatocyte necrosis, sinusoidal fibrosis and eccentric luminal narrowing or phlebosclerosis. Deposition of fibrin and von Willebrand factor and fibrous vessel obliteration were also reported earlier by the same author.

Since VOD is a complication occurring during the early post-transplant phase, patients suffer from severe thrombocytopenia and impaired coagulation at the time of symptoms. Therefore, percutaneous biopsy is associated with a high risk of severe or even fatal bleeding complications, which has to be weighed against the potential benefit of having a biopsy sample for histological analysis. Additionally, due to a patchy pattern of histologic VOD alterations and the coincidence of hepatic VOD and GvHD, biopsy may yield false negative results. The risk of severe bleeding complications can be reduced by using transvenous techniques, e.g. transjugular biopsy.

Other proposals for diagnosing VOD have been made. Several ultrasound studies have been published. Gray scale and Doppler ultrasound criteria, including hepatosplenomegaly, gall bladder wall thickening, ascites, paraumbilical vein recanalisation and modulation of intrahepatic vessel diameters and flow characteristics have been published by several groups. A sensitivity and specificity of 83% and 87%, respectively, at the most accurate cut-off level of the total score was described by Lassau. An enhancement of the wedge pressure in the hepatic vein (>9 mm Hg) has been demonstrated to be a highly specific parameter for VOD.
Several laboratory parameters can be helpful in the case of patients with hyperbilirubinemia and suspected VOD. High levels of plasminogen activator inhibitor 1 (PAI-1), the main inhibitor of the fibrinolytic system, was found to be very specific and sensitive for diagnosing VOD, whereas normal levels were found in patients with GvHD and other causes of hyperbilirubinemia. These results have been confirmed by prospective weekly PAI-1 screening of transplant recipients in our centre (unpublished data) and point to a possible involvement of hypofibrinolysis in the pathogenesis of VOD.

Protein C, the natural anticoagulant, is reduced in most symptomatic patients and has been described to be indicative of VOD even before conditioning. Nevertheless, it is unclear at the present time whether low protein C levels are a cause or symptom of VOD.

Procollagen III peptide levels have also been reported to be raised in VOD patients. A reduction of antithrombin and protein S as well as increases of von Willebrand factor, F VIII, fibrinogen, tissue factor or F VII have been described in smaller surveys.

A typical phenomenon in VOD patients is a high requirement for platelet transfusions and persistent thrombocytopenia after engraftment. The pathophysiology is not understood but the low platelet count is not due to diminished hepatic thrombopoietin synthesis according to data recently published by Oh et al.

Prophylaxis

Recommendations for the prophylaxis of VOD are contradictory. Heparin, either in unfractionated or low molecular weight form, is used in some centres. Attal et al. showed, in a randomised trial with 161 patients, a significant reduction of VOD in the group treated with unfractionated heparin (2.5 vs. 13.7%, p<0.01) but the study contained only a small proportion of patients (11%) at high risk of VOD. In the EBMT survey and other large investigations the use of heparin in patients with or without risk factors did not influence the incidence or mortality of VOD. Larger randomised trials with precisely defined inclusion criteria are warranted to elucidate the role of heparin in the prophylaxis of VOD definitively.

Prostaglandin E and ursodeoxycholic acid have been used in some studies and beneficial effects have been reported, but the surveys have been too small to draw definite conclusions.

Pentoxyphylline, which inhibits TNFα, has shown positive effects in smaller non-randomised trials, but these results were not confirmed in randomised trials.

Therapy

Thrombolytic therapy with recombinant tissue plasminogen activator (rtPA) was successfully used in single patients and small surveys. In another report VOD was resolved in two patients who received rtPA in conjunction with antithrombin. In one case report VOD, which was associated with severe capillary leak syndrome, was successfully treated with a combination of rtPA and C1-esterase inhibitor concentrate.

Whereas fast resolution of symptoms occurred in some patients after thrombolytic therapy with rtPA, severe bleeding complications were observed in others. The analysis of the 42 patients treated in Seattle—the largest analysis so far—yielded disappointing results. Symptoms improved in 29% of the patients, but severe bleeding episodes were observed in ten patients. Requirement for mechanical ventilation, supplemental oxygen and dialysis before thrombolytic therapy were indicators of no response. These data are in accordance with the experience at other centres, showing that patients with multiorgan failure are not candidates for thrombolytic therapy.

Several shunting techniques including transjugular intrahepatic portosystemic shunts (TIPS), have been proposed for the management of severe VOD and, in isolated cases, liver transplants have been performed. Nevertheless, none of these therapeutic procedures can be regarded as standard therapy.

Recently, Richardson et al. reported on their experience with defibrotide, a polydeoxyribonucleotide, known to enhance tPA and thrombomodulin and lower PAI-1 expression in endothelial cell cultures. Nineteen patients with severe VOD were treated. The drug was well tolerated and in 8 patients of this high risk group a reversal of hyperbilirubinemia and other VOD symptoms was achieved; 6 patients survived beyond day 100. No severe haemorrhage occurred. These data point to the need for further investigation of defibrotide, a drug which holds promise of improving VOD therapy in the future.

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References

A more extensive list of all references can be obtained upon request from the corresponding author.


Donor selection-matching for mismatches

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Selection for voluntary unrelated donors (VUD) for unrelated bone marrow transplantation (BMT) generally relies on matching for HLA-A, B and DR antigens. Initial studies have shown that recipients of transplants from unrelated donors matched for these HLA antigens have a lower risk of acute graft-versus-host disease (GvHD) than recipients of marrow from mismatched VUD, but this risk is still much higher than that observed for HLA-identical sibling transplants. Improvement of overall survival was not apparent in HLA-A, B, DR matched unrelated recipients when compared with mismatched related recipients. Thus, the effect of HLA matching on survival after unrelated transplantation has been controversial. It is now becoming clear that multiple factors could be associated with these results such as a) the presence of unidentified HLA-A, B and DR disparities due to the low resolution HLA typing techniques used, b) incompatibility for other HLA loci such as HLA-C, DQ and DP, c) mismatched minor transplantation antigens and d) non-HLA factors.

At present, typing of most unrelated individuals for class I antigens is still achieved with serology. In addition to serological typing, cellular assays, e.g. the MLC reaction have served for a secondary definition of class II molecular diversity. During the past 10 years, over 800 new HLA alleles have been recognised by DNA sequencing analysis and many more probably remain to be identified. With the discovery of these new alleles, it became obvious that serological specificities comprise multiple undetected molecular subtypes. For example, there are currently over 100 recognised HLA-A alleles and of these only a fraction can be typed by routine serology. Improvements in DNA-based methods for the detection of the many HLA alleles have provided the opportunity to investigate the relationship between HLA disparity and transplant complications.

Tissue typing techniques are limited by the extensive polymorphism of HLA genes. A pairwise comparison of the nucleotide sequences of known HLA alleles indicates that genetic recombination has played a key role in the generation of HLA diversity. Inter-allelic conversion or double recombination is the principal mechanism which generates HLA diversity. For HLA class I alleles the highest frequencies of substitutions occur within exons 2 and 3, which encode the α1 and α2 domains of the HLA molecules and for practical purposes 97% of HLA class I alleles can be identified by sequence-based analysis of exons 2 and 3. In contrast, for HLA class II alleles, the highest frequency of substitutions occurs mainly in exon 2 of the β chain.

Molecular typing methods include restriction fragment length polymorphism analysis (RFLP), sequence-specific primer amplification (SSP), hybridisation with sequence-specific oligonucleotide probes (SSO), heteroduplex analysis, single strand conformation polymorphism (SSCP), and direct nucleotide sequencing. The techniques most commonly used are SSP and SSO. SSP utilises group and/or allele specific sequences for PCR primer design and SSO typing is based on the identification of allele specific sequences using oligoprobes, which either alone or in certain combinations allow allele identification. Heteroduplex analysis and SSCP allow comparison of the conformation of DNA molecules, and these are used as supplementary methods by tissue typing laboratories for allelic subtyping. Direct sequencing in principle allows the most accurate typing of HLA alleles.

We have recently described a novel high resolution DNA-based typing technique which offers a level of resolution similar to direct sequencing techniques, without the problem of heterozygous ambiguities.

The method, now known as Reference Strand-mediated Conformation Analysis (RSCA), analyses the conformation of HLA DNA duplexes. Chimaeric duplexes are formed between a locus-specific fluorescently labelled reference strand (FLR) and the locus specific allele from the sample to be typed. The duplexes formed are resolved by polyacrylamide gel electrophoresis (PAGE) in an automated DNA sequencing instrument with a laser-based fluorimetric detection system. Only duplexes formed with labelled reference sense strands are observed, i.e. two bands for a homozygous sample (labelled reference homoduplex and labelled reference/allele duplex) and three bands for a heterozygous sample (labelled reference homoduplex and two labelled reference/allele duplexes). This method is simple and easy to use, in contrast to the other DNA based methods described previously, which require a large number of group and/or allele specific probes or primer mixes for HLA class I alleles and thus cannot achieve such high level of resolution.
As these incompatibilities may be invisible in routine matching techniques, cellular assays have been developed in an attempt to confirm patient/donor identity. Limiting dilution analysis has proved to be a sensitive tool for the detection and investigation of T lymphocytes of defined specificity. The cytotoxic T lymphocyte and helper T lymphocyte precursor (CTLp and HTLp) assays use limiting dilution analysis to quantify the frequency of donor cytotoxic T and helper T cell precursors capable of responding to mismatched HLA antigens present on the patient’s cells. High CTLp frequencies correlate with class I mismatches usually undetected by conventional typing, whereas HTLp appears capable of detecting class II differences.

How much do we have to match? Many studies have attempted to evaluate the clinical contribution of 0, 1, 2 and multiple mismatches, but by using high resolution DNA typing it is becoming clear that once a mismatch is detected for one locus, there is a high probability of an associated mismatch for another locus. This is understandable given the strong linkage disequilibria of HLA. We have observed a high number of associated hidden mismatches between HLA-C and either HLA-A or B mismatches and there seems to be a clear additive effect of additional mismatches in relation to GvHD and TRM. As HLA types are increasingly defined to higher degrees of resolution, so the probability of finding a completely matched VUD is reduced. Until the high resolution DNA typing, an understanding of HLA matching and mismatching will be imprecise and the identity of donor/recipient pairs are resolved by high resolution DNA typing. Therefore, the route to take must be to choose the donor who is most acceptable from those matched to the allelic level, for HLA and other polymorphic genes which can affect transplant outcome. This decision will only be possible when a definitive hierarchy of factors has been established with respect to transplant outcome and many scientists are currently working towards this goal.

References

Cytomegalovirus (CMV) infection remains a major cause of morbidity and mortality after allogeneic stem cell transplantation. Due to the broad application of antiviral prophylaxis and preemptive therapy, a decrease in early onset and a subsequent increase in late onset CMV disease has been observed. Transplantation from unrelated donors or related donors mismatched for one or more HLA class I or II alleles is clearly associated with a prolonged and more profound deficiency of CD3+, CD4+ and CD8+ T-cell populations when compared with recipients of a BMT from a related donor. Thus, these patients are at increased risk of early and late onset CMV disease, especially when receiving T-cell depleted grafts. Sensitive diagnostic assays using nucleic acid amplification and hybridisation techniques have been commercialised and will allow standardisation of CMV diagnosis in antiviral drug trials. Quantification of the viral load will be increasingly considered for initiation and, in patients with persistence of high viral titres despite antiviral therapy, screening for antiviral drug resistance. Apart from ganciclovir, clinical data are emerging that foscarnet can be given safely even after allogeneic SCT. Additional drugs such as lobucavir and cidofovir have been used for specific indications. Furthermore, immunotherapeutic approaches are being increasingly evaluated for the control of CMV infection following allogeneic stem cell transplantation.

Pathophysiology of cytomegalovirus infection

After primary infection, cytomegalovirus (CMV) establishes latency in the host characterised by the persistence of viral genomes without production of infectious virus. Despite the fact that latent CMV is obviously transmitted through transfusion of blood products, bone marrow grafts and solid organs, cells harbouring latent virus were not identified until recently. Reactivation of CMV in long-term cultures of allogeneically stimulated adherent monocyte-derived macrophages was demonstrated, with infectious virus detected 26-61 days after allogeneic stimulation. The cells harbouring the virus were found to be CD14 and CD83 positive, thus providing evidence that myeloid lineage cells are the source of latent CMV, which might reactivate upon allogeneic stimulation. Latency-associated CMV transcripts were found in CD33+ subpopulations co-expressing myeloid and dendritic cell markers. Co-culture of latently infected CD33+ cells with permissive human fibroblasts showed reactivation of infectious virus after 3-4 weeks of culture.

CMV has been reported to cause myelosuppression after transplant. A significant reduction in the number of haematopoietic progenitor cells was reported, and an association of particular CMV genotypes with death due to myelosuppression described. CMV has evolved diverse mechanisms for evading host cellular immunity: CTL lysis is inhibited by multiple glycoproteins which inhibit MHC class I expression, and US6 which inhibits TAP-peptide-complex translocation, the CMV encoded class I homologue UL18 blocks NK cell lysis, and CMV also inhibits MHC class II expression via compromise of the JAK/STAT pathway.

The predominance of CMV disease in patients with impaired cellular immunity indicates the pivotal role of cell-mediated immunosurveillance. Patients lacking CMV-specific CD8+ and CD4+ T cells are at an increased risk of developing CMV disease upon CMV post-transplant reactivation.

Transplantation from unrelated donors or related donors mismatched for one or more HLA class I or II alleles is clearly associated with a prolonged and more profound deficiency of CD3+, CD4+ and CD8+ T-cell populations when compared to a BMT from a related donor. Thus, these patients are at increased risk of early and late onset CMV disease, especially when receiving T-cell depleted grafts. Consequently, fatal opportunistic infections after successful engraftment occur in 12-28% of unrelated transplant recipients compared to in only 4-15% after HLA-matched sibling BMT.

Risk factors and manifestations of CMV disease

In spite of recent developments in diagnosis and treatment, CMV infection remains one of the most important opportunistic infections in recipients of an allogeneic stem cell transplant (SCT). The incidence of CMV infection increases with severity and duration of immunosuppression and approaches 70% in allogeneic SCT recipients being either CMV-seropositive and/or receiving a transplant from a CMV-sero-
positive donor. CMV disease is associated with a high mortality (70%) in this patient population, even when treated with antiviral chemotherapy (ganciclovir, famciclovir, foscarnet) plus CMV hyperimmunoglobulin.\(^2\)

The development of sensitive screening assays that allow early pre-emptive initiation and monitoring of antiviral therapy, as well as antiviral chemoprophylaxis, have helped to reduce early post-transplant CMV-associated mortality significantly. However, patients receiving a transplant from a matched unrelated donor and T-cell depleted grafts are still at a high risk of developing early onset CMV disease. T-cell depletion by 2-3 logs as achieved by CD\(^34^+\) selection using immunoabsorption techniques is associated with an increased risk of CMV reactivation, but not CMV disease, at least in patients receiving a stem cell graft from an HLA-identical sibling donor.

The epidemiology of CMV with the introduction of pre-emptive and prophylactic antiviral strategies has changed markedly, with increasing reports of CMV disease occurring late (>100 days post-transplant), but less frequently during the first 100 days post-transplant. In a minority of CMV-seropositive patients, CMV disease occurs prior to engraftment and, probably due to the frequent presence of co-pathogens, has been associated with a very high mortality.

Thus, late onset CMV disease occurring at least 100 days post-transplant has now become the leading CMV-related complication after allogeneic SCT. In two studies, late onset CMV disease was associated with chronic graft-versus-host disease and long-term ganciclovir application. Clinical manifestations of CMV infection that had been only rarely observed early post-transplant, e.g. CMV retinitis, have been described in patients developing late onset CMV disease.

Diagnosis of CMV infection

Qualitative PCR and antigenaemia assays have been used in recent years to screen patients undergoing allogeneic SCT. Due to the low incidence of CMV disease in autograft recipients, monitoring by sensitive assays should be limited to patients after allogeneic SCT and possibly to autograft recipients at very high risk of CMV disease. T-cell depletion by 2-3 logs as achieved by CD\(^34^+\) selection using immunoabsorption techniques is associated with an increased risk of CMV reactivation, but not CMV disease, at least in patients receiving a stem cell graft from an HLA-identical sibling donor.

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To increase the value of nucleic acid amplification techniques for positively predicting the occurrence of CMV disease, materials other than whole blood or buffy coat, such as plasma or serum, were used. A study showed plasma PCR to be less sensitive than PCR on whole blood and antigenaemia. In a limited number of patients the results of a commercially available PCR assay on plasma samples were comparable to those of a pp65 antigenaemia assay in a cohort of immunocompromised patients including recipients of an allogeneic SCT.

To improve the positive predictive value of PCR assays further, reverse transcriptase (RT) PCR assays for detection of immediate-early and late viral mRNAs have been evaluated in a limited number of studies. However, since these techniques are more time-consuming and less sensitive, despite having a higher positive predictive value than amplification of DNA, further studies are warranted before these assays can be recommended for routine screening of clinical samples. Recently, an assay based on the amplification of late viral pp67 mRNA was commercialised and a first clinical study has demonstrated its potential for early diagnosis of CMV infection and monitoring of antiviral therapy.

In conclusion, major progress has been achieved concerning quantification of the viral load in the blood, which might help to improve monitoring of antiviral therapy. The availability of commercialised sensitive assays will allow standardisation of CMV diagnosis in upcoming multicentre antiviral drug trials.

Prophylaxis of CMV infection

To prevent the acquisition of exogenous virus in seronegative patients receiving a transplant from a seronegative donor is of major importance. Primary infection can be prevented efficiently by transfusion of blood products from CMV-seronegative donors. Alternatively, leukocyte-depleted blood products have been shown to be safe, if a leukocyte depletion of at least three logs can be provided.\(^5\)

Antiviral prophylaxis to suppress reactivation of CMV if either patient and/or donor is seropositive is a very efficient approach to prevent CMV infection and disease. High-dose intravenous acyclovir at a dosage of 500 mg/m\(^2\) three times a day until day +30 slightly reduced the probability of and delayed the onset of CMV infection. Most importantly, survival could be significantly improved by high dose intravenous compared to low dose oral acyclovir. Although prolongation of acyclovir prophylaxis beyond day +30 did not further reduce the risk of developing CMV infection, survival seemed to be further improved.\(^6\) Valaciclovir, a valine ester of acyclovir that is rapidly metabolised
to produce high acyclovir levels, applied prophylactically was found to reduce CMV disease in PCR positive patients with advanced AIDS.

Prophylaxis with intravenous ganciclovir in allogeneic BMT recipients at risk, given from the time of engraftment until day +100 post-transplant, has been assessed in 2 studies. In both studies, a significant reduction of the incidence of CMV infection and in one study also of CMV disease was demonstrated. Moreover, in both studies survival of patients receiving ganciclovir was comparable to the survival of patients in the high-dose acyclovir trial. However, ganciclovir prophylaxis was associated with significant toxicity, specifically therapy-related neutropenia and secondary bacterial infections. Hyperbilirubinemia > 6 mg/dL before day 20, low marrow cellularity at day 21, and a serum creatinine > 2 mg/dL after day 20, but not CMV infection or disease, were reported as major risk factors for ganciclovir-related neutropenia. Oral ganciclovir is currently being evaluated as a new antiviral modality for CMV prophylaxis.

Antiviral therapy for documented CMV infection (pre-emptive therapy)

As the outcome of patients treated for overt CMV disease is poor, pre-emptive or early antiviral therapy has been introduced as a therapeutic strategy. In two large studies early treatment with ganciclovir based on a positive virus culture assay, was evaluated. Ganciclovir was either administered at the time of first CMV excretion from blood, urine or throat washing samples or from a bronchoalveolar lavage sample taken at day 35 post-transplant. In both studies, pre-emptive antiviral therapy reduced CMV disease and transplant-related mortality. However, 12-13% of patients presented with CMV disease before or coincident with CMV excretion, leading to a 10% CMV-related mortality.

Thus, more sensitive techniques were used to detect CMV infection prior to the onset of CMV disease. CMV was detected up to 20 days earlier by PCR than by the culture technique. Comparison of PCR and culture-based pre-emptive antiviral therapy demonstrated that PCR screening allowed antiviral therapy to be started significantly earlier than culture results. Additionally, stopping and withholding antiviral therapy was found to be safe in the PCR-negative patients. The incidences of CMV disease and CMV-related mortality were decreased, and the duration of antiviral therapy significantly shorter in the PCR-monitored group leading to a reduced incidence of ganciclovir-related side effects such as neutropenia and non-viral infections. Overall survivals at day 100 and 180 post-transplant were significantly improved in the PCR-monitored group. Boeckh et al. compared antigenaemia guided antiviral therapy and ganciclovir prophylaxis in 226 CMV-seropositive allogeneic marrow transplant recipients. Fourteen percent in the antigenaemia-ganciclovir group developed CMV disease before day 100 compared to 2.7% in the ganciclovir group. Low-grade antigenaemia progressed to CMV disease only in patients with grades III and IV acute graft-versus-host disease. The incidence of CMV disease until day 180, CMV-related death, transplant survival, and neutropenia were not significantly different between the 2 groups at day 180 after transplantation. Ganciclovir at engraftment was associated with a higher rate of early invasive fungal infection and late CMV disease resulting in a similar survival rate in both groups.

Foscarnet (PFA), a pyrophosphate analogue with in vitro activity against all known human herpes viruses, does not cause myelosuppression but is associated with significant nephrotoxicity. In earlier studies, foscarnet was found to prevent CMV disease in recipients of an allogeneic SCT, and using adequate prehydration even a combination of ganciclovir and foscarnet could be safely administered to high risk patients. For CMV prophylaxis following allogeneic SCT, foscarnet (60 mg/kg/day) was administered with low toxicity to patients who could not receive ganciclovir for delayed engraftment or ganciclovir-induced neutropenia. However, in 15% of patients CMV was detected while they were receiving prophylaxis and 2 (5%) died of CMV disease. The results of the phase III EBMT protocol comparing pre-emptive therapy with ganciclovir versus foscarnet based on antigenaemia or PCR assay results will further elucidate the role of foscarnet in treating patients undergoing allogeneic stem cell transplantation with documented CMV infection. Lobucavir, a desoxyguanine nucleoside analogue with broad-spectrum antiviral activity, has been shown to be active against CMV. As lobucavir phosphorylation can occur in the absence of viral phosphorylases, lobucavir may be another alternative drug to treat ganciclovir-resistant strains of CMV.

Cidofovir has been shown to be an effective and, due to its long half-life allowing a weekly administration, convenient systemic treatment for CMV retinitis. Promising results with the administration of this drug also in marrow transplant recipients were reported at the EBMT-Congress in Hamburg. Antiviral resistance has been reported more frequently, and prolonged administration of antiviral compounds has been shown to predispose for selection of resistant viral strains. Thus, molecular screening for mutations in the UL97 and DNA polymerase gene might be indicated in patients with persistence of high viral load in the blood despite antiviral therapy. Moreover, new antiviral compounds are needed to increase the therapeutic armamentarium against CMV.

Immunotherapy of CMV infection and disease

Transplantation from unrelated donors or related donors mismatched for one or more HLA class I or II alleles is clearly associated with a prolonged and more
profound deficiency of CD3+, CD4+ and CD8+ T-cell populations in recipients when compared to a transplant from a related donor. Thus, these patients are at increased risk of early and late onset CMV disease, especially when receiving T-cell depleted grafts. Consequently, fatal opportunistic infections after successful engraftment occur in 12-28% of unrelated transplant recipients compared with in only 4-15% after HLA-matched sibling BMT.

Adoptive immunotherapy with small numbers of unirradiated donor leukocytes was recently found to be associated with rapid restoration of CD3+, CD4+ and CD8+ T-cell numbers, antigen-specific T-cell responses, and resolution of CMV- and EBV-associated disease after unrelated T-cell depleted BMT.

The prophylactic transfer of CMV-specific CD8+ T-cell clones early post-transplant has been shown to provide protection against CMV disease. However, long-term in vivo persistence of transferred CTLs required the development of an endogenous CMV-specific T helper response. A phase II study is currently evaluating co-administration of CD4+ CMV-specific Th clones and CD8+ CMV specific CTL clones.

Alternatively, peptide vaccines are a possible approach. Diamond et al. mapped an HLA-A*0201 restricted nonamer peptide out of the immunodominant pp65 protein. This peptide was used to amplify a memory CTL response in CMV-seropositive individuals. Lipid modification of the amino terminus of the nonamer peptide resulted in its ability to simulate peptide-specific primary T-cell responses without the use of adjuvant in a transgenic mouse model.

Conclusions
Pre-emptive as well as prophylactic antiviral treatment strategies have helped to reduce CMV-associated mortality significantly in recipients of an allogeneic BMT. Pre-emptive antiviral therapy, compared to antiviral prophylaxis, has the advantage of being able to be used in patients stratified according to individual risk factors (active CMV infection, viral load) and thus helps to reduce the number of patients treated and also the duration of antiviral therapy, which might have important implications concerning side effects and emergence of antiviral resistance. In the future, immunotherapeutic strategies, e.g. donor vaccination and transfer of donor-derived CMV-specific T-cells to patients in a prophylactic or therapeutic setting will be more broadly applied and evaluated for feasibility and efficacy following allogeneic stem cell transplantation.

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References
Improved results in marrow transplantation from unrelated donors

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UD-BMT and HLA disparity

Major improvement has been achieved by molecular typing for HLA class II antigens: in an EBMT review of 366 patients, 210 were matched for DRB1. Their TRM was 49%; 31 were DRB1 mismatched and their TRM was 79% (p=0.00002).5 More recently HLA class I antigens are being matched by molecular typing and it is now possible to assess the influence of mismatching at class I or class II:6

- risk of aGvHD is highest with class II mismatching;
- mismatching at one single allele for class I or class II does not affect survival;
- mortality is increased for mismatching at more than one class I allele and by simultaneous mismatching for class I and class II alleles.

In a recent analysis of the Italian Group for Bone Marrow Transplantation (GITMO) TRM was predicted by HLA matching: event free survival was 55% for DRB1 matched pairs and 15% for DRB1 mismatched patients (p=0.0006).7

At present a minimum requirement for an UD-BMT in adults would be class I identity in serology (including split antigens) and class II identity in molecular biology (DRB1). A more stringent requirement would be identity at the molecular level for class I and class II.

The report from the Seattle group of a comparable mortality in the presence of one single antigen mismatch should be confirmed by other groups.

An additional approach is matching for ancestral haplotypes: a pilot study is ongoing within the GITMO for UD-BMT in thalassaemia, with encouraging results (La Nasa, personal communication). However, this implies knowledge of ancestral haplotypes and a geographical environment which donor and recipients are likely to share (Sardinia is one example).

The relevance of HLA-C mismatching has also been studied: an increased risk of graft failure has been reported in HLA-C mismatched, otherwise matched pairs.6 These data need to be confirmed: one should remember that approximately 50% of HLA-A, B, DRB1 matched pairs have disparities at the HLA-C locus.

In vitro functional tests for donor selection

The mixed lymphocyte reaction (MLR) is no longer considered a reliable test for identifying unrelated donors, and the test is not required to have access to...
donors. Cytotoxic T cell precursors (CTL-P) have been extensively studied in some centres, and a high frequency is known to be associated with mismatching at class I. Helper T cell precursors (HTL-P) have also been studied, but conclusive results on their predictive value in GvHD is lacking. The same can be said of the mixed skin-lymphocyte reaction.

How transplant related mortality can be reduced

TRM remains high in UD-BMT and the encouraging results achieved in chronic myeloid leukaemia (CML) patients grafted within one year from diagnosis (74%) do not necessarily apply to all patients, especially those with advanced disease, with a long interval from diagnosis to BMT and the elderly. If we want to reduce transplant mortality we need to:

1. improve immunologic recovery;
2. improve haemopoietic recovery;
3. reduce GvHD.

To achieve these results, the transplant can be modified in several ways, and some areas of current investigation are outlined:

- selection of donor and patient;
- modified conditioning regimens;
- manipulation of the graft;
- high cell dose;
- low lymphocyte content;
- expansion of marrow stromal cells;
- pre and post-BMT GvHD prophylaxis;
- early treatment of acute GvHD;
- aggressive CMV prophylaxis and treatment.

UD transplant programme in Genoa

We have developed a transplant regimen which can be summarised as follows:

- selection of donor and patient;
- HLA-A, HLA-B matched by serology and molecular biology sequence-specific oligonucleotide probes (SSOP, sequencing);
- DRB1 matched: high resolution molecular biology (SSOP, sequencing);
- DRB3, 4, 5 matched (not prerequisite);
- DQA, DQB, DPA, DPB : mismatch accepted (especially for advanced phase);
- modified conditioning regimens;
- cyclosporin A from day -7 (CyA-7);
- CY 120mg/kg +TBI 9-9 - 12 Gy; [CY=cyclophosphamide; TBI= fractionated total body irradiation (2 Gy x2 x3 for 38 patients and 3.3 Gy x3 for 22 patients)];
- manipulation of the graft;
- in this programme the graft is given unmanipulated, but care is taken to have the highest possible cell dose;
- pre and post-BMT GvHD prophylaxis;
- rabbit ATG 4 days is being compared to no ATG in a prospective ongoing randomised trial in adult patients, with the collaboration of the Italian Cooperative BMT Group (GITMO);
- CyA 2 mg/kg i.v. is given day -1 day +20; then orally;
- methotrexate (MTX) is given as a short course (day +1, +3, +6, +11);
- early treatment of acute GvHD;
- patients are treated at first signs of acute GvHD with prednisolone 2 mg/kg: non-responders are randomised within day +5 to receive 5 mg/kg of prednisolone or 5 mg/kg of prednisolone+ATG low dose (1.25 mg/kg on alternate days x 5 doses);
- aggressive CMV prophylaxis and treatment;
- all patients receive foscarnet 30 mg/kg x 2 day -7 day +30; then 90 mg/kg/day day +31 day +100 (5 days/week);
- pre-emptive treatment of CMV-antigenaemia is given by adding ganciclovir (10 mg/kg/day x 15 days) to foscarnet.

Results of the transplant programme in Genoa

We have transplanted 60 patients from DRB1 matched unrelated donors in this programme. Twelve of the patients had acute leukaemia and 48 had chronic myeloid leukaemia (CML); 34 patients were in early phase. Their median age was 29 years (range 18-49). The median interval between diagnosis and BMT was 1,248 days (range 262-3,064).

Actuarial transplant related mortality

The overall TRM is 28% (Figure 1), being 20% for patients in early phase of the disease (n=34). There is an effect of age (TRM 20% vs TRM 35%; p=0.2) at a cut-off age of 35, and an effect of diagnosis-BMT interval (TRM 20% vs TRM 27%; p=0.5).

Survival

The actuarial 5 year survival is 69% for all patients (n=60). For patients with CML (n=48), including 27 in first chronic phase and 21 in accelerated phase CML, the actuarial survival is 75%.

Quality of graft function

We analysed the quality of graft function as represented by platelet counts (>109/L) on day +30, +50, +100; for SIB transplants platelet counts (>109/L) were 122 (range 34-385), 113 (48-283), 99 (31-291);
for UD-BMT they were 38 (2-257), 50 (10-160), 45 (10-247). The difference (Rank Sum test) was very significant on day +30 (p<0.001), on day +50 (p<0.0001) and on day +100 (p=0.02) suggesting that graft function after UD-BMT is significantly poorer than after SIB-BMT.

**Graft-versus-host disease**

Acute GvHD (aGvHD) occurred at a median interval from BMT of 15 days (range 7-45) (and day 14 in a control group of SIB-BMT). aGvHD was scored as grade 0-I, grade II, grade III-IV in 50%, 36% and 12% of SIB-BMTs, respectively, vs 25%, 43%, 31% in UD-BMTs (p<0.0001). Chronic GvHD was scored as extensive in 23% of SIB-BMT and 36% of UD-BMT (p=0.005).

**CMV infections**

The actuarial risk of CMV antigenaemia at 1 year was 61% for SIB-BMT with high dose acyclovir prophylaxis and 69% for UD-BMT with foscarnet prophylaxis.

**Relapse**

The actuarial risk of relapse was 15%.

**Conclusions**

If we compare SIB-BMT with UD-BMT, the latter exhibit:
- slower haemopoietic recovery;
- slower immunologic recovery;
- higher incidences of acute and chronic GvHD;
- higher rate of transplant mortality.

The 2,942 patients reported to the EBMT had an overall 5 year actuarial TRM of 45%; it was 33% for 1,506 HLA identical siblings and 41% for 150 unrelated donors: an 80% survival for 30 1st chronic phase CML patients receiving MUD grafts was 37% and 19% for patients with more advanced phase CML: an update on a larger number of patients in the course of their disease.12

TRM has decreased over the past decades by a factor of 2, but still remains a major concern for physicians advising a patient whether he should or should not undergo an allogeneic BMT. Improved survival with the use of alternative haematologic therapy is also a reality.

We believe that several combined strategies can be applied to improve the outcome of UD-BMT: selection of donor/recipient pairs seems to be crucial, and lots of work is going on in that direction in laboratories. Clinical protocols with the use of less intensive conditioning, high cell dose, improved management of GvHD and CMV infections are also being explored.

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**References**

Among the most remarkable nutritional breakthroughs of the past decade was the conclusive discovery that periconceptional supplementation with the vitamin folic acid could prevent up to 70% of neural tube defects (NTDs). This finding, the result of major double-blind randomised clinical trials carried out in the UK and Hungary, was the culmination of more than ten years of research and speculation.1,2 Seven years have elapsed since then and in spite of an explosion of research in the area, no clear-cut folate related mechanism has emerged and there is still widespread debate and uncertainty on the choice of strategy to adopt in order to substantially reduce the incidence of NTDs.

This article reviews recent work in the area from the following two perspectives: 1. what is known about the underlying folate responsive mechanism and 2) what folic acid/folate intake is required to prevent the occurrence of NTDs.

The underlying folate responsive mechanism

The first question is whether folic acid acts by preventing dietary deficiency or by treating a metabolic block. Current opinion is that it is unlikely that NTDs result from maternal folate deficiency. Several studies have confirmed that NTD affected mothers do not have clinically low blood folate status although their blood folate concentrations tend to be lower than mothers with non-affected pregnancies, which is consistent with a folate related aetiology.3 Moreover, folate malabsorption has not been demonstrated in NTD affected mothers. Ongoing research, therefore, has focused on the developing embryo and the possibility of genetic variants in folate dependent enzymes giving rise to an inadequate supply of essential metabolites or excessive levels of toxic products. Figure 1 illustrates pathways of folate metabolism in mammalian cells, which may be important during the neurulation process in embryonic cells. There are numerous ways in which these pathways may be disturbed which could potentially result in abnormal closure of the neural tube.

Inadequate folate or vitamin B₁₂ to overcome a metabolic deficiency

A number of studies have examined folate and vitamin B₁₂ status in relation to NTDs. These include the evaluation of (a) levels in maternal blood and/or in amniotic fluid during NTD affected pregnancies, (b) levels in the blood of non-pregnant women who had previously had an NTD affected pregnancy and (c) levels in blood taken from NTD affected (spina bifida) patients and their families. The general consensus is that NTD affected pregnancies tend to be associated with lower blood folate and possibly also lower vitamin B₁₂ levels, particularly during the first trimester.4 Several reports have also demonstrated low amniotic vitamin B₁₂ or transcobalamin levels, indicating abnormal metabolism in the foetal compartment.

Increased homocysteine levels

For a number of reasons, much of the interest in the mechanism of NTDs has involved the role that homocysteine might play. Firstly, while homocysteine is an essential intermediate in folate dependent pathways (Figure 1), it is toxic both in vivo and in vitro. Intricate control mechanisms within the cell are designed to maintain low levels. Abnormally high homocysteine levels within the embryo could have a primary effect on the neurulation process via a toxic effect on membrane function, protein structure, etc. Alternatively, high intracellular homocysteine could act in a secondary manner by causing the level of S-adenosylhomocysteine within the cell to rise. Since S-adenosylhomocysteine is a potent inhibitor of almost all methyltransferase reactions, this would have the effect of inhibiting the production of essential methylated products such as methylated proteins, DNA, etc (Figure 1). Finally, an alteration of homocysteine metabolism associated with NTDs may merely point to an undefined malfunction in a folate related event. Apart from congenital deficiencies in specific homocysteine metabolising enzymes, the most common cause of plasma homocysteine accumulation is an inadequate supply of folate or vitamin B₁₂.

To date, the evidence that elevated homocysteine is an important factor in the aetiology of NTDs is quite weak. Mildly elevated plasma homocysteine concentrations have been detected in maternal blood during NTD affected pregnancies even after adjusting for folate and vitamin B₁₂ status.5 Amniotic fluid homocysteine is also moderately raised in NTD pregnan-
Some animal models have been used to examine the teratogenicity of homocysteine, but they give conflicting results and are difficult to interpret. Nevertheless, there has been particular interest in determining the influence of the three enzymes involved in the metabolism of homocysteine. These are methionine synthase, cystathionine-β-synthase (CBS) and 5,10-methylenetetrahydrofolate reductase (MTHFR).

**Methionine synthase**

Methionine synthase occupies a central role in folate metabolism (Figure 1). It is an essential intermediate in the incorporation of plasma folate into the cell because 5-methyltetrahydrofolate, the circulating form of the vitamin, must be demethylated via methionine synthase before it can be polyglutamated and retained by the cell. In addition, the reaction is coupled with the recycling of homocysteine to methionine, thus maintaining the supply of methyl groups through 5-adenosylmethionine for the essential methylation of DNA, proteins, etc. When cells are folate replete these methyl groups are supplied to folate cofactors from serine or glycine and converted to 5-methyltetrahydrofolate through MTHFR. The activity of the enzyme methionine synthase is the only event known to be influenced by both folate and vitamin B12 status. Reduced activity of this enzyme also results in higher than normal homocysteine levels. Thus, an attractive hypothesis for the underlying aetiology of NTDs has been either a direct or indirect involvement of this enzyme. The methionine synthase gene has recently been cloned and some mutational analyses carried out. One reasonably prevalent mutation (D919G) was examined in 56 NTD cases, 69 case mothers and 364 controls but there was no evidence of an association between this amino acid change and NTDs. While the result is disappointing, it is possible that other mutations in the methionine synthase gene may exist in populations with a high NTD prevalence. Very recently, the cDNA was cloned and mapped for methionine synthase reductase, an enzyme which provides the reducing system necessary to maintain a properly functioning methionine synthase. This enzyme provides an exciting new point of interest for mutational analysis in relation to NTDs.

**Cystathionine-β-synthase (CBS)**

This vitamin B6 dependent enzyme is the first step on the irreversible catabolic pathway for homocysteine (Figure 1). Inborn errors of CBS cause massive elevations of plasma homocysteine resulting in a variety of functional and clinical abnormalities. The frequency of two relatively common mutations in the CBS gene was examined in NTD cases and controls in...
the Irish population. Neither the severely dysfunctional G307S allele nor the 68 bp insertion/1278T allele was present at increased frequency in the NTD cases suggesting that this enzyme does not play a significant role in the pathogenesis of NTDs.

5,10 methylenetetrahydrofolate reductase

The enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) is also in a pivotal position in relation to folate and homocysteine metabolism. It irreversibly converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, thereby functioning as a control point wherein one-carbon units are channelled away from the DNA synthesis cycle and into the methylation cycle (Figure 1). The genetic profile of this enzyme has been more thoroughly explored than the other two homocysteine metabolising enzymes. Between 5% and 15% of Caucasian populations are homozygous for a thermolabile variant of the enzyme (C677T) which confers reduced enzyme activity and mildly increased plasma homocysteine concentrations when folate status is low. Homozygosity for this variant has now been clearly shown to be a risk factor for spina bifida, albeit accounting for no more than 12% of the population attributable risk. Analysis of the data suggests that case TT genotype (rather than maternal or paternal) is of paramount importance in determining risk, although it is not known how the variant functions to confer this risk in the developing embryo. Mothers who are homozygotes for the thermolabile (TT) variant are also at increased risk of giving birth to an NTD affected infant. However, in the general population homozygosity for this variant is associated with reduced red cell folate status, a known risk factor for NTDs. Thus, it seems likely that the enzyme may act merely as a risk factor for low folate status in these women. A second variant (A1298C), recently discovered in the MTHFR gene, was not more frequent in NTD cases. Nevertheless, it is possible that there are other mutations or combinations of mutations in this gene which contribute to folate responsive NTD risk.

Other enzyme defects

In recent years a number of genetic mutations have been described which cause NTDs in the mouse. Only a few of these can be prevented by treating the pregnant dam with folic acid, however, these models may be important aids in elucidating the mechanisms involved in neural tube formation. Because NTDs are sporadic even within families with high recurrence rates it is probable that the underlying aetiology is a combination of genetic and environmental factors present at the time of neural tube closure.

What intake of folate is needed to prevent NTDs?

The results of the MRC and Hungarian trials presented Public Health decision makers with a difficult dilemma – to decide on the most effective strategy to adopt in order to provide women of reproductive age with sufficient folic acid to prevent NTDs. In the randomised controlled trials, doses of 4 mg folic acid per day decreased the recurrence and 800 µg per day decreased the occurrence of NTDs. However, in earlier non-randomised studies, doses of approximately 400 µg per day were effective. Thus, while recommendations for the prevention of NTD recurrence were straightforward (periconceptional supplementation with 4 or 5 mg folic acid per day) because these mothers were already in a specific high-risk group, the approach to adopt for the rest of the population was more contentious. At present, Department of Health advice in many countries is that women who are planning a pregnancy should increase their folate consumption by 400 µg per day. However, campaigns to increase public awareness on the benefits of folic acid have been largely ignored. To make matters worse, an increased intake of 400 µg of food folate per day is much less effective in changing folate status than taking a supplement of 400 µg folic acid. After much debate and concern that high folic acid consumption may mask the symptoms of pernicious anaemia in the elderly, the Food and Drug Administration (FDA) in the US adopted a population strategy to increase the folic acid intake of all inhabitants from 1st January 1998 by mandating that cereal grain be fortified with 1.4 mg of folic acid per kg of grain. This strategy is expected to add approximately 100 µg per day of folic acid to the average diet, which is unlikely to mask pernicious anaemia but may be too low to prevent many NTDs. A major difficulty has been the lack of information on the minimum amount of folic acid needed to prevent NTDs. Daly et al. addressed this question by examining the relationship between NTD risk and early pregnancy maternal red cell folate levels. They found an eight-fold higher risk among women whose levels were less than 150 µg/L compared with those above 400 µg/L and concluded that if the average red cell folate of the population could be increased such that all pregnant women had values above 400 µg/L, then the risk of NTDs could be reduced by as much as 60%. In a recent randomised trial Daly et al. showed that doses of 400 µg/day would place all women over the protective red cell folate concentration of 400 µg/L but at the expense of unnecessarily high exposure for some people. Delivery of 200 µg/day would also prevent many NTDs and 100 µg/day would be beneficial. They called for immediate fortification at this low level; however, many experts believe that their conclusions are over-optimistic and that fortification at higher levels is the only long-term solution.

References

2. Czeizel AE, Dudas I. Prevention of the first occurrence


7. Shields DC, Kirke PN, Mills JL, et al. The “thermola-


Homocysteine and ischaemic heart disease

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A link between increasing serum homocysteine concentration and increasing risk of atherosclerotic disease was first shown 30 years ago, and a large body of evidence has since been accumulated on the subject. Serum homocysteine can be lowered by folic acid supplementation, so if the relationship between homocysteine and cardiovascular disease is causal, folic acid supplementation would lower cardiovascular mortality. Randomised controlled trials of folic acid supplementation and ischaemic heart disease are underway, but the results are not expected for some time. In this review we examine the evidence on homocysteine and circulatory diseases, ischaemic heart disease in particular, assessing the likelihood that the relationship is one of cause and effect and quantifying the reduction in mortality to be expected through folic acid supplementation. Our review draws on our previous paper on homocysteine and heart disease, and also draws on its citation list: to comply with the limitation of 10 references here we identify papers cited in our previous paper by their reference numbers there.

Serum homocysteine in Western populations

Homocysteine is an intermediate compound generated from the demethylation of methionine. It is metabolised in two ways. The first is the transsulfuration pathway, in which cystathionine β-synthase irreversibly binds homocysteine to serine to form cystathionine. The second is the remethylation cycle, which involves two major enzymes, 5,10-methylene-tetrahydrofolate reductase (MTHFR) and methionine synthase. Specific genetic defects leading to deficiencies in the activities of all three enzymes have been identified and these are discussed below. The activities of the three enzymes, and hence the blood concentration of homocysteine, also have environmental determinants, and are dependent on three of the B vitamins - folate, B6 and B12. Most of the evidence is on folic acid, which has been shown to have an important effect in lowering homocysteine.

Serum homocysteine concentration follows a Gaussian distribution skewed to the right and increases with age. In a cohort of men recruited in London (the BUPA study), the average concentration was 12 µmol/L (at an average age of 53 years), similar to the Western average at this age, and the 5th, 25th, 50th, 75th and 95th centiles were 7.5, 9.5, 11.1, 13.3 and 17.8 µmol/L respectively. In non-Western communities levels tend to be lower.

Epidemiological evidence on homocysteine and ischaemic heart disease

The majority of the epidemiological studies are of retrospective design; homocysteine was measured in subjects with known ischaemic heart disease (generally survivors of myocardial infarction) and in control subjects, adjusting (or matching) for age, sex and other confounding factors. Boushey and her colleagues reviewed 15 such studies in 1995 (ref. #10 cited in1). These studies show a strong and continuous relationship between homocysteine and ischaemic heart disease. An analysis of eight retrospective studies yielded the summary estimate that the odds ratio of ischaemic heart disease for a 5 µmol/L increase in serum homocysteine was 1.84 (95% confidence interval 1.52-2.23) - that is, an excess risk of 84% (52-123%). In the remaining retrospective studies the relation could not be quantified in this way because the necessary data were not published or because homocysteine was measured only after methionine loading. No substantive source of systematic error in this estimate from the retrospective studies can be identified. Retrospective studies are generally considered less rigorous than prospective or cohort studies, but the major source of bias affecting retrospective studies in general, recall bias, does not apply because a biochemical measurement was made to quantify the exposure, and the retrospective studies were conducted sufficiently long after the ischaemic event to avoid the known acute change in serum homocysteine after myocardial infarction.

The evidence from the cohort studies is less consistent. The results of eight published cohort studies are summarised in Table 1 (these studies were of nested case-control design: blood was collected from healthy subjects at the outset and serum frozen, and at the end of follow-up homocysteine was measured in...
In addition to these eight cohorts recruited from the general population, there are three additional published cohort studies, one of progression of peripheral arterial disease, one of mortality in patients with known coronary artery disease, and one of thrombotic events in patients with systemic lupus erythematosus, and these all demonstrated an association between homocysteine and occlusive vascular disease (refs. #18, 19 and 21 cited in1). Additional confirmatory observational evidence is provided by the association of ultrasound-detected carotid artery disease with raised serum homocysteine concentration, and the presence of a cross-country association (refs #31, 32 and 11 cited in1).

In quantifying the association in the cohort studies, we take the result of our own study (BUPA) as representative of the positive studies because it is large, its results were reported in some detail, and its result - an odds ratio of 1.41 (95% confidence interval 1.20-1.65; \( p = 0.001 \)), or 1.33 (1.22-1.59) after adjustment for other heart disease risk factors, is close to the median of the positive cohort studies (Table 1). As with the retrospective studies, the true association would be somewhat greater because of the effect of regression dilution bias (the dilution of the effect of a risk factor in a cohort when based on single measurements that fluctuate in an individual over time). The bias could be allowed for using data from a study recording homocysteine measurements on two or more occasions in the same individuals, but at present no such data are published.

The retrospective studies and the positive cohort studies show a dose-response relationship between serum homocysteine and ischaemic heart disease that is continuous across the range of homocysteine levels in Western populations. Table 2 shows the estimates from the eight retrospective studies combined and from the BUPA prospective study of the risk of IHD according to homocysteine level, with homocysteine levels divided into four categories (<10, 11-20, 21-30, and 31-60 µmol/L). The continuous dose-response relationship provides strong evidence against the view that only greatly elevated levels of homocysteine increase the risk of ischaemic heart disease.

**Genetic evidence on homocysteine and ischaemic heart disease**

There are autosomal-recessive inborn errors of metabolism affecting each of the three major enzymes

### Table 1. Odds ratio of ischaemic heart disease (IHD) events for a 5 µmol/L increase in serum homocysteine levels: results from eight prospective studies (nested case-control analysis) of persons without disease at study entry, adjusted (or matched) for age and sex.

<table>
<thead>
<tr>
<th>Study</th>
<th>Sex</th>
<th>No of subjects</th>
<th>Estimated average age at IHD event, yrs</th>
<th>Odds ratio (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tromso Study (Ames et al.;* Norway)</td>
<td>90% men</td>
<td>122 with IHD, 478 without IHD</td>
<td>53</td>
<td>1.54 (1.21-1.95)</td>
</tr>
<tr>
<td>BUPA Study (Wald et al.;* Britain)</td>
<td>men</td>
<td>229 with IHD, 1129 without IHD</td>
<td>58</td>
<td>1.41 (1.20-1.65)</td>
</tr>
<tr>
<td>British Regional Heart Study (Whincup et al.†)</td>
<td>men</td>
<td>110 with IHD, 118 without IHD</td>
<td>54</td>
<td>1.38 (1.02-1.86)</td>
</tr>
<tr>
<td>Rotterdam (Bots et al.;* the Netherlands)</td>
<td>66% men</td>
<td>104 with IHD, 533 without IHD</td>
<td>75</td>
<td>1.34 (1.10-1.69)</td>
</tr>
<tr>
<td>US Physicians (Stampfer et al.)*</td>
<td>men</td>
<td>271 with IHD, 271 without IHD</td>
<td>62</td>
<td>1.29 (1.01-1.67)</td>
</tr>
<tr>
<td>Atherosclerosis Risk in Communities (ARIC) Study (Folsom et al.† USA)</td>
<td>women</td>
<td>174 with IHD, 395 without IHD</td>
<td>59</td>
<td>1.00 (0.93-1.07)*</td>
</tr>
<tr>
<td>North Karelia Study (Altham et al.;* Finland)</td>
<td>men and women</td>
<td>191 with IHD, 269 without IHD</td>
<td>61</td>
<td>1.00 (0.77-1.29)*</td>
</tr>
<tr>
<td>MRFIT (Evans et al.† USA)</td>
<td>men</td>
<td>230 with IHD, 474 without IHD</td>
<td>61</td>
<td>0.95 (0.79-1.12)*</td>
</tr>
</tbody>
</table>

* The Tromso, US Physicians, North Karelia and MRFIT studies are cited as refs. #12-15 respectively in our earlier review; † Calculated from published data; IHD death only (other studies recorded non-fatal myocardial infarction in addition).
in homocysteine metabolism, and in each defect homoyzogotes have very high serum homocysteine levels (about 10-50 times higher than the general population) and very high risk of premature cardiovascular disease. Heterozygotes for the three disorders have serum homocysteine levels about three times the population average and high risk of cardiovascular disease. The only biochemical change that is common to all three genetic disorders, homocysteine level; no other metabolite is consistently high or low in all three. Given that circulatory disease is also common to all three genetic disorders, homocysteine is almost certainly the cause of the disease and not merely a marker of some other cause.

Another genetic defect affecting about 10% of the population (being homozygous for a thermolabile form of MTHFR) offers a useful natural experiment to test the homocysteine hypothesis. This variant leads to moderate increases in homocysteine (2.8 µmol/L on average in subjects homozygous for the mutation compared to those without the mutation in a meta-analysis of eight studies) and a moderate increase in risk of ischaemic heart disease (the summary odd's ratio was 1.22, 95% confidence interval 1.01-1.47). A significantly elevated risk was also shown in studies identifying the abnormal phenotype rather than the genotype.

Combining the epidemiological and genetic evidence

Table 3 shows estimates from three types of study on the magnitude of the relationship between homocysteine and ischaemic heart disease. The estimate from the retrospective studies is greater than that from the cohort study; this may be partly attributable to the fact that the subjects in the retrospective studies were about seven years younger on average and relative risk decreases with age. The magnitude of the association in both the retrospective studies and the cohort study would be a little lower after adjustment for the confounding effects of other heart disease risk factors (the BUPA result changing from an odds ratio of 1.41 to 1.33), but this is offset by the fact that the inability to adjust the results for the regression dilution bias (because of the unavailability of published data on serum homocysteine measured on two or more occasions in the same subjects) has led to underestimation. The estimate taken from the studies of subjects homozygous for the thermolabile MTHFR defect is applicable to a similar age group (about 60) as the cohort study estimate, and is similar in magnitude to this estimate. This quantitative agreement between the cohort study and the MTHFR genetic studies is remarkable and offers compelling evidence for homocysteine being directly responsible for the excess heart disease mortality associated with higher levels. At the age of 60, a 5 µmol/L increase in serum homocysteine is therefore likely to increase the risk of ischaemic heart disease by approximately 40%.

Conclusions and implications for prevention

The cohort studies taken together favour an association between homocysteine and heart disease although the negative results of three studies cannot satisfactorily be explained. The retrospective studies indicate a strong relationship, and it is not possible to identify any likely source of error in these studies. The three cohort studies on patients with existing circulatory disease, and the additional observational evidence, all show associations between homocysteine and occlusive vascular disease. There is also epidemiological evidence for a direct relationship between blood folate concentration and risk of cardiovascular disease. The genetic evidence for a cause and effect relationship, discussed above, is strong. Results of animal and in vitro experimental studies show that increases in blood homocysteine levels increase the extent of vascular and platelet damage (refs. #52-55 cited in). Taken together, the epidemiological, genetic, and experimental evidence make a compelling case for a causal relationship between homocysteine and ischaemic heart disease. The genetic and epidemiological evidence both indicate that the dose-response relationship is continuous across the range of serum homocysteine levels in Western populations.

### Table 2. Odds ratio of ischaemic heart disease according to level of serum homocysteine: results from eight retrospective studies and the prospective BUPA study. (Data from Wald et al.).

<table>
<thead>
<tr>
<th>Serum homocysteine (µmol/L)</th>
<th>Odds ratio (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BUPA study</td>
</tr>
<tr>
<td>&lt;10</td>
<td>1.0 (0.5-1.9)</td>
</tr>
<tr>
<td>11-20</td>
<td>1.9 (1.0-3.5)</td>
</tr>
<tr>
<td>21-30</td>
<td>2.2 (0.9-5.7)</td>
</tr>
<tr>
<td>31-60</td>
<td>5.7 (1.1-28.5)</td>
</tr>
</tbody>
</table>

### Table 3. Estimates from three sources of the increase in risk of ischaemic heart disease for a 5 µmol/L increase in serum homocysteine.

<table>
<thead>
<tr>
<th>Odds ratio (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retrospective studies*</td>
</tr>
<tr>
<td>Cohort study*</td>
</tr>
<tr>
<td>Genetic evidence (subjects with thermolabile MTHFR)*</td>
</tr>
</tbody>
</table>

*estimate from the BUPA cohort.
Serum homocysteine can be reduced by increasing consumption of folic acid.\textsuperscript{2,4} Folate is best given as folic acid (either as supplements or as food fortification) because the bioavailability is greater than that of the conjugated folates that occur naturally in food.\textsuperscript{4} The homocysteine-lowering effect appears to plateau at folic acid intakes of 1 mg/day or less,\textsuperscript{2} but there are few trial data on intakes of 0.4 mg/day or less (an appropriate level for food fortification). A supplement of 0.4 mg/day has been shown by Ward and colleagues to reduce average homocysteine levels in middle-aged subjects by 1.9 µmol/L (ref. #61 cited in\textsuperscript{1}), and a study of breakfast cereal fortification at this level showed a similar reduction in homocysteine.\textsuperscript{3} The cohort study result indicates that this reduction in homocysteine is equivalent to a reduction in mortality from ischaemic heart disease of 10% (95% confidence interval 4-16%), and adjustment for the regression dilution bias would increase this estimate. There is a need to confirm the size of the effect of this level of folic acid supplementation on serum homocysteine levels, and to determine whether there is a homocysteine threshold below which folic acid ceases to reduce serum homocysteine concentration further. This could be accomplished by a relatively small and short-term randomised study of folic acid supplementation.

The knowledge that increasing folic acid intake has an important effect in preventing neural tube defects justifies fortifying staple food with folic acid. The expected benefit that this would have in reducing the risk of heart disease is an important associated benefit affecting everyone in the community.

References

Homocysteine, platelet function and thrombosis

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It was almost 30 years ago that hyperhomocysteinaemia was first associated with arteriosclerosis and since then a growing body of evidence has established its independent contribution to cardiovascular disease. It is now clear that 15-40% of patients with coronary, cerebral or peripheral arterial disease have increased plasma levels of homocysteine, and that, regardless of the severity and/or the genetic/nutritional background leading to hyperhomocysteinaemia, subjects with elevated (>20 µM/L) plasma levels of homocysteine have a tendency to arterial and venous thrombotic events. When severely increased in plasma (>100 µM/L), homocysteine can leak into the urine causing homocystinuria. In this severe form of hyperhomocysteinaemia, premature arteriosclerosis, and arterial and venous thrombosis are common findings. Biochemical measurements of urinary metabolites and clinical trials with aspirin, indicate that enhanced biosynthesis of thromboxane A2 (TXA2) by platelets is a major contributor to the risk of thrombosis associated with several risk factors. In subjects with homocystinuria, we have reported an abnormally high in vivo TXA2 biosynthesis, as reflected by the excretion of its major enzymatic urinary metabolites. The possibility of a platelet origin for the abnormally high TXA2, was suggested by the results of studies with 50 mg/day of aspirin. In the following paragraphs, we shall review the effects of homocysteine on platelets.

Activation of the coagulation process and/or endothelial dysfunction are known to trigger specific effects on platelet biochemistry that in turn enhance the biosynthesis of TXA2. A hypercoagulable syndrome and endothelial dysfunction are triggered by homocysteine. Thus, we shall also review data concerning the effects of high levels of homocysteine on blood coagulation/fibrinolysis and endothelial cells.

Homocysteine and platelet function

In homocystinuric patients, platelet survival has been reported abnormally low. This was in line with the results obtained in non-human primates. In keeping with this, increased platelet stickiness was demonstrated in the blood of homocystinuric patients and after the addition of homocysteine to plasma. However, subsequent studies did not confirm these observations. Increased platelet aggregation after homocysteine exposure has been documented. This concept has been challenged as well. Discrepancies also exist about the effect of homocysteine thiolactone (HTL), the cyclic oxidation product of homocysteine, on platelet function. Early studies suggested that HTL had only a very small effect on platelet aggregation. However, at variance with the inactive salt (hydrochloride form), the free base of HTL fosters platelet aggregation. On the other hand, in synergism with other methyltransferase inhibitors, HTL inhibits platelet aggregation. A study that sheds some light on such discrepancies is that of Stamler et al. Their experiments indicate that homocysteine does not cause platelet aggregation per se. This is consistent with the antiplatelet effects of other low molecular weight thiols with physical and chemical characteristics similar to cysteine and glutathione. Instead, homocysteine can increase platelet adhesion to endothelial cells (EC) as a consequence of its toxic effect on the endothelium itself. EC produces endothelium-derived relaxing factor (EDRF) which reacts with homocysteine to form S-nitroso-homocysteine (SNOHO). The latter is a strong antiplatelet agent with a 15-min half-life (for comparison, EDRF half life is about 5-30 sec). Therefore in normal conditions, the toxicity of homocysteine is abolished by the formation of SNOHO. However when homocysteine levels saturate the available amounts of EDRF, unmodified homocysteine becomes available. This causes endothelial injury, with a consequent reduction of the EDRF production, followed by reduced formation of SNOHO and in turn, of the antiplatelet potential.

These data suggests a role for an oxidant stress in the risk of thrombosis related to elevated levels of homocysteine. In keeping with this, we have reported >2 SD increase from the control mean of the urinary excretion of 11-dehydro-thromboxane B2 and of 2,3-dinor-thromboxane B2 (TXB2), major enzymatic derivatives of TXA2 in 11 homocystinuric patients. The abnormally high excretion of this valuable index of in vivo platelet activation, was independent of the presence of major cardiovascular risk factors. On the other hand, the fact that two metabolites resulting from two independent pathways were both increased in a similar manner strongly argued for these changes to
be a reflection of increased biosynthesis of TXA₂. Both the profound suppression by low-dose aspirin and the slow recovery after drug withdrawal were compatible with a platelet source of the abnormally high TXA₂ biosynthesis. To understand the mechanisms of the enhanced TXA₂ biosynthesis in homozygous cystathionine β-synthase deficiency (CBSD), 500 mg of the antioxidant drug probucol was given to seven patients for 3 weeks. In vitro probucol prevents the oxidative modification of low-density lipoproteins. This treatment resulted in a 40-60% drop in TX metabolite excretion, which did not correlate with a reduction in blood cholesterol levels. Since oxidation of lipoproteins – which can induce platelet TXA₂ formation – is facilitated by homocysteine, inhibition of TXA₂ production by probucol is consistent with the possibility that oxidized lipoproteins contribute to an increased arachidonic acid metabolism in platelets of patients with CBSD. It is worth stressing that lipid peroxidation can be initiated not only by hydrogen peroxide, but also by superoxide and hydroxyl radicals, which can be generated during oxidation of thiols.

**Endothelial cells**

The data with endothelial cells suggest a major role for some free radical species in the endothelial injury mediated by homocysteine. As for platelet activation, the possibility therefore exists that an oxidant stress may be involved in the thrombogenic potential of homocysteine. The possibility that hydrogen peroxide is responsible for the cellular damage induced by homocysteine has been analysed in detail by Starkebaum and Harlan. Copper-catalysed auto-oxidation of cysteine in alkaline media leads to the reduction of oxygen and the generation of hydrogen peroxide. In view of this, Starkebaum and Harlan showed that, in a cell-free system, increasing concentrations of copper (1-50 μM) increased homocysteine oxidation in a dose-dependent fashion. The addition of catalase to the system reduced oxygen consumption by nearly one half, thus suggesting that H₂O₂ was formed during the reaction. However, H₂O₂ did not seem to accumulate in the presence of homocysteine, suggesting that homocysteine itself can scavenge H₂O₂. Interestingly, while at concentrations of 0.05-5 μM, Cu²⁺ increased the rate of H₂O₂ formation, at concentrations above 5 μM, H₂O₂ formation was reduced. The effect of the higher concentrations may be the result of Cu²⁺ catalysed reduction of H₂O₂ to water. The relationship between copper, homocysteine and endothelial injury was documented by the observation that a dose-dependent lysis of cultured bovine aortic endothelial cells could only be observed when homocysteine (up to 5 mM) was added in the presence of copper (2 μM).

**Coagulation/ fibrinolysis**

Ex vivo data from patients with homocystinuria have shown a variety of abnormalities of the coagulation system that suggest a hypercoagulable state in this setting. Reduced levels of antithrombin (AT), of factor VII, and protein C have been reported. In vitro studies provided a biochemical background for a hypercoagulable syndrome in hyperhomocysteinaemia. Factor V activity and prothrombin activation have been shown to be increased by the addition of 0.5-10 mM homocysteine to cultured bovine aortic endothelial cells. While 8 hrs were required to detect an increase in factor V activity in the presence of 10 mM homocysteine, 24 to 30 hrs were needed in the presence of 0.1 or 0.5 mM homocysteine. These effects appeared to depend on the effect of homocysteine on the natural anticoagulant protein C. A direct effect of homocysteine on protein C activation was subsequently shown. Incubation of bovine or human umbilical vein cultured endothelial cells (HUVEC) with 7.5 to 10 mM homocysteine for 6 to 9 hrs produced a 90% inhibition of protein C activation. This effect could be partially explained by a competitive inhibition by homocysteine of the thrombomodulin-thrombin interaction. Hayashi et al. gained additional insight into the mechanism by which homocysteine impairs thrombomodulin activity in HUVEC. They found a time- and dose-dependent inhibitory effect of homocysteine on thrombomodulin cofactor activity. Thrombomodulin activity, measured as protein C activation, was reduced to 5 or 10% of the baseline values after incubation with 10 mM homocysteine when the activity was determined on the cell surface or in whole cell extracts respectively. Thus, their data suggest that the effect of homocysteine on thrombomodulin activity is due to a reduction of the native thrombomodulin, which is followed by a compensatory increase in the expression of the thrombomodulin gene and of the total thrombomodulin level. Finally, in a cell-free system, they demonstrated that homocysteine is able to inhibit the binding of thrombomodulin to thrombin and concluded that this is caused by a decreased binding capacity of the reduced thrombomodulin. Also tissue factor (TF), a central protein of the extrinsic pathway of the coagulation, has been indicated as another possible target for the thrombogenic action of homocysteine. Incubation of HUVEC with 10 mM homocysteine for 8 hours increased TF activity by six-fold. A clear dose dependency of this effect (0.1-10 mM homocysteine) was demonstrated. Homocysteine induced TF activity was inhibited by N-ethylmaleimide in HUVEC, thus indicating that the sulphur group was instrumental in the observed phenomenon. Finally, the ability of homocysteine to induce TF mRNA, measured by a quantitative polymerase chain reaction technique, revealed an almost 4-fold increase in the TF mRNA, when comparing HUVEC and fibroblasts after 3 hrs incubation with 10 mM homocysteine. The effect of homocysteine on AT has been explored with emphasis on the interaction between AT and heparin-like glycosaminoglycans in porcine aortic
endothelial cells. The data showed that the maximal AT binding capacity to heparin sulphate was reduced to 30% of normal after a 24-hour incubation with 1 mM homocysteine. This effect was dependent on sulphydryl groups and appeared to involve the generation of hydrogen peroxide, being prevented by catalase, but not by superoxide dismutase.

The interference of homocysteine with the fibrinolytic system has been addressed by Hajjar. She demonstrated a 65% decrease in cellular binding sites for tissue plasminogen activator (t-PA), following treatment of cultured HUVEC with 1.5 to 7.5 mM homocysteine. The author also provided evidence that this was due to a reduction of the binding sites for t-PA on the 40 kDa receptor protein. Interestingly, the receptor capacity to bind plasminogen was not altered, thus suggesting that the receptor had been altered only in the specific domain responsible for the binding of t-PA, and that the COOH-terminal domain, which binds plasminogen, remained unmodified. Along the same line, Harpel et al., also focused on the potential modulation of fibrinolysis by homocysteine. They studied the interaction of plasmin-modified fibrin and lipoprotein(a), Lp(a). Because of its homology to kringle IV of plasminogen, Lp(a) interferes with fibrinolysis by competing with plasminogen binding sites. Harpel et al. demonstrated that homocysteine can enhance the binding of Lp(a) to fibrin, especially to plasmin-treated fibrin. This binding was inhibited by ε-aminocaproic acid, thus indicating lysine binding site specificity, and was also increased by cysteine, glutathione and N-acetylcysteine. Using gel electrophoresis and immunoblotting, the authors observed changes in the mobility of the apo(a) moiety after exposure to homocysteine, and therefore concluded that homocysteine could be responsible for altering the structure of apo(a), possibly exposing additional binding sites for the fibrin surface. As a consequence, the thrombotic potential of Lp(a) would be increased by homocysteine.

Perspectives

High levels of thrombin in the circulation and a sustained tendency to thrombosis occur in hypercoagulable states. In addition to its role in the conversion of fibrinogen to fibrin, thrombin is a potent inducer of platelet activation and TXA2 biosynthesis. The data presented above suggests a hypercoagulable syndrome in patients with severe hyperhomocysteinaemia. This raises the possibility that anticoagulation should be taken into consideration in preventing thrombosis in these patients. On the other hand, since a homocysteine-mediated oxidant stress may trigger platelet activation, the latter leading to a hypercoagulable state, the question is whether lowering plasma homocysteine will per se correct the the tendency to thrombosis of these patients. The latter strategy could be strengthened by the addition of antioxidants. Whether this is the case, will need to be established in large, prospective studies in appropriate experimental and clinical settings.

References

Transgenic models of T-cell prolymphocytic leukaemia

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T-cell prolymphocytic leukaemia (T-PLL) is a rare form of mature T-cell leukaemia. It occurs in two populations, in elderly (mean age: 69 years) and in young patients suffering from ataxia telangiectasia (AT). This leukaemia is characterised by an aggressive syndrome with lymphocytosis, lymphadenopathy, hepatosplenomegaly and skin lesions. Prolymphocytes are typically cells with a high nucleocytoplasmic ratio, a basophilic cytoplasm devoid of granules, and a single prominent nucleolus. The immunophenotype is that of mature T lymphocytes, but leukaemic cells bearing both CD4 and CD8 are not infrequent. Interestingly, a preleukaemic stage of T-PLL has been identified in AT patients. Recurrent chromosomal aberrations associated with T-PLL involve on one hand the TCRα/D or TCRβ gene, and on the other, the Xq28 or 14q32.1 regions. Their molecular characterisation led to the identification of the MTCP1 and TCL1 genes, respectively. MTCP1 is organised into seven exons spread over approximately 10 kb. Alternative splicing generates two types of transcripts encoding two entirely different proteins, p8MTCP1 and p13MTCP1. Both products were upregulated in leukaemic cells bearing a t(X;14) translocation, none showed similarities with known oncogenes, and in vitro transformation of a fibroblastic cell line was negative. Direct evidence of the oncogenic activity of MTCP1 was thus required.

Characterisation of CD2-p13 transgenic mice

Transgenic mice were generated in which expression of p13MTCP1 was controlled by the CD2 regulatory sequences (CD2-p13 mice). This construct was chosen because it confers a level of expression independent of the insertion site of the transgene. Three founders were obtained and bred. These transgenic lines expressed p13MTCP1 in thymuses and in spleens at different levels, in correlation with the number of transgene copies in the line. No expression was detected in the non-lymphoid organs.

Elderly CD2-p13 transgenic mice develop T-cell prolymphocytic leukaemia

Although no immunologic abnormality or tumours were detected in the first year of life of the transgenic mice, the survey of the animal cohort was maintained for a long period of time. Keeping in mind that the follow-up of AT patients has shown that leukaemias with MTCP1 or TCL1 rearrangements have a long and indolent pre-clinical course. It was not until 15 months that the first mice suffering from leukaemic syndromes were detected. Because the spontaneous death of the transgenic animals could impair the characterisation of the leukaemia, the cohort was sacrificed at 18 to 20 months of age, and compared to non-transgenic age-matched siblings.

A typical leukaemia was characterised by lymphoid cells with an irregular nucleus, condensed chromatin, a unique and prominent nucleolus, and a basophilic cytoplasm devoid of granules. Spleen and liver were consistently invaded, even in the absence of organomegaly. The immunophenotype of the leukaemic cells was CD3+CD8+CD4+CD25- in all cases but one which was CD4+CD8+. The rarity of CD4+ T-cell leukaemia was one of the rare differences between the human and murine T-PLLS and could not be explained by a lower level of expression of p13MTCP1 in the CD4+ subset. Some leukaemias were also characterised by smaller cells, as has also been described in some human T-PLL.

It should be noted that the analysis of a cohort of old mice (18-20 months) revealed a large number of tumours not linked to the transgene (arising in transgenic and control mice). Solid tumours were easily discarded from the analysis, but coincidental haematological diseases needed to be identified by precise and complete diagnosis. Tissue samples from each transgenic and control mouse were reviewed by a pathologist, blood smears were reviewed by a cytologist, splenocytes were analysed by FACS with B and T cell markers and clonal rearrangements of the TCRβ and IGH were searched for by Southern blotting. M urine T-PLLS were unambiguously demonstrated in the three transgenic lines and never in the control mice, whereas up to 10 percent of various B cell and non-lymphoid malignancies were found in transgenic and control animals. The incidence of T-PLL was 100, 50 and 21 percent in the three lines, and correlated with the level of expression of the transgene (see Table 1).
Natural history of the disease

Systematic analysis of the transgenic mice gave the opportunity to study the disease before it was clinically or biologically symptomatic. Although no longitudinal follow-up was performed, a plausible reconstitution of the malignant invasion could be proposed. No immunological abnormality was detected before the emergence of a clonal population. A proliferative advantage of the polyclonal T-cell population is deduced from their susceptibility to develop leukaemia but needs to be defined. Clonal populations in the spleen were found in animals without macroscopic abnormality or lymphocytosis. However, lymphoid secondary organs were disorganised and nodular invasion of the liver was constant. Even in animals with very large spleens and livers, malignant populations were confined to these organs, bone marrow was found normal when analysed, and lymphocytosis was inconstant at this stage. It was only in the animals with the most florid diseases that malignant cells were found in most organs including bone marrow, and that lymphocytosis could reach 2,000 G/L.

MTCP1 is an oncogene

The presence of T-cell leukaemia in the three transgenic lines, and in none of the non-transgenic siblings, demonstrated that p13\textsuperscript{MTCP1} is an oncogene. In data not shown here, the alternative product of MTCP1, p8\textsuperscript{MTCP1}, was also expressed in transgenic animals using a similar construct. No phenotype was demonstrated in these transgenic animals. Thus, over-expression of p8\textsuperscript{MTCP1} in leukaemias is probably coincidental whereas expression of p13\textsuperscript{MTCP1} is a causal factor of leukaemogenesis. Similar experiments were performed by Virgilio et al. for the TCL1 gene.\textsuperscript{7} TCL1 has four exons and encodes a protein product of 14 kD, p14\textsuperscript{TCL1}, sharing significant homologies with p13\textsuperscript{MTCP1}. Both p14\textsuperscript{TCL1} and p13\textsuperscript{MTCP1} proteins have a closely related β-barrel structure. Animals transgenic for TCL1 were generated using another T-cell specific promoter, lck. CD8 \textsuperscript{T-cell leukaemias similar to those arising in the CD2-p13 animals were detected after a very long incubation period. It is remarkable that the two animal models of T-PLL are so superimposable.

The use of transgenic animals clearly demonstrated the role of a new oncogene family, MTCP1/TCL1, in the malignant transformation of mature T-cell lymphocytes.

Interests in generating a murine model for T-PLL

The blood disorder arising in CD2-p13 transgenic mice shares most of the clinical and biological features of the human T-PLL. Its late onset also mimics the human T-PLL, which generally arises in the elderly. The only differences are the more restricted immunophenotype of the leukaemic cells and the absence of detectable skin lesions in mice. As it is not the general rule that a human disease is mimicked so closely in transgenic mice, we can infer that the oncogenic activity of MTCP1 is very specific for a precise stage of the differentiation of the T cell lineage. Given the parallel between human and murine diseases, we can hypothesise that the initial (pre-diagnosis) stage of T-PLL is similar to that observed in mice. It is thus possible that a smouldering invasion of spleen and liver precedes for years or decades the emergence of lymphocytosis. Diagnosis would be made only in individuals who survived long enough to allow the disease to progress until its florid stage.

The latency period before emergence of the malignancies, longer than in most murine models of oncogenesis, is in agreement with the necessity of additional genetic events in the development of the T-PLL. To date, studies of the human disease have demonstrated the importance of the inactivation of the ATM gene\textsuperscript{8} and of the duplication of the long arm of chromosome 8.\textsuperscript{1} The role of these genetic abnormalities could be tested by generating double mutant animals.

The striking similarity of the human and murine T-PLLs makes CD2-p13 mice a valuable model to investigate the biological functions of the family of oncoproteins formed by p13\textsuperscript{MTCP1} and p14\textsuperscript{TCL1}, to identify the secondary events necessary for the malignant phenotype, and to test therapeutics.

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References


A murine transgenic model of human cold agglutinin disease

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In 1900, the group working with Metchnikoff suggested the concept of autoimmunisation by demonstrating the presence of autoantibodies in normal conditions; which was opposed to the concept of horror autoxicus raised by Ehrlich. Nearly at the same time, Landsteiner described the rules governing blood compatibility. He showed that a subject with group A blood will never be able to produce anti-A antibodies. These results provided strong support to Ehrlich’s idea. The influence of the Ehrlich’s ideas was so strong that the experiments from Metchnikoff’s school were forgotten, and when Donath and Landsteiner described for the first time an autoantibody, the biphasic haemagglutinin responsible for paroxysmal cold haemoglobinuria, they failed to call it autoantibody. In 1949 Burnet and Fenner proposed the clonal deletion theory. This theory was strongly influenced by the experiments carried out by Owen with dizygotic calves sharing a single placenta. These two calves were mixing their red blood cells but, despite the fact that they were expressing different blood groups, they were unable to produce allo-antibodies against the blood groups from the other calf. The interpretation of these experiments and the experiments from Medawar’s group, demonstrating that injection of new born mice with spleen or marrow cells from an unrelated strain of mice enables these animals to accept skin-grafts from the second strain, led both groups to conclude that during embryonic life the immune system learns to tolerate self antigens. The clonal deletion theory was a magnificent theory explaining tolerance and autoimmunity in a simple way. Autoimmunity, according to this theory, will only arise in the case of somatic mutations, which is an unusual phenomenon.

However, in 1956 Witebsky and Rose were able, for the first time, to induce an experimental autoimmune disease mediated by autoantibodies: autoimmune thyroiditis. They succeeded in inducing this disease by injecting thyroglobulin in the presence of Freund adjuvant. During the last several years, considerable data have accumulated raising doubts concerning the clonal deletion theory as a general explanation for tolerance since: 1) autoimmune diseases can be induced by injecting organ extracts; 2) numerous autoantibodies have been demonstrated under normal conditions; 3) the presence of normal autoreactive B cells can also be demonstrated; 4) autoantibodies have been induced from normal B lymphocytes upon mitogenic stimulation. Since they were able to produce autoantibodies, the precursor B-cells producing these autoantibodies should exist.

In collaboration with B. Guilbert and S. Avrameas, we demonstrated the existence of high levels of natural autoantibodies (NAA) in normal conditions and that the B cell repertoire secretes these so-called NAA. Overall, these results indicate that in normal serum, a substantial proportion of circulating IgS are indeed NAA and, that the precursors of this autoreactive repertoire account for a substantial part of normal B cells. As these autoantibodies express recurrent idiotopes, express V genes frequently in germline configuration and predominately in early life, they are the expression of the germline repertoire. Interestingly, this repertoire has been found in species phylogenetically distant from mammals such as fish and batrachians. They are autoantibodies since they bind autoantigens. However, they are not self-specific, since nobody has been able to demonstrate this type of autoantibodies against very critical self-antigens such as the A and B red blood cells groups. On the contrary, these autoantibodies bind public epitopes shared by all individuals belonging to a given species and even antigens that are well conserved during evolution. However, this is also the characteristic of pathogenic autoantibodies observed in autoimmune diseases. For instance anti-red blood cell autoantibodies recognise public antigens, anti-DNA from SLE patients recognise human, rat and murine DNA, anti-AChR autoantibodies recognise human and even fish receptors (for reviews see refs. #1-4).

Thus, we have evolved from Ehrlich’s horror autoxicus notion, to Burnet’s forbidden does hypothesis, to reach, now, the view that autoimmunity is a normal physiological phenomenon. But, how can we reconcile the experiments from Metchnikoff with the experiments from Ehrlich and Landsteiner, since these results have never been challenged.

Experiments with transgenic mice allow us to integrate all this experimental evidence. Nemazee’s group has created double transgenic mice expressing H-2Kk and anti-H2k transgenes. This is a very critical situation, since the transgene is recognising a determinant of polymorphism, that is a real-self antigen. According to Burnet’s prediction, B cells expressing the anti-
H2k transgene were stringently downregulated through programmed cell death (PCD) and receptor editing. However, in the case of Ig transgenes with autoantibody activity, which are not directed against critical self polymorphisms, the transgene is not deleted; it is simply downregulated or energised. In double transgenic mice expressing HEL and anti-HEL transgenes, B-cells were downregulated. B cells were also downregulated through energy and receptor editing in transgenic mice expressing pathogenic anti-DNA transgenes.

These experiments allow us to understand the apparent discrepancy between Ehrlich, Landsteiner and Metchnikoff’s research. Indeed, a subject expressing the A or B group will never produce typical autoantibodies against these determinants and no autoimmune haemolytic anaemias displaying autoantibodies with such specificities have been reported. However, the production of autoantibodies against public antigens, such as the I group, is a common phenomenon. Haemolytic anaemia autoantibodies are mainly directed against these public antigens. So, the B cell repertoire that is going to be directed against polymorphic determinants will be probably submitted to very stringent negative selection, i.e. deletion or stringent receptor editing mechanisms, whilst the repertoire that is directed against public determinants is probably not deleted and is an important component of the normal immune repertoire.

Construction of a murine model for human cold agglutinin disease

Autoimmune haemolytic anaemia (AIHA) induced by cold agglutinins (CA) is due to autoantibodies usually expressing the uk isotype, that recognise carbohydrate epitopes on human red blood cells (RBC) and cause haemagglutination at temperatures below 37°C and, optimally, in the cold (i.e. 4°C). The serum of healthy adult individuals frequently exhibits low titres of CA. Transient pathological increase of CA, defined as the titre at the highest temperature causing RBC agglutination, correlates better than its titre at 4°C with the pathogenic potential of a CA.

Different specificities of CA have been discerned, the most frequent being anti-I/i, which recognises developmentally regulated polyglycosyl ceramides epitopes, that constitute the precursor molecule of the ABO antigen system. Less frequently, CA directed against sialylated motifs can be observed: anti-Pr, anti-Sia-1b (anti-Gd), anti-Sia-b (anti-Fl) anti-Sia-I (anti-Vo).

CA disease is a rare disease (approximate incidence of 5 cases per million population) predominantly occurring in the elderly. A substantial proportion of cases develop in patients with B-cell lymphomas, Waldenström’s macroglobulinaemia or chronic lymphocytic leukaemia. The fact that CA disease can also evolve in two steps, the first one limited to AIHA, which subsequently can evolve to a tumoural disease, makes this disease a paradigmatic model of the relationship between autoimmune and malignant diseases. Pioneer work demonstrated that CA shared recurrent idiotopes and this was confirmed by the demonstration that anti-Il/i exclusively express the VH 4-34 gene, frequently associated with members of the VkIII family. Since the vast majority of CA express a unique VH gene, therapeutic strategies based on the preparation of anti-idiotypic vaccines directed against recurrent idiotopes may be devised and should allow the manufacture of vaccine that could be used in most cases of CA. This is particularly important because there is no effective therapy for this disease.

One of the major obstacles limiting progress in management strategies for this disease is the absence of an animal model. In a previous work, we studied one human CA displaying the rare anti-Sia-1b (Gd) specificity (CAGAS) at the molecular level. In contrast to classical anti-I/i CAs, which do not bind to mouse red blood cells, CAGAS agglutinates murine erythrocytes (MRBC) with better affinity than it binds human red blood cells (HRBC) and is able to haemolysie MRBC in the presence of complement. These characteristics make CAGAS a suitable candidate for the construction of a transfectoma and a transgenic model of autoimmune haemolytic anaemia.

We have introduced CA\textsuperscript{GAS} VH and V, domains into eukaryotic expression vectors and transfected them into the non-secreting mouse myeloma X63 cell line. Clones expressing complete engineered pentameric IgM k CA\textsuperscript{GAS} (eCA\textsuperscript{GAS}) recapitulating the characteristics of serum CA (sCA\textsuperscript{GAS}), could be obtained. The i.p. injection of eCA\textsuperscript{GAS} to normal BALB/c mice induced a typical haemolytic anaemia, as demonstrated by the presence of spontaneous cold agglutination of RBC, induction of anaemia and significant reticulocytosis.

Of interest, conspicuous bilateral ear loss was observed in one of these animals. In addition, i.p. injection of X63 transfected line into BALB/c nude mice induced ascites, typical haemolytic anaemia and shortening of the mean RBC survival. These findings validated the practical interest of constructing a transgenic mouse model expressing eCA\textsuperscript{GAS}.

Transgenic mice for either the CA\textsuperscript{GAS} heavy or light chain gene were then produced.

Expression of the human H chain alone resulted in a block in B lymphocyte maturation at the pro-B stage, and did not induce allelic exclusion. Double-transgenic (dTg) mice, obtained by mating the mice of the
two monotransgenic lines, produced significant amounts of serum CA.GAS, though the agglutination titre was insufficient to induce AIHA. In these mice, co-expression of the human H and L chains mostly released the pro-B cell block but the majority of mature cells expressing the human IgM in the bone marrow were subsequently eliminated by deletion, or submitted to receptor editing. Hybridomas secreting CA.GAS could be derived from splenic lymphocytes of these mice, indicating the presence of splenic B cells displaying this specificity. Interestingly, peritoneal cavity B cells, were enriched in B cells co-expressing the complete transgene. In addition, a large proportion of splenic B cells co-expressed the human H chain with a murine one, and probably secreted mixed IgM with a lower than expected agglutination capacity.

Indeed, this model demonstrates that CA.GAS is definitively pathogenic for mice, thus constituting the first reported case of a CA pathogenic for both human and mouse. When transgenic mice were constructed, it could be demonstrated that despite significant deletion and receptor editing, the transgenic clone secreting the CA still secreted significant amounts of the CA, although not sufficient to induce the disease.

We are trying to induce the disease using the following strategies:

a) mating the double transgenic mice, secreting the CA at levels insufficient to induce the disease, with Bcl-2 transgenic mice. The anti-apoptotic effect of the Bcl-2 transgene may increase the survival of B cells expressing the transgene. In addition, as lymphoma tumours occur in this type of mouse, a lymphoma expressing the CA transgene may be obtained. In that case, the disease should be observed;

b) since Mycoplasma has been reported to induce the disease in humans, through polyclonal stimulation of the normal B cell population producing CA, work is in progress in our laboratory to infect the transgenic animals with this micro-organism;

c) LPS has been reported to be able to rescue immature B cells in the bone marrow and allow them to migrate to the peripheral compartment. Since in this transgenic model, a significant number of B cells are deleted in the bone marrow, it is reasonable to test whether LPS is able to rescue these cells and to allow their migration to the peripheral compartment.

We hope that these different approaches will lead to the establishment of a full-blown CA disease, as was observed in the transfectoma model.

References

Acute promyelocytic leukaemia (APL) has aroused interest well outside the haematological field during the last ten years. Two features, both of which are unique to APL, have attracted the attention of various sectors of biomedical research: i) the remission of the disease obtained with retinoic acid (RA) treatment, whose mechanism of action consists of inducing the APL blasts to differentiate; ii) the presence in the APL blasts of fusion proteins that involve one of the retinoic acid receptors (RAR). RARα is a member of the super-family of nuclear hormone receptors that are involved in development and differentiation. In the last 5 years crucial insights have been gained into the issues connected with the molecular basis of APL and RA treatment, raising further questions about the cellular and molecular mechanisms underlying the leukaemogenic activities of RARα-fusion proteins and the physiological functions of the RARα-translocation partners. Present knowledge, however, allows us to speculate that understanding of such mechanisms will not only be relevant to APL, but to the processes of leukaemogenesis and growth/differentiation control in general.

The RARα-fusion proteins are leukaemogenic

APL is cytogenetically characterised by a reciprocal translocation that constantly involves chromosome 17, which breaks within the locus encoding for RARα. Usually the chromosome partner is chromosome 15, with the break located within the PM L locus, and less frequently, chromosomes 11 and 5 with breaks in the PLZF or NuMa and NPM loci, respectively. The hybrid genes so formed encode a PM L/RARα, PLZF/RARα, NuMa/RARα or NPM/RARα fusion protein, all of which retain the same portion of RARα. The RARα-translocations are primary chromosome aberrations and are often the only cytogenetic anomaly in the neoplastic metaphases. Experimental evidences for leukaemogenic potential is, however, only available for PM L/RARα and PLZF/RARα. Mice transgenic for PM L/RARα and PLZF/RARα manifest myeloid differentiative alterations with the phenotypic features of promyelocytic leukaemia.

RARα-fusion proteins affect differentiation

Despite clinical similarities, RA induces differentiation of leukaemic blasts and disease remission only in PM L/RARα APLs whereas PLZF/RARα APLs are ATRA resistant. Recent experimental evidence, obtained with PM L/RARα and PLZF/RARα model systems, has shown that RARα-fusion proteins interfere directly with the programme of terminal differentiation and are involved in both disease pathogenesis and response to RA: i) before the onset of leukaemias, the PM L/RARα transgenic mice show a pre-leukaemic condition characterised by increased and poorly differentiated haematopoietic precursors in the bone marrow; ii) expression of PM L/RARα or PLZF/RARα in haematopoietic precursor cell lines blocks differentiation induced by physiological stimuli; iii) RA induces differentiation of PM L/RARα-expressing cells, but not of PLZF/RARα-, both in the patients and in transgenic mice; iv) expression of PM L/RARα, but not PLZF/RARα, in resistant or poorly RA-sensitive cell lines restores a differentiative response to RA. It appears, therefore, that PM L/RARα and PLZF/RARα block differentiation at physiological concentrations of RA, while only PM L/RARα favours it at pharmacological doses.

RARα-fusion proteins regulate transcription

RA-induced degradation of PM L/RARα, which occurs through the activation of caspase and proteosomal pathways, has been proposed as a critical mechanism accounting for the response of APL blasts to this agent. However, the inhibition of the PM L/RARα proteolysis obtained by using various caspase inhibitor peptides does not impair the RA effect on APL blast differentiation, indicating that PM L/RARα is actively involved in conferring ATRA sensitivity.

The potential of PM L/RARα and PLZF/RARα to interfere with haematopoietic differentiation and to mediate RA sensitivity is thought to involve transcriptional regulation of RA target genes. Retinoic acid receptors are transcription factors involved in the control of terminal myeloid differentiation. They behave as ligand-dependent transcriptional regulators, repressing transcription in the absence of ligand and activating transcription in its presence. The different effects on transcription are carried out through recruitment of coregulators: unliganded receptors bind corepressors (NCoR and SMRT) that are found within a complex containing histone deacetylase
(HDAC) activity, whereas liganded receptors recruit coactivators with histone acetylase activity (HATs). Chromatin remodelling activities (such as the NURD and hBRG1 complexes) have also shown to be required for transcriptional regulation by retinoid receptors and other members of the nuclear hormone receptor superfamily, suggesting a hierarchy of promoter structure modifications in RA target genes carried out by multiple co-regulatory complexes.2,5

Both PM L/RARα and PLZF/RARα retain the ability of RARα to regulate transcription of RA-target genes. PM L/RARα and PLZF/RARα fusion proteins recruit the N-CoR/HD complex through their RARα moiety.6 PLZF/RARα contains a second, ATRA-resistant, binding site in the PLZF N-terminal region. High doses of ATRA release HD activity from PM L/RARα, but not from PLZF/RARα. Mutation of the N-CoR binding site abrogates the ability of PM L/RARα to block differentiation, whereas inhibition of HD activity switches the transcriptional and biological effects of PLZF/RARα from inhibitor to activator of the RA-signalling pathway. Therefore, recruitment of HD and regulation of RA-target genes are crucial to the transforming potential of APL-fusion proteins while the different effects of ATRA on the stability of the PM L/RARα- and PLZF/RARα--corepressor complexes determine the differential response of APLs to ATRA.

**RARα fusion proteins affect survival**

An additional biological activity which may contribute to the leukaemogenic potential of RARα fusion proteins is their interference with cell survival. Expression of PM L/RARα in haematopoietic cells inhibits programmed cell death, while expression of the fusion protein into non-haematopoietic cells induces apoptosis.7 These effects depend on the integrity of the incorporated PM L sequences, and may be the consequence of the interference of PM L/RARα with the growth-suppressive function of the wild-type PM L protein. Indeed, forced expression of PM L in a variety of cell lines induces growth arrest through mechanisms which involve induction of apoptosis; targeted disruption of the PM L locus in mice increases the rate of spontaneous or induced carcinogenesis.8 However, the molecular mechanisms through which PM L/RARα deregulates the PM L intracellular pathways are not clear. PM L localises within distinct nuclear compart-

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### References

Diagnosis

A unique t(15;17) chromosome aberration resulting in the PM/L/RARα gene fusion and an exquisite sensitivity to the differentiating agent all-trans retinoic acid (ATRA) are the main distinguishing features of acute promyelocytic leukaemia (APL). Given in combination with anthracycline-containing chemotherapy, ATRA has been shown to provide the best treatment results in this disease. Because the presence of the PM/L/RARα hybrid in leukaemic cells is known to predict response to ATRA, and due to frequent life-threatening haemorrhagic diatheses, rapid diagnosis at the genetic level is recommended for promptly initiating APL-tailored therapy.1

Demonstration of the disease’s genetic hallmark can be carried out at chromosome, protein, DNA or RNA levels, using conventional karyotyping and/or fluorescent in situ hybridisation (FISH), anti-PML antibodies, Southern blotting and reverse-transcriptase polymerase chain reaction (RT-PCR), respectively. Each of these procedures has its own advantages and pitfalls (Table 1). In particular, karyotyping may occasionally give false-negative results (i.e. absence of t(15;17) in cases later found to contain cryptic PM/L/RARα rearrangement) and like Southern blotting is time-consuming, requiring a few days for execution. RT-PCR allows a rapid and highly sensitive diagnosis, but it is prone to artefacts and technically difficult if not performed in experienced laboratories. Recently, immunohistochemical analysis of PML staining with monoclonal antibodies has proven useful for specific diagnosis, which is established following the identification of the so-called microspeckled PML protein distribution consequent to the translocation.2

In light of its widespread availability for rapid, specific and low cost diagnosis, this procedure might be recommended as a convenient tool for identification of the disease’s hallmark, particularly in centres not equipped or trained for more sophisticated analyses. Apart from confirming morphologically typical APLs, this assay would clarify diagnosis in cases with uncertain cytological features and its application could be extended to all acute myeloid leukaemias (AMLs).

In cases with the typical hypergranular morphology, however, treatment initiation need not be postponed pending the results of genetic studies. These patients may in fact immediately receive specific therapy and ATRA could be subsequently withdrawn in those rare cases lacking the genetic abnormality despite having typical morphology. If not available on site, RT-PCR and karyotypic characterisation might be requested from specialised centres in order to obtain further potentially relevant information (e.g. additional chromosome abnormalities, variant translocations, PM/L/RARα isoform type) and to define the correct RT-PCR strategy to be used in the individual patient for minimal residual disease (MRD) monitoring.

Front line treatment

Following the advent of ATRA, large trials using this agent in variable combinations with chemotherapy (CHT) for newly diagnosed APL have been carried out in Europe (APL ‘93, GIMEMA, and PETHEMA studies), the USA (New York Memorial Sloan-Kettering Cancer Center, US Intergroup and MD Anderson studies), the UK (MRC), China and Japan (JALSG). Overall, nearly 2,000 patients were included in these trials.3 The advantage of ATRA inclusion in front line treatment was established in 1993 by the APL European group in a randomised study comparing ATRA followed by CHT (ATRA→CHT) with CHT alone.6 Subsequent studies were aimed in most cases at establishing the optimal ATRA and CHT combination. Other issues which were addressed were the importance of genetic diagnosis and the role of maintenance therapy including or not ATRA. The main results may be summarised as follows:

1. resistant leukaemia to regimens variably combining ATRA and CHT is virtually absent in patients with genetically confirmed diagnosis. Failure to achieve haematological complete remission (CR, reported in 7-20% of cases) is mainly due to early death from haemorrhage or infectious complications;

2. the simultaneous ATRA+CHT combination provides the best results in terms of CR and disease-free survival (DFS), and seems more effective in diminishing the occurrence of overt ATRA syndrome. This latter is also successfully counteracted, however, by strict adherence to the recom
Table 1. Technical approaches for genetic diagnosis in APL.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Main advantages</th>
<th>Main pitfalls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Karyotype</td>
<td>Additional genetic information (lesions other than t(15;17))</td>
<td>Frequent poor quality metaphases and cryptic translocations (false negative)</td>
</tr>
<tr>
<td>Southern blot</td>
<td>Specific</td>
<td>Time consuming; hybridisation with &gt;1 probe is often required</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Highly sensitive</td>
<td>Frequent poor RNA yield and amplification artefacts</td>
</tr>
<tr>
<td>Immunohistochemistry (PML staining pattern)</td>
<td>Rapid, simple and low-cost</td>
<td>Provides no information on the PML breakpoint type</td>
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Conclusions and future perspectives

Despite considerable improvement in diagnosis and management, a sizeable proportion of APL patients who receive state-of-art front line treatment still die of early complications or following disease recurrence. With regard to early death, a special effort should be made to define risk categories better (hyperleukocytosis, older age, severity of the coagulopathy, other unknown factors) in order to promptly reinforce adequate supportive care and to evaluate the feasibility of a tailored (less intensive?) initial treatment.

At least 60% of newly diagnosed patients become long-term survivors and are probably cured with ATRA+CHT. It is conceivable to hypothesise that a relevant fraction of these patients are being overtreated and that they might, therefore, be spared this risk. On the other hand, we are still unable to identify those cases (approximately 20%) who will ultimately relapse after initial therapy and are, therefore, in need of treatment intensification. The definition of risk categories at diagnosis for more appropriate treatment stratification remains a big challenge for future clinical investigation in this disease. Finally, the place of novel drugs which have proven effective in relapse, such as arsenic trioxide, and the advantage of anticipating salvage treatment at the time of molecular recurrence remain to be established.

Acknowledgements

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References

Several new therapeutic approaches have recently been clinically tested in patients with advanced cancer, including the use of arsenicals and various drugs that affect gene transcription via inhibition of histone deacetylase. Although our interest in these agents arose from our programme in acute promyelocytic leukaemia (APL), we expect that these agents will prove useful for a number of other cancers. The following pages summarise some aspects of our group’s clinical work in these areas.

Arsenicals as cancer treatment

Like many others, we were intrigued by reports emanating from the People’s Republic of China in the early 1990s that intravenous arsenic had re-emerged as a cancer treatment. Arsenic has been used as a medicinal for many centuries, both in topical and injectable forms (as escharotic agents) as well as oral forms that were first described in the 1700s. Fowler’s solution given orally was used in the 19th and early 20th centuries as a treatment to reduce extreme leukocytosis in patients with chronic myelocytic leukaemia (CML). In the West, however, this use dwindled after the widespread application of radiotherapy in the 1930s and the advent of other cytotoxic drugs after the 2nd World War.

The recent upsurge of interest stems from reports from Harbin China that an intravenously injectable formulation induced remissions in patients with APL. Since we perceived an enormous regulatory hurdle to direct application of this therapy in the United States, we first tested an organic arsenical, melarsoprol, which was already formulated for human use and was immediately available on an investigational basis because of its use for the treatment of African trypanosomiasis due to T. brucei.

Laboratory and clinical studies of melarsoprol

We evaluated both As$_2$O$_3$ and melarsoprol for possible antileukaemic activity in vitro. In these studies, melarsoprol exhibited broad antileukaemic activity against both myeloid and lymphoid cells. Given the breadth of this activity, we then initiated a clinical study to evaluate the pharmacokinetics, safety, and potential efficacy of melarsoprol in patients with relapsed leukaemia. Using the anti-trypanosomal dose and schedule, patients received escalating intravenous doses daily for 3 days, repeated weekly for 3 weeks. Doses were 1 mg/kg on day 1, 2 mg/kg on day 2, and 3.6 mg/kg on day 3 and on all days thereafter, up to a maximum daily dose of 200 mg. Eight patients [5 AML, 1 APL, 1 CML, and 1 CLL] were treated. Mean peak plasma concentrations of the parent drug were obtained immediately after injection. These concentrations ranged from 1.2 µg/mL on day 1 to 2.4 µg/mL on day 3, were dose proportional, and decayed with a $t_1/2$ $\approx$ 15 minutes. A minor clinical response (regression of splenomegaly and lymphadenopathy) was observed in a patient with chronic lymphocytic leukaemia. One patient with t(11;17)-variant APL who had been refractory to chemotherapy and all-trans retinoic acid had slight improvements in both his leukocyte and platelet counts with the first course of treatment. He then received a second 3-week course without further improvement and was removed from the study.

Unfortunately, central nervous system (CNS) toxicity proved limiting. Three patients experienced generalised grand mal seizures during the second week of therapy. Other neurologic reactions included vertigo (2 patients) and paraesthesia (1 patient), both of which resolved after discontinuing therapy. The patient who received two complete treatment cycles experienced moderately severe peripheral neuropathy, with bilateral lower extremity paraesthesia that caused moderate functional impairment, but which resolved approximately 5 weeks after the last dose. Other common reactions were nausea, vomiting, and irritation at the injection site. We concluded that the antitrypanosomal dosing schedule of melarsoprol is associated with excessive CNS toxicity, and that verification of the striking preclinical activity in patients with leukaemia requires development of an alternative dosing schedule.

Arsenic trioxide

We and others have recently confirmed that arsenic trioxide induces complete remissions (CR) in a high proportion of patients with APL. In our initial pilot study, 12 heavily pretreated patients were treated...
with arsenic trioxide at doses ranging from 0.06 to 0.2 mg/kg/day until leukemic cells were eliminated from the marrow. Patients who achieved CR were eligible to receive up to 5 additional courses of therapy, with each course given for a cumulative total of 25 days at a daily dose of 0.15 mg/kg/d every 3-6 weeks. Marrow leukemic cells were eliminated in 11 of 12 patients after a median treatment duration of 33 days (range, 12-39 days) (one patient who had an intracranial haemorrhage on day 1 died on day 5). The median cumulative dose during induction was 360 mg (range, 160 to 515 mg). CR by all criteria was attained at a median time of 47 days (range, 24-83 d). Impressively, 8 of 11 patients tested converted RT-PCR assays for PM L/RAR-α to negative at or before the end of the second course, while 3 patients who remained positive relapsed early and appeared to have rapidly developed arsenic resistance. Several patients have received up to 6 cycles of arsenic trioxide therapy without showing evidence of cumulative toxicity.

We subsequently initiated a confirmatory multicentre study in patients with relapsed or refractory APL using the same treatment schedules at a fixed daily dose of 0.15 mg/kg/d. To date, 28 patients have been accrued, and 19 of 21 evaluable patients (90%) have achieved complete remission. A new study of arsenic trioxide at doses ranging from 0.06 to 0.2 mg/kg/day until leukemic cell lifespan. Patients who achieved CR were eligible to receive up to 5 additional courses of therapy, with each course given for a cumulative total of 25 days at a daily dose of 0.15 mg/kg/d every 3-6 weeks. Marrow leukemic cells were eliminated in 11 of 12 patients after a median treatment duration of 33 days (range, 12-39 days) (one patient who had an intracranial haemorrhage on day 1 died on day 5). The median cumulative dose during induction was 360 mg (range, 160 to 515 mg). CR by all criteria was attained at a median time of 47 days (range, 24-83 d). Impressively, 8 of 11 patients tested converted RT-PCR assays for PM L/RAR-α to negative at or before the end of the second course, while 3 patients who remained positive relapsed early and appeared to have rapidly developed arsenic resistance. Several patients have received up to 6 cycles of arsenic trioxide therapy without showing evidence of cumulative toxicity.

**Adverse reactions to arsenic trioxide**

Adverse effects due to arsenic trioxide have generally been mild. These reactions have included skin rash, light headache during the infusion, fatigue, mild hyperglycaemia, musculoskeletal pain, and EKG changes (particularly QT prolongation). In the past, all-trans retinoic acid (RA) was shown to induce terminal differentiation of APL cells in vivo, and leukocytosis was commonly observed during this process, probably as a result of a transient increase in leukemic cell lifespan. Similarly, previous studies by us and others have shown that arsenic treatment is associated with induction of partial, non-terminal immunophenotypic cytodifferentiation.

In clinical studies of APL patients treated with arsenic trioxide at this centre, leukocytosis (defined as a total peripheral blood leukocyte count ≥ 10×10⁹ cells/L) was observed in 13 of 23 patients (57%). The median baseline leukocyte count for all treated patients (2.6×10⁹/L [range, 0.7-65.2]) was not substantially different from the cohort of patients who subsequently developed leukocytosis (3.5×10⁹/L [range, 1.3-65.2]). The median peak leukocyte count of the latter group was 60.3×10⁹/L (range, 17.1-247.0), which occurred at a median of 19 days from the start of arsenic therapy (range, 4-24 days). No other cytotoxic therapy was administered, and the leukocytosis resolved in all patients.

Distinct from leukocytosis, approximately 50% of APL patients who receive all-trans RA for induction develop one or more signs of the RA syndrome, a potentially lethal complication. The RA syndrome is a loosely defined constellation of problems that include fever, weight gain, dyspnoea, pulmonary oedema, pleural effusions, musculoskeletal pain, or joint effusions. Signs/symptoms of the RA syndrome were observed in 17 of the 23 patients (35%) treated with arsenic trioxide. Several patients were presumptively treated with high-dose dexamethasone. All patients with this problem have recovered, and interruption of arsenic therapy was not required. These data show that even non-terminal cytodifferentiation induced by arsenic trioxide is associated with induction of leukocytosis and the RA syndrome in APL. These effects appear to be related to intrinsic biological responsiveness to differentiating agents.

**Pharmacokinetics of arsenic trioxide and studies in other cancers**

We have conducted several dose-ranging studies to examine the pharmacokinetics and biological effects of arsenic trioxide in patients with other types of haematologic cancers and solid tumours. Pharmacokinetic analysis of blood and urine for elemental arsenic content showed that arsenic is distributed in both plasma and red blood cell fractions of whole blood. Parallel elimination curves suggest that these 2 compartments are freely exchangeable. The mean area under the plasma × concentration curve (AUC) on day 1 after a dose of 0.15 mg/kg was ~ 400 ng.hr/mL. Approximately 20% of the administered dose was recovered in urine within the first 24 hrs. The terminal half-life appears to be quite prolonged.

In an ongoing dose-ranging study in patients with haematologic cancers other than APL, we have employed a daily IV dosing schedule for a cumulative total of 25 days per treatment course, with additional cycles administered every 3-5 weeks. Dose levels have ranged from 0.1 to 0.25 mg/kg/day. A separate study in patients with solid tumours is using a daily × 5 days schedule, with additional cycles in responding patients administered every 4 weeks. Doses in that study have ranged from 0.15 to 0.3 mg/kg/day. Over this range of doses, the drug has continued to be relatively well-tolerated. However, skin rash, diarrhoea, QTc prolongation on EKG, and fatigue (but not leukocytosis or RA syndrome) have emerged as increasingly prominent side-effects in these other diseases.

**Targeting of histone deacetylase as anticancer therapies**

Acetylation of DNA-associated histones is linked to gene-specific activation, whereas histone deacetylation is associated with transcriptional repression. Recent studies have shown that inhibitors of histone deacetylase (HDAC) can relieve transcriptional repression caused by certain oncogenes. These inhibitors include trichostatin A, trapoxin, depudecins, bicyclic...
depsipeptides, hybrid polar compounds and various butyrates.

Since sodium phenylbutyrate was immediately available for investigational use in human subjects, we tested whether this drug, as well as the concept of HDAC inhibition, could be effective in vitro and then clinically in patients with highly resistant APL. Having documented that sodium phenylbutyrate (PB) inhibited HDAC, we found that high concentrations of PB alone decreased cell growth but had no effect upon differentiation of NB4 cells; however, the combination with all-trans RA caused a substantial increase in differentiation.

We then treated a child who had relapsed after numerous cytotoxic drugs, all-trans RA, allogeneic bone marrow transplantation, and arsenic trioxide. Blood and marrow mononuclear cells were assayed for histone hyperacetylation by immunohistochemistry and Western blot analysis. Minimal residual disease was assessed by RT-PCR for PML/RARα. After reconfirming that the patient was clinically resistant to RA, PB was added to the treatment regimen. The RA dose ranged from 45-90 mg/m²/d in 2 equally divided doses; the PB dose ranged from 150-210 mg/kg IV BID infused IV over 1 hr. By day 23, the patient’s marrow showed complete elimination of visible leukaemic cells, and she achieved a complete clinical and cytogenetic remission shortly thereafter. By the 2nd treatment course, the previously positive RT-PCR assay for PML/RARα had converted to negative. Peak PB levels in plasma were 2.5 mM; however, these levels had decayed to baseline levels by 4 hrs. Immunofluorescence and Western blot analysis showed that PB clinical treatment caused a time-dependent increase in histone hyperacetylation in blood and bone marrow mononuclear cells.10

These preliminary data suggest that although PB is a relatively weak HDAC inhibitor, the drug can reversibly increase chromatin hyperacetylation in target cells, possibly restoring RA sensitivity in retinoid-resistant APL patients. The concept of HDAC inhibition, if it can be selectively induced, may prove useful in other neoplastic diseases associated with oncogenic repression of gene transcription due to recruitment of histone deacetylases. This concept is currently being tested in a broader study.

Acknowledgements
Numerous colleagues have collaborated in studies by our group. I am especially indebted to Drs. Pier Paolo Pandolfi, Victoria Richon, Steven Soignet, Paul Marks, Elizabeth Calleja, and Peter M aslak for their contributions to these efforts.

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References
procedures surrounding the test need to be standardised and the criteria for a normal and an abnormal test result need to be defined, using for example receiver-operator curve analysis. In addition the inter- and intraobserver variability of the test should be assessed. Ideally, the development of the diagnostic test will pass into the next phase only after all these aspects have been adequately investigated. Phase 2 would then consist of studies in which the diagnostic accuracy of the new test is evaluated in a large group of consecutive patients with a suspicion of the disease for which the test is intended. The outcomes of the new test must be blindly and independently compared with the results of the gold standard test for the relevant disease. The sensitivity, specificity and positive and negative predictive values of the test can then be determined. In case of insufficient accuracy, one might need to return to phase 1 to re-evaluate the test procedures and the definition of normal and abnormal results.

Only after the requirements of both phases 1 and 2 have been adequately met, can the development process advance into phase 3 in which the role of the new test in the diagnostic process is evaluated. In this phase of development management studies are performed, in which therapeutic decisions are made based on the result of the new diagnostic test. A new diagnostic test should only be implemented in routine clinical use after all three phases of development have been properly performed with good results.

**Pulmonary angiography**

At present pulmonary angiography is regarded as the gold standard diagnostic test for pulmonary embolism. There are generally accepted standardised procedures for the performance of the test and criteria for a normal and an abnormal test result have been formulated. Furthermore, the observer variability of the test has been assessed and has been found to be minimal. Assessment of the sensitivity and specificity is not possible since pulmonary angiography is itself the reference method. The ability of pulmonary angiography to confirm or exclude pulmonary embolism is, however, considered to be high. Several studies in patients who underwent pulmonary angiography for the suspicion of acute pulmonary embolism have shown that it is safe to withhold anticoagulant treatment from those patients who have a normal test result. In a total of 840 patients with suspected pulmonary embolism and a normal pulmonary angiogram, anti-coagulant therapy was withheld and patients were followed-up for a minimum of 3 months. The overall incidence of recurrent pulmonary embolism was 1.9% (95% CI 1.4% - 3.2%) and the incidence of fatal pulmonary embolism was 0.3% (95% CI 0.09% - 1.08%). On the basis of these studies it is deemed safe to consider pulmonary embolism excluded in the case of a normal pulmonary angiography and proven in the case of an abnormal result.

**Ventilation-perfusion scintigraphy**

Ventilation-perfusion scintigraphy is the most widely used first line diagnostic test when pulmonary embolism is suspected. The criteria for the interpretation of lung scans have been a matter of debate for many years. The most clinically applicable classification is that of three categories. These are: 1) a normal perfusion scan on the basis of which pulmonary embolism is excluded; 2) a high probability lung scan, defined as one or more defects of at least segmental size with associated local normal ventilation, which strongly indicates the presence of emboli; and 3) the remaining lung scan results which are considered to be non-diagnostic. The interobserver variability of ventilation-perfusion scintigraphy using the various classifications has been investigated in several studies and has been found to be acceptable.

Numerous studies have compared lung scintigraphy with pulmonary angiography. The overall positive predictive value of a high-probability lung scan has been shown to be 88% (95% CI 84% - 91%). This is generally considered to be enough evidence to accept the diagnosis of pulmonary embolism. However, pulmonary embolism cannot be considered proven or excluded on the basis of a non-diagnostic lung scan. Studies have shown that the prevalence of angiographically proven pulmonary embolism is approximately 25% in patients with such a scan result. Further diagnostic investigation is therefore warranted in these patients.

Management studies using ventilation-perfusion scintigraphy in the clinical work-up of patients suspected of having pulmonary embolism have been performed. In these studies a normal perfusion lung scan was used to reject the diagnosis and anticoagulant treatment was withheld. In a total of 693 patients with a normal perfusion lung scan in whom anticoagulants were withheld 0.3% (95% CI 0.2% - 0.4%) suffered a thromboembolic event during a follow-up period of at least 3 months. Hence, it is deemed safe to withhold anticoagulants in patients with a normal perfusion lung scan.

**D-dimer**

In recent years the measurement of D-dimer, a degradation product of fibrin, has been suggested for use in the diagnostic work-up of patients with clinically suspected pulmonary embolism. Absence of an elevated concentration of D-dimer has been suggested to have a high negative predictive value for venous thromboembolism. As a result, numerous D-dimer tests (quantitative and qualitative) have been developed which are now widely commercially available.

Proper evaluation of the interassay and intra- and interobserver variation has only been performed for the minority of the available D-dimer tests. The studies which have been performed have shown that this variation is rather high. Several D-dimer tests have been compared to a gold standard diagnostic method
to determine the accuracy of the tests, albeit often in non-consecutive series of patients. The high sensitivity and moderate specificity of the D-dimer assays seem to qualify the test as potentially useful for the exclusion of venous thromboembolism. However, the sensitivity of D-dimer tests varies widely among the studies, ranging from 61% to 100% and data are still limited. The cut-off values of the D-dimer assays can be varied so as to increase the sensitivity leading, however, to a decrease in specificity, which could limit the clinical usefulness. When a receiver operator curve (ROC) analysis is performed, the various quantitative D-dimer assays appear to be nearly identical. The determination of optimal cut-off values for the individual D-dimer assays is, therefore, essential. Management studies have evaluated the use of D-dimer integrated in a diagnostic strategy in combination with other non-invasive tests, such as ventilation-perfusion lung scanning and clinical decision rules. In this setting, management with D-dimer assays seems safe and cost-effective. However, one needs to interpret these data with great caution. The use of D-dimer assays within a strategy combined with other diagnostic tests seems safe, while the exclusion of pulmonary embolism on basis of a normal D-dimer test alone seems unjustified.

Spiral computed tomography

Spiral computed tomography (spiral CT) is a relatively new diagnostic technique for patients with suspected pulmonary embolism, first described in 1992. In one study the interobserver agreement was found to be relatively high (kappa 0.77). However, this does not appear to be true for pulmonary emboli that are confined to the subsegmental arteries. In addition, no accepted criteria for normal and abnormal test results have been formulated. The first study that compared spiral CT with angiography was performed in 42 patients and found a sensitivity of 100% and a specificity of 96%. There have been many subsequent studies comparing spiral CT with angiography or lung scintigraphy. The overall sensitivity and specificity are 91% (95% CI 87-94) and 93% (95% CI 90-96), respectively. However, the sensitivity varies from 54% to 100% and the specificity from 67% to 100% in these studies. This can in part be explained by differences in patient selection in the various studies, the higher sensitivities and specificities generally being found in the studies with highly selected patient groups. Furthermore, different criteria for the diagnosis of pulmonary embolism and various procedures for the performance of the test were used.

One management study implementing the spiral CT in the diagnostic work-up of patients suspected of pulmonary embolism has been performed. In this study a recurrence rate of venous thromboembolism of 5.5% (95% CI 1.3 to 9.7) during a follow-up period of 3 months was found in patients with a normal spiral CT and a normal ultrasound. This finding illus-

Discussion

The introduction of a new diagnostic test is usually accompanied by a great amount of enthusiasm concerning the potential indications for its use. A new test is often presented as an improvement in the diagnostic arsenal. This all too frequently results in the wide acceptance and large-scale implementation of a new diagnostic test directly following its introduction. This often occurs without adequate foundation on properly performed studies. However, after the initial enthusiasm, the limitations and the true value in clinical practice become clear. In the past this has often resulted in the almost complete disappearance of new and initially promising tests from the diagnostic armamentarium. One may assume that with proper evaluation these tests could have been found valuable within the proper setting. As example of the problems associated with the introduction of new diagnostic methods we have assessed 4 tests currently available for the diagnosis of pulmonary embolism using the suggested guidelines for the proper evaluation of diagnostic tests.

Pulmonary angiography, presently considered the gold standard method, has been evaluated quite extensively throughout the various phases mentioned earlier. Both the role in the diagnostic work-up of a patient with suspected pulmonary embolism and the consequences of its result are known. Ventilation-perfusion scintigraphy has also been shown to be of use in the diagnosis of pulmonary embolism. With the performance of proper studies in comparison to pulmonary angiography and numerous clinical follow-up studies, the value and limitations of the test have become clear. Both pulmonary angiography and ventilation-perfusion scintigraphy have therefore been properly evaluated according to the suggested guidelines and are well-established diagnostic methods.

When considering the evaluation of D-dimer assays, it is apparent that all phases of development have been traversed for some of the available assays. However, there are also some troubling aspects in the development of this test. Not all of the assays have been properly evaluated in phase 1 and 2 studies, and the results of the studies that have been performed should not be directly extrapolated to other D-dimer assays. Furthermore, a wide spread in the sensitivity and specificity has been found between studies, further complicating the choice of an optimal cut-off point. Optimal cut-off levels for each individual D-dimer assay need to be determined. The observation that the ROC curves of the various quantitative D-dimers are nearly identical would seem to support this. The management studies that have so far been performed show promising results. Use of the D-
dimer within certain diagnostic strategies appears safe and cost-effective. However, further studies are warranted before the widespread use of D-dimer assays in routine clinical practice is justified. The use of a D-dimer assay as a single test for the exclusion of pulmonary embolism, a practice that is gaining ground, is certainly not founded on adequate studies.

When the studies evaluating spiral CT scanning are critically assessed, one must conclude that the phases of development have been passed in a less orderly fashion. Firstly, consensus does not exist concerning the optimal method of performance of the test. Furthermore, accepted criteria by which the presence or absence of pulmonary embolism can be evaluated are still lacking. This translates to the wide variation found in the performance of the test when compared to pulmonary angiography and ventilation-perfusion scanning. A more important cause of this variation is, however, that these comparisons were not consistently performed in representative groups of patients with suspected pulmonary embolism. This can be seen by the wide range of the prevalence of pulmonary embolism in the various studies (33-88%). The only management study which has so far been performed using spiral CT scanning in the management of patients with suspected pulmonary embolism by Ferretti et al., has shown disappointing results (recurrence rate 5.4%, upper 95% CI 9.7%). Further phase 1 studies in which methodology and criteria are standardised, and phase 2 studies in which spiral CT scanning is compared with pulmonary angiography or ventilation-perfusion scintigraphy are, therefore, needed before further management studies or implementation in routine clinical practice should occur.

In conclusion, at present pulmonary angiography and ventilation-perfusion scintigraphy are the only properly evaluated diagnostic tests for pulmonary embolism. Although there are many new developments in this field, such as D-dimer assays and the spiral CT scan which are certainly promising, further studies are needed to determine their real value and safety in the diagnostic work-up of patients suspected of having a pulmonary embolism.

The diagnostic tests for pulmonary embolism exemplify the problems often surrounding the introduction of new tests. Due to the initial enthusiasm, a new test is often implemented in clinical use before proper evaluation has occurred. Studies are also often performed without adequate data from earlier phases of development. We would suggest that guidelines for the proper evaluation of diagnostic tests before their implementation in routine clinical practice are necessary. The criteria that need to be met by consecutive phases of development need to be generally accepted and implemented.

References

9. de Monyé W, Sanson BJ, Büluer HR, Pattynama PM, Huisman MV. The value of rapid quantitative D-dimer assays versus gold standard for suspected pulmonary embolism and the influence of embolus size on accuracy. Submitted for publication.
Diagnosis

A unique t(15;17) chromosome aberration resulting in the PM L/RARα gene fusion and an exquisite sensitivity to the differentiating agent all-trans retinoic acid (ATRA) are the main distinguishing features of acute promyelocytic leukaemia (APL). Given in combination with anthracycline-containing chemotherapy, ATRA has been shown to provide the best treatment results in this disease. Because the presence of the PM L/RARα hybrid in leukaemic cells is known to predict response to ATRA, and due to frequent life-threatening haemorrhagic diatheses, rapid diagnosis at the genetic level is recommended for promptly initiating APL-tailored therapy.1-4

Demonstration of the disease’s genetic hallmark can be carried out at chromosome, protein, DNA or RNA levels, using conventional karyotyping and/or fluorescent in situ hybridisation (FISH), anti-PML antibodies, Southern blotting and reverse-transcriptase polymerase chain reaction (RT-PCR), respectively. Each of these procedures has its own advantages and pitfalls (Table 1). In particular, karyotyping may occasionally give false-negative results (i.e. absence of t(15;17) in cases later found to contain cryptic PM L/RARα rearrangement) and like Southern blotting is time-consuming, requiring a few days for execution. RT-PCR allows a rapid and highly sensitive diagnosis, but it is prone to artefacts and technically difficult if not performed in experienced laboratories. Recently, immunohistochemical analysis of PM L staining with monoclonal antibodies has proven useful for specific diagnosis, which is established following the identification of the so-called microspeckled PM L protein distribution consequent to the translocation.5 In light of its widespread availability for rapid, specific and low cost diagnosis, this procedure might be recommended as a convenient tool for identification of the disease’s hallmark, particularly in centres not equipped or trained for more sophisticated analyses. Apart from confirming morphologically typical APLs, this assay would clarify diagnosis in cases with uncertain cytological features and its application could be extended to all acute myeloid leukaemias (AMLs).

In cases with the typical hypergranular morphology, however, treatment initiation need not be postponed pending the results of genetic studies. These patients may in fact immediately receive specific therapy and ATRA could be subsequently withdrawn in those rare cases lacking the genetic abnormality despite having typical morphology. If not available on site, RT-PCR and karyotypic characterisation might be requested from specialised centres in order to obtain further potentially relevant information (e.g. additional chromosome abnormalities, variant translocations, PM L/RARα isoform type) and to define the correct RT-PCR strategy to be used in the individual patient for minimal residual disease (MRD) monitoring.

Front line treatment

Following the advent of ATRA, large trials using this agent in variable combinations with chemotherapy (CHT) for newly diagnosed APL have been carried out in Europe (APL ‘93, GIMEMA, and PETHEMA studies), the USA (New York Memorial Sloan-Kettering Cancer Center, US Intergroup and MD Anderson studies), the UK (MRC), China and Japan (JALSG). Overall, nearly 2,000 patients were included in these trials.6 The advantage of ATRA inclusion in front line treatment was established in 1993 by the APL European group in a randomised study comparing ATRA followed by CHT (ATRA→CHT) with CHT alone.6 Subsequent studies were aimed in most cases at establishing the optimal ATRA and CHT combination. Other issues which were addressed were the importance of genetic diagnosis and the role of maintenance therapy including or not ATRA. The main results may be summarised as follows:

1. resistant leukaemia to regimens variably combining ATRA and CHT is virtually absent in patients with genetically confirmed diagnosis. Failure to achieve haematological complete remission (CR, reported in 7-20% of cases) is mainly due to early death from haemorrhage or infectious complications;
2. the simultaneous ATRA+CHT combination provides the best results in terms of CR and disease-free survival (DFS), and seems more effective in diminishing the occurrence of overt ATRA syndrome. This latter is also successfully counteracted, however, by strict adherence to the recom-
### Table 1. Technical approaches for genetic diagnosis in APL

<table>
<thead>
<tr>
<th>Technique</th>
<th>Main advantages</th>
<th>Main pitfalls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Karyotype</td>
<td>Additional genetic information (lesions other than (15;17))</td>
<td>Frequent poor quality metaphases and cryptic translocations (false negative)</td>
</tr>
<tr>
<td>Southern blot</td>
<td>Specific</td>
<td>Time consuming; hybridisation with &gt;1 probe is often required</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Highly sensitive</td>
<td>Frequent poor RNA yield and amplification artefacts</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>Rapid, simple and low-cost</td>
<td>Provides no information on the PML breakpoint type</td>
</tr>
</tbody>
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1. The benefit of cytarabine administered in addition to anthracyclines during induction and/or consolidation is unclear. Studies in which cytarabine has been omitted from induction (GIMEMA) or both induction and consolidation (MD Anderson and PETHema) have reported results comparable or even superior (at least in terms of reduced toxicity) to those obtained in studies including this agent. Whilst the effectiveness of anthracycline-based monochemotherapy for remission induction of APL was established as long as 26 years ago by the pioneering work of Jean Bernard, the omission of cytarabine from overall treatment (i.e. including consolidation) is a recent attempt and long-term results of the MD Anderson and PETHema groups are awaited to resolve this issue. However, no study has as yet compared different consolidation options including or not cytarabine in phase III trials;

2. The benefit of cytarabine administered was confirmed in the analysis of the GIMEMA study which adopted the same randomisation designed by the European APL '93 study (unpublished observations). The advantage of using CHT maintenance with methotrexate and 6-mercaptopurine had been suggested in France and the USA in the past decade; hence, both the APL '93 and the GIMEMA groups included four randomisation arms in their studies to compare ATRA vs. CHT vs. ATRA+CHT vs. observation. The final analysis of these studies should be available soon.

4. Two large randomised studies (APL'93 and US Intergroup) have demonstrated a benefit conferred by ATRA-containing maintenance therapy. This is also confirmed in the analysis of the GIMEMA study which adopted the same randomisation designed by the European APL '93 study (unpublished observations). The advantage of using CHT maintenance with methotrexate and 6-mercaptopurine had been suggested in France and the USA in the past decade; hence, both the APL '93 and the GIMEMA groups included four randomisation arms in their studies to compare ATRA vs.

5. The prognostic outcome of elderly APL patients has dramatically improved after the advent of ATRA. In fact, complete remission and event-free survival rates of >80% and >50% have been reported in recent trials for patients aged >60.

#### Molecular assessment of response and MRD monitoring

Besides refining diagnosis, PM/L/RARα RT-PCR studies offer the additional advantage of identifying patients with distinct transcript isoforms (bcrl or long type, bcr2 or variable type and bcr3 or short type) and, particularly, of providing a sensitive tool to assess response to treatment. Due to technical difficulties in distinguishing long and variable forms, in most reported studies these two types are included in a common group referred to as long transcript, and this latter compared to the short type. Contrary to earlier reports indicating a worse outcome in patients with the short form, recent trials have shown no significant differences in DFS. It has to be emphasised, however, that the former analyses were done in patients treated with ATRA alone for remission induction, whereas the latter derive from trials in which patients received CHT added to ATRA.

Several groups have analysed the response to treatment by RT-PCR tests performed at various times during and after treatment. Using assays with sensitivity levels of approximately \(10^{-4}\), up to 50% of patients in haematological remission after induction have detectable PM/L/RARα transcript in their marrow cells. No correlations were found between PCR status at the time of remission achievement and relapse risk in the GIMEMA and MRC studies. The vast majority (up to 95%) of patients tested after completion of consolidation were PCR-negative in the New York Memorial Sloan-Kettering, MRC, GIMEMA and PETHema series. Interestingly, detection of residual disease at the end of the third CHT course (of 4 given) predicted an increased relapse risk associated with poorer survival in the MRC study.

The clinical value of post-treatment molecular monitoring has been emphasised in a recently reported prospective study of the GIMEMA group. This showed that conversion from PCR-negativity to PCR-positivity after consolidation therapy is almost always associated with subsequent haematological relapse. Based on these results, patients in this trial who convert to PCR-positivity in two successive marrow samples are now defined as being in molecular relapse and given anticipated salvage treatment.
Conclusions and future perspectives

Despite considerable improvement in diagnosis and management, a sizeable proportion of APL patients who receive state-of-art front line treatment still die of early complications or following disease recurrence. With regard to early death, a special effort should be made to define risk categories better (hyperleukocytosis, older age, severity of the coagulopathy, other unknown factors) in order to promptly reinforce adequate supportive care and to evaluate the feasibility of a tailored (less intensive?) initial treatment.

At least 60% of newly diagnosed patients become long-term survivors and are probably cured with ATRA+CHT. It is conceivable to hypothesise that a relevant fraction of these patients are being overtreated and that they might, therefore, be spared this risk. On the other hand, we are still unable to identify those cases (approximately 20%) who will ultimately relapse after initial therapy and are, therefore, in need of treatment intensification. The definition of risk categories at diagnosis for more appropriate treatment stratification remains a big challenge for future clinical investigation in this disease. Finally, the place of novel drugs which have proven effective in relapse, such as arsenic trioxide, and the advantage of anticipating salvage treatment at the time of molecular recurrence remain to be established.

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References

Several new therapeutic approaches have recently been clinically tested in patients with advanced cancer, including the use of arsenicals and various drugs that affect gene transcription via inhibition of histone deacetylase. Although our interest in these agents arose from our programme in acute promyelocytic leukaemia (APL), we expect that these agents will prove useful for a number of other cancers. The following pages summarise some aspects of our group's clinical work in these areas.

**Arsenicals as cancer treatment**

Like many others, we were intrigued by reports emanating from the People's Republic of China in the early 1990s that intravenous arsenic had re-emerged as a cancer treatment. Arsenic has been used as a medicinal for many centuries, both in topical and injectable forms (as escharotic agents) as well as oral forms that were first described in the 1700s. Fowler's solution given orally was used in the 19th and early 20th centuries as a treatment to reduce extreme leukocytosis in patients with chronic myelocytic leukaemia (CML). In the West, however, this use dwindled after the widespread application of radiotherapy in the 1930s and the advent of other cytotoxic drugs after the 2nd World War.

The recent upsurge of interest stems from reports from Harbin China that an intravenously injectable formulation induced remissions in patients with APL. Since we perceived an enormous regulatory hurdle to direct application of this therapy in the United States, we first tested an organic arsenical, melarsoprol, which was already formulated for human use and was immediately available on an investigational basis because of its use for the treatment of African trypanosomiasis due to T. brucei.

**Laboratory and clinical studies of melarsoprol**

We evaluated both As$_2$O$_3$ and melarsoprol for possible antileukaemic activity in vitro. In these studies, melarsoprol exhibited broad antileukaemic activity against both myeloid and lymphoid cells. Given the breadth of this activity, we then initiated a clinical study to evaluate the pharmacokinetics, safety, and potential efficacy of melarsoprol in patients with relapsed leukaemia. Using the anti-trypanosomal dose and schedule, patients received escalating intravenous doses daily for 3 days, repeated weekly for 3 weeks. Doses were 1 mg/kg on day 1, 2 mg/kg on day 2, and 3.6 mg/kg on day 3 and on all days thereafter, up to a maximum daily dose of 200 mg. Eight patients [5 AML, 1 APL, 1 CML, and 1 CLL] were treated. Mean peak plasma concentrations of the parent drug were obtained immediately after injection. These concentrations ranged from 1.2 µg/mL on day 1 to 2.4 µg/mL on day 3, were dose proportional, and decayed with a $t_1/2$ of 15 minutes. A minor clinical response (regression of splenomegaly and lymphadenopathy) was observed in a patient with chronic lymphocytic leukaemia. One patient with t(11;17)-variant APL who had been refractory to chemotherapy and all-trans retinoic acid had slight improvements in both his leukocyte and platelet counts with the first course of treatment. He then received a second 3-week course without further improvement and was removed from the study.

Unfortunately, central nervous system (CNS) toxicity proved limiting. Three patients experienced generalised grand mal seizures during the second week of therapy. Other neurologic reactions included vertigo (2 patients) and paraesthesia (1 patient), both of which resolved after discontinuing therapy. The patient who received two complete treatment cycles experienced moderately severe peripheral neuropathy, with bilateral lower extremity paraesthesia that caused moderate functional impairment, but which resolved approximately 5 weeks after the last dose. Other common reactions were nausea, vomiting, and irritation at the injection site. We concluded that the antitrypanosomal dosing schedule of melarsoprol is associated with excessive CNS toxicity, and that verification of the striking preclinical activity in patients with leukaemia requires development of an alternative dosing schedule.

**Arsenic trioxide**

We and others have recently confirmed that arsenic trioxide induces complete remissions (CR) in a high proportion of patients with APL. In our initial pilot study, 12 heavily pretreated patients were treated...
with arsenic trioxide at doses ranging from 0.06 to 0.2 mg/kg/day until leukemic cells were eliminated from the marrow. Patients who achieved CR were eligible to receive up to 5 additional courses of therapy, with each course given for a cumulative total of 25 days at a daily dose of 0.15 mg/kg/d every 3-6 weeks. Leukemic cells were eliminated in 11 of 12 patients after a median treatment duration of 33 days (range, 12-39 days; one patient who had an intracranial haemorrhage on day 1 died on day 5). The median cumulative dose during induction was 360 mg (range, 160 to 515 mg). CR by all criteria was attained at a median time of 47 days (range, 24-83 d). Impressively, 8 of 11 patients tested converted RT-PCR assays for PM L/RAR-α to negative at or before the end of the second course, while 3 patients who remained positive relapsed early and appeared to have rapidly developed arsenic resistance. Several patients have received up to 6 cycles of arsenic trioxide therapy without showing evidence of cumulative toxicity.

We subsequently initiated a confirmatory multicentre study in patients with relapsed or refractory APL using the same treatment schedules at a fixed daily dose of 0.15 mg/kg/d. To date, 28 patients have been accrued, and 19 of 21 evaluable patients (90%) have achieved complete remission. A new study of arsenic trioxide plus all-trans retinoic acid (RA) was shown to induce terminal differentiation of APL cells in vivo, and leukocytosis was commonly observed during this process, probably as a result of a transient increase in leukaemic cell lifespan. Similarly, previous studies by us and others have shown that arsenic treatment is associated with induction of partial, non-terminal immunophenotypic cytodifferentiation.

In clinical studies of APL patients treated with arsenic trioxide at this centre, leukocytosis (defined as a total peripheral blood leukocyte count ≥10×10⁹ cells/L) was observed in 13 of 23 patients (57%). The median baseline leukocyte count for all treated patients (2.6×10⁹/L [range, 0.7-65.2]) was not substantially different from the cohort of patients who subsequently developed leukocytosis (3.5×10⁹/L [range, 1.3-65.2]). The median peak leukocyte count of the latter group was 60.3×10⁹/L (range, 17.1-247.0), which occurred at a median of 19 days from the start of arsenic therapy (range, 4-24 days). No other cytotoxic therapy was administered, and the leukocytosis resolved in all patients.

Distinct from leukocytosis, approximately 50% of APL patients who receive all-trans RA for induction therapy develop one or more signs of the RA syndrome, a potentially lethal complication. The RA syndrome is a loosely defined constellation of problems that include fever, weight gain, dyspnoea, pulmonary oedema, pleural effusions, musculoskeletal pain, or joint effusions. Signs/symptoms of the RA syndrome were observed in 8 of the 23 patients (35%) treated with arsenic trioxide. Several patients were presumptively treated with high-dose dexamethasone. All patients with this problem have recovered, and interruption of arsenic therapy was not required. These data show that even non-terminal cytodifferentiation induced by arsenic trioxide is associated with induction of leukocytosis and the RA syndrome in APL. These effects appear to be related to intrinsic biological responsiveness to differentiating agents.

**Pharmacokinetics of arsenic trioxide and studies in other cancers**

We have conducted several dose-ranging studies to examine the pharmacokinetics and biological effects of arsenic trioxide in patients with other types of haematologic cancers and solid tumours. Pharmacokinetic analysis of blood and urine for elemental arsenic content showed that arsenic is distributed in both plasma and red blood cell fractions of whole blood. Parallel elimination curves suggest that these 2 compartments are freely exchangeable. The mean area under the plasma concentration curve (AUC) on day 1 after a dose of 0.15 mg/kg was ~ 400 ng.hr/mL. Approximately 20% of the administered dose was recovered in urine within the first 24 hrs. The terminal half-life appears to be quite prolonged.

In an ongoing dose-ranging study in patients with haematologic cancers other than APL, we have employed a daily IV dosing schedule for a cumulative total of 25 days per treatment course, with additional cycles administered every 3-5 weeks. Dose levels have ranged from 0.1 to 0.25 mg/kg/day. A separate study have ranged from 0.15 to 0.3 mg/kg/day. Over 5 days schedule, with additional cycles in responding patients administered every 4 weeks. Doses in that study have ranged from 0.15 to 0.3 mg/kg/day. Over this range of doses, the drug has continued to be relatively well-tolerated. However, skin rash, diarrhoea, QTc prolongation on EKG, and fatigue (but not leukocytosis or RA syndrome) have emerged as increasingly prominent side-effects in these other diseases.

**Targeting of histone deacetylase as anticancer therapies**

Acetylation of DNA-associated histones is linked to gene-specific activation, whereas histone deacetylation is associated with transcriptional repression. Recent studies have shown that inhibitors of histone deacetylase (HDAC) can relieve transcriptional repression caused by certain oncoproteins. These inhibitors include trichostatin A, trapoxin, depudecins, bicyclic...
depsipeptides, hybrid polar compounds and various butyrates.

Since sodium phenylbutyrate was immediately available for investigational use in human subjects, we tested whether this drug, as well as the concept of HDAC inhibition, could be effective in vitro and then clinically in patients with highly resistant APL. Having documented that sodium phenylbutyrate (PB) inhibited HDAC, we found that high concentrations of PB alone decreased cell growth but had no effect upon differentiation of NB4 cells; however, the combination with all-trans RA caused a substantial increase in differentiation.

We then treated a child who had relapsed after numerous cytotoxic drugs, all-trans RA, allogeneic bone marrow transplantation, and arsenic trioxide. Blood and marrow mononuclear cells were assayed for histone hyperacetylation by immunohistochemistry and Western blot analysis. M infantilis disease was assessed by RT-PCR for PM L/RAR-α. By reconfirming that the patient was clinically resistant to RA, PB was added to the treatment regimen. The RA dose ranged from 45-90 mg/m²/d in 2 equally divided doses; the PB dose ranged from 150-210 mg/kg IV BID infused IV over 1 hr. By day 23, the patient’s marrow showed complete elimination of visible leukaemic cells, and she achieved a complete clinical and cytogenetic remission shortly thereafter. By the 2nd treatment course, the previously positive RT-PCR assay for PM L/RAR-α had converted to negative. Peak PB levels in plasma were 2.5 mM; however, these levels had decayed to baseline levels by 4 hrs. Immunofluorescence and Western blot analysis showed that PB clinical treatment caused a time-dependent increase in histone hyperacetylation in blood and bone marrow mononuclear cells.10

These preliminary data suggest that although PB is a relatively weak HDAC inhibitor, the drug can reversibly increase chromatin hyperacetylation in target cells, possibly restoring RA sensitivity in retinoid-resistant APL patients. The concept of HDAC inhibition, if it can be selectively induced, may prove useful in other neoplastic diseases associated with oncogenic repression of gene transcription due to recruitment of histone deacetylases. This concept is currently being tested in a broader study.

Acknowledgements

Numerous colleagues have collaborated in studies by our group. I am especially indebted to Drs. Pier Paolo Pandolfi, Victoria Richon, Steven Soignet, Paul Marks, Elizabeth Calleja, and Peter M aslak for their contributions to these efforts.

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References

procedures surrounding the test need to be standard-ised and the criteria for a normal and an abnormal test result need to be defined, using for example receiver-operator curve analysis. In addition the inter- and intraobserver variability of the test should be assessed. Ideally, the development of the diagnostic test will pass into the next phase only after all these aspects have been adequately investigated. Phase 2 would then consist of studies in which the diagnostic accuracy of the new test is evaluated in a large group of consecutive patients with a suspicion of the disease for which the test is intended. The outcomes of the new test must be blindly and independently compared with the results of the gold standard test for the relevant disease. The sensitivity, specificity and positive and negative predictive values of the test can then be determined. In case of insufficient accuracy, one might need to return to phase 1 to re-evaluate the test procedures and the definition of normal and abnormal results.

Only after the requirements of both phases 1 and 2 have been adequately met, can the development process advance into phase 3 in which the role of the new test in the diagnostic process is evaluated. In this phase of development management studies are performed, in which therapeutic decisions are made based on the result of the new diagnostic test.

A new diagnostic test should only be implemented in routine clinical use after all three phases of development have been properly performed with good results.

**Pulmonary angiography**

At present pulmonary angiography is regarded as the gold standard diagnostic test for pulmonary embolism. There are generally accepted standardised procedures for the performance of the test and criteria for a normal and an abnormal test result have been formulated. Furthermore, the observer variability of the test has been assessed and has been found to be minimal. Assessment of the sensitivity and specificity is not possible since pulmonary angiography is itself the reference method. The ability of pulmonary angiography to confirm or exclude pulmonary embolism is, however, considered to be high. Several studies in patients who underwent pulmonary angiography for the suspicion of acute pulmonary embolism have shown that it is safe to withhold anticoagulant treatment from those patients who have a normal test result. In a total of 840 patients with suspected pulmonary embolism and a normal pulmonary angiogram, anti-coagulant therapy was withheld and patients were followed-up for a minimum of 3 months. The overall incidence of recurrent pulmonary embolism was 1.9% (95% CI 1.4% - 3.2%) and the incidence of fatal pulmonary embolism was 0.3% (95% CI 0.09% - 1.08%). On the basis of these studies it is deemed safe to consider pulmonary embolism excluded in the case of a normal pulmonary angiography and proven in the case of an abnormal result.

**Ventilation-perfusion scintigraphy**

Ventilation-perfusion scintigraphy is the most widely used first line diagnostic test when pulmonary embolism is suspected. The criteria for the interpretation of lung scans have been a matter of debate for many years. The most clinically applicable classification is that of three categories. These are: 1) a normal perfusion scan on the basis of which pulmonary embolism is excluded; 2) a high probability lung scan, defined as one or more defects of at least segmental size with associated local normal ventilation, which strongly indicates the presence of emboli; and 3) the remaining lung scan results which are considered to be non-diagnostic. The interobserver variability of ventilation-perfusion scintigraphy using the various classifications has been investigated in several studies and has been found to be acceptable.

Numerous studies have compared lung scintigraphy with pulmonary angiography. The overall positive predictive value of a high-probability lung scan has been shown to be 88% (95% CI 84%-91%). This is generally considered to be enough evidence to accept the diagnosis of pulmonary embolism. However, pulmonary embolism cannot be considered proven or excluded on the basis of a non-diagnostic lung scan. Studies have shown that the prevalence of angiographically proven pulmonary embolism is approximately 25% in patients with such a scan result. Further diagnostic investigation is therefore warranted in these patients.

Management studies using ventilation-perfusion scintigraphy in the clinical work-up of patients suspected of having pulmonary embolism have been performed. In these studies a normal perfusion lung scan was used to reject the diagnosis and anticoagulant treatment was withheld. In a total of 693 patients with a normal perfusion lung scan in whom anticoagulants were withheld 0.3% (95% CI 0.2% - 0.4%) suffered a thromboembolic event during a follow-up period of at least 3 months. Hence, it is deemed safe to withhold anticoagulants in patients with a normal perfusion lung scan.

**D-dimer**

In recent years the measurement of D-dimer, a degradation product of fibrin, has been suggested for use in the diagnostic work-up of patients with clinically suspected pulmonary embolism. Absence of an elevated concentration of D-dimer has been suggested to have a high negative predictive value for venous thromboembolism. As a result, numerous D-dimer tests (quantitative and qualitative) have been developed which are now widely commercially available. Proper evaluation of the interassay and intra- and interobserver variation has only been performed for the minority of the available D-dimer tests. The studies which have been performed have shown that this variation is rather high. Several D-dimer tests have been compared to a gold standard diagnostic method.
to determine the accuracy of the tests, albeit often in non-consecutive series of patients. The high sensitivity and moderate specificity of the D-dimer assays seem to qualify the test as potentially useful for the exclusion of venous thromboembolism. However, the sensitivity of D-dimer tests varies widely among the studies, ranging from 61% to 100% and data are still limited.7,8 The cut-off values of the D-dimer assays can be varied so as to increase the sensitivity leading, however, to a decrease in specificity, which could limit the clinical usefulness. When a receiver operator curve (ROC) analysis is performed, the various quantitative D-dimer assays appear to be nearly identical.9 The determination of optimal cut-off values for the individual D-dimer assays is, therefore, essential.

Management studies have evaluated the use of D-dimer integrated in a diagnostic strategy in combination with other non-invasive tests, such as ventilation-perfusion lung scanning and clinical decision rules.10 In this setting, management with D-dimer assays seems safe and cost-effective. However, one needs to interpret these data with great caution. The use of D-dimer assays within a strategy combined with other diagnostic tests seems safe, while the exclusion of pulmonary embolism on basis of a normal D-dimer test alone seems unjustified.

Spiral computed tomography
Spiral computed tomography (spiral CT) is a relatively new diagnostic technique for patients with suspected pulmonary embolism, first described in 1992.11 In one study the interobserver agreement was found to be relatively high (kappa 0.77).12 However, this does not appear to be true for pulmonary emboli that are confined to the subsegmental arteries. In addition, no accepted criteria for normal and abnormal test results have been formulated. The first study that compared spiral CT with angiography was performed in 42 patients and found a sensitivity of 100% and a specificity of 96%.13 There have been many subsequent studies comparing spiral CT with angiography or lung scintigraphy. The overall sensitivity and specificity are 91% (95% CI 87-94) and 93% (95% CI 90-96), respectively. However, the sensitivity varies from 54% to 100 % and the specificity from 67% to 100% in these studies.4 This can in part be explained by differences in patient selection in the various studies, the higher sensitivities and specificities generally being found in the studies with highly selected patient groups. Furthermore, different criteria for the diagnosis of pulmonary embolism and various procedures for the performance of the test were used.

One management study implementing the spiral CT in the diagnostic work-up of patients suspected of pulmonary embolism has been performed.14 In this study a recurrence rate of venous thromboembolism of 5.5% (95% CI 1.3 to 9.7) during a follow-up period of 3 months was found in patients with a normal spiral CT and a normal ultrasound. This finding illus-

Discussion
The introduction of a new diagnostic test is usually accompanied by a great amount of enthusiasm concerning the potential indications for its use. A new test is often presented as an improvement in the diagnostic arsenal. This all too frequently results in the wide acceptance and large-scale implementation of a new diagnostic test directly following its introduction. This often occurs without adequate foundation on properly performed studies. However, after the initial enthusiasm, the limitations and the true value in clinical practice become clear. In the past this has often resulted in the almost complete disappearance of new and initially promising tests from the diagnostic armamentarium. One may assume that with proper evaluation these tests could have been found valuable within the proper setting. As example of the problems associated with the introduction of new diagnostic methods we have assessed 4 tests currently available for the diagnosis of pulmonary embolism using the suggested guidelines for the proper evaluation of diagnostic tests.

Pulmonary angiography, presently considered the gold standard method, has been evaluated quite extensively throughout the various phases mentioned earlier. Both the role in the diagnostic work-up of a patient with suspected pulmonary embolism and the consequences of its result are known. Ventilation-perfusion scintigraphy has also been shown to be of use in the diagnosis of pulmonary embolism. With the performance of proper studies in comparison to pulmonary angiography and numerous clinical follow-up studies, the value and limitations of the test have become clear. Both pulmonary angiography and ventilation-perfusion scintigraphy have therefore been properly evaluated according to the suggested guidelines and are well-established diagnostic methods.

When considering the evaluation of D-dimer assays, it is apparent that all phases of development have been traversed for some of the available assays. However, there are also some troubling aspects in the development of this test. Not all of the assays have been properly evaluated in phase 1 and 2 studies, and the results of the studies that have been performed should not be directly extrapolated to other D-dimer assays. Furthermore, a wide spread in the sensitivity and specificity has been found between studies, further complicating the choice of an optimal cut-off point. Optimal cut-off levels for each individual D-dimer assay need to be determined. The observation that the ROC curves of the various quantitative D-dimers are nearly identical would seem to support this. The management studies that have so far been performed show promising results. Use of the D-
dimer within certain diagnostic strategies appears safe and cost-effective. However, further studies are warranted before the widespread use of D-dimer assays in routine clinical practice is justified. The use of a D-dimer assay as a single test for the exclusion of pulmonary embolism, a practice that is gaining ground, is certainly not founded on adequate studies.

When the studies evaluating spiral CT scanning are critically assessed, one must conclude that the phases of development have been passed in a less orderly fashion. Firstly, consensus does not exist concerning the optimal method of performance of the test. Furthermore, accepted criteria by which the presence or absence of pulmonary embolism can be evaluated are still lacking. This translates to the wide variation found in the performance of the test when compared to pulmonary angiography and ventilation-perfusion scanning. A more important cause of this variation is, however, that these comparisons were not consistently performed in representative groups of patients with suspected pulmonary embolism. This can be seen by the wide range of the prevalence of pulmonary embolism in the various studies (33-88%). The only management study which has so far been performed using spiral CT scanning in the management of patients with suspected pulmonary embolism by Ferretti et al., has shown disappointing results (recurrence rate 5.4%, upper 95% CI 9.7%). Further phase 1 studies in which methodology and criteria are standardised, and phase 2 studies in which spiral CT scanning is compared with pulmonary angiography or ventilation-perfusion scintigraphy are, therefore, needed before further management studies or implementation in routine clinical practice should occur.

In conclusion, at present pulmonary angiography and ventilation-perfusion scintigraphy are the only properly evaluated diagnostic tests for pulmonary embolism. Although there are many new developments in this field, such as D-dimer assays and the spiral CT scan which are certainly promising, further studies are needed to determine their real value and safety in the diagnostic work-up of patients suspected of having a pulmonary embolism.

The diagnostic tests for pulmonary embolism exemplify the problems often surrounding the introduction of new tests. Due to the initial enthusiasm, a new test is often implemented in clinical use before proper evaluation has occurred. Studies are also often performed without adequate data from earlier phases of development. We would suggest that guidelines for the proper evaluation of diagnostic tests before their implementation in routine clinical practice are necessary. The criteria that need to be met by consecutive phases of development need to be generally accepted and implemented.
Clinical significance of non clinical end-points in studies on the prevention of venous thromboembolism

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The end-point(s) in studies on the prevention of venous thromboembolism

The large majority of studies on the prevention of venous thromboembolism are carried out in patients undergoing surgery. Indeed, post-operative pulmonary embolism is the most common cause of preventable death in high risk patients, such as those undergoing major orthopaedic and cancer surgery. The definition of the proper end-point to be used in clinical trials on the prevention of venous thromboembolism has been a matter of debate for a long time. Mortality is the ideal end-point for clinical trials on the prevention of pulmonary embolism. However, post-operative mortality, although high in epidemiological terms, is relatively low in statistical terms making the study sample size quite large. Furthermore, the overall mortality during the peri-operative period is influenced by factors unrelated to venous thromboembolism. This background noise reduces the specificity of the study intervention and amplifies the sample size required to demonstrate the study hypothesis. An alternative end-point to death is death due to venous thromboembolism. Unfortunately, this end-point has a limited diagnostic accuracy.

The ideal end-point is both reliably measurable and clinically relevant. Unfortunately, accuracy and clinical relevance are not necessarily associated as far as study end-points are concerned. This is indeed the case in clinical studies on the prevention of post-operative venous thromboembolism. Studies targeting accuracy use venography to measure the end-point. However, patients with venography-detected post-operative deep vein thrombosis (DVT) are almost invariably asymptomatic and the clinical relevance of venography-detected DVT is still unclear. Studies targeting clinical relevance are based on clinically overt events. Unfortunately, the clinical diagnosis of DVT and pulmonary embolism has a low accuracy, both in terms of sensitivity and specificity. Furthermore, studies on the prevention of venous thromboembolism with clinical end-points require a large number of patients because of the low number of events. In this paper the relative advantages and disadvantages of clinical and non-clinical end-points will be discussed.

Accuracy of venography as end-point measurement in clinical trials on prevention of venous thromboembolism

Contrast venography of the lower limbs is the method of choice to measure the end-point in clinical trials on the prevention of DVT. Venography is not routinely performed in the large majority of the hospitals. Indeed, the introduction and validation of real time B-mode ultrasonography in patients with clinical suspicion of DVT have reduced the use of venography in the diagnosis of this disease. In clinical studies on prophylaxis of venous thromboembolism the study centres are selected on the basis of the availability of venography. A standardised technique is usually adopted to improve the interpretation of the results. However, a certain degree of inter-observer variability may be difficult to eliminate and can confound the results of multicentre clinical trials. We determined the inter-observer agreement between the local and central assessment of venographies in a multicentre trial comparing enoxaparin and placebo in the prevention of DVT after elective neurosurgery. The central and local adjudication panels were both blind with respect to the assigned treatment. The central panel was unaware of the local adjudication. Venographies were adjudicated as positive, negative or inadequate for adjudication and positive venographies as proximal or distal DVT. Inter-observer agreement was assessed according to the Cohen's inter-observer variability index (K index). All 266 venographies (8 monolateral) were considered adequate for adjudication by both the central and local panels. A disagreement was found in 25 cases, K index = 0.75. Fourteen venographies adjudicated as negative centrally were considered positive locally (3 were proximal DVT). Eleven venographies adjudicated as positive centrally (1 was a proximal DVT) were considered negative locally. Enoxaparin was found to be more effective than placebo according to both the central and local adjudication: 16.9% versus 32.6% (relative risk, RR = 0.52, 95% CI 0.33-0.82) according to central adjudication; 18.5% versus 33.3% (RR = 0.56, 95% CI 0.36-0.87) according to local adjudication. It was concluded that there
was a good inter-observer agreement between the central and local adjudication in clinical study centres frequently involved in clinical studies on the prevention of DVT. Disagreement was mainly confined to distal DVT. The cost and work load of central assessment of venographies in multicentre trials on DVT prevention seems not to be justified.

Role of real time B-mode ultrasonography

Real time B-mode ultrasonography in asymptomatic patients is insufficiently accurate. The limited sensitivity of this test might be compensated for by enrolling additional patients if the diagnostic technique is highly specific. This is the case for proximal DVT but not for distal DVT. High quality studies using compression, duplex and colour Doppler ultrasound show sensitivities of 42-70% and positive predictive values of 35-83% for proximal DVT. For calf DVT, the accuracy is undoubtedly lower.

Limitations of non-clinical end-points in studies on the prevention of venous thromboembolism

The value of non-clinical outcomes to measure the efficacy of antithrombotic agents in studies on the prevention of venous thromboembolism is not clear. Studies based on venography-detected asymptomatic DVT overestimate the event rates compared with studies based on clinical end-points. When comparing the efficacy of one agent versus another, the absolute benefit of the more efficacious agent may be overestimated if the event rate is high. The long-term outcome of asymptomatic patients with untreated venography-detected DVT is unknown. Some authors consider this event only a surrogate end-point of mortality or morbidity due to venous thromboembolism. Other authors believe that venography-detected DVT has an undoubted pathophysiological relevance and is predictive of clinical outcome.

Clinically overt events as end-points in clinical trials on prevention of venous thromboembolism

Symptomatic outcomes are of greatest concern to patients and their physicians. Prevention of these outcomes is the primary goal of antithrombotic prophylaxis. To minimise bias, randomised controlled clinical trials on prevention of post-operative venous thromboembolism are usually designed taking into account the following essential requirements. Only patients developing symptoms presumed to be due to venous thromboembolism are considered for study end-points. The criteria for diagnosis of venous thromboembolic events are based on validated objective diagnostic testing. Investigators blind to the study intervention review suspected outcome events. Ideally the study is double blind, so that there is no referral bias whereby patients (or physicians) might be have (or investigate) differently.

Limitations of clinically overt events as end-points in clinical trials on prevention of venous thromboembolism

Although objectively confirmed venous thromboembolic events are the most appropriate outcome for prevention studies, they present some limitations. Reduced accuracy and validity of clinically overt events, as study end-points, primarily affect the generalisation (external validity) of the study results. The internal validity of the comparison between different treatment interventions will not be compromised, as long as the design of the clinical trial is methodologically sound.

The results of clinical outcome study may be confounded by the non-antithrombotic pharmacological effect of the study drug. This is clearly illustrated by studies assessing the value of aspirin in the prevention of venous thromboembolism in orthopaedic surgery. In the first studies objective testing was performed to confirm the diagnosis in symptomatic patients only. These studies were randomised but were not blind and adopted clinical diagnosis for the evaluation of the occurrence of DVT. The results of these trials indicated that aspirin was effective in the prophylaxis of DVT. However, since aspirin has a significant analgesic effect and pain is one of the most frequent clinical symptoms of DVT, the efficacy of aspirin was probably overestimated. Indeed, aspirin might have concealed pain without preventing the underlying DVT and the subsequent risk of pulmonary embolism. Subsequent trials, conducted by the same investigators and by others, adopted correct methods of DVT adjudication and indicated that aspirin has a modest efficacy, being much less effective than a number of other available prophylactic methods.

Major bleeding as an end-point in clinical trials on prevention of venous thromboembolism

The major side effect of antithrombotic therapy is bleeding. This unexpected event may cause considerable patient morbidity and may even be fatal. Typically, major bleeding has been considered to be a secondary outcome of trials of antithrombotic therapy. However, several recent studies have considered symptomatic objectively documented venous thromboembolism and major bleeding as a composite outcome measure to evaluate the effectiveness of antithrombotic therapy. This approach may be adequate to assess the benefits and risks of individual antithrombotic agents.

Conclusions

Clinically overt events confirmed by objective testing are the most relevant outcomes to be used to determine the rates of unfavourable events in studies designed to set the standard of care. However, low sensitivity and specificity hamper clinical diagnosis of DVT and pulmonary embolism. The gold standards
for the diagnosis of asymptomatic DVT and pulmonary embolism are bilateral venography and pulmonary angiography, respectively. Both these tests are invasive and difficult to interpret. The clinical value of asymptomatic venography-detected DVT is still unknown. However, until it can be demonstrated that asymptomatic venography-detected DVT are entirely without deleterious consequences, it seems prudent to prevent them. The accuracy of non-invasive diagnostic techniques to assess study end-points has still to be adequately validated.

References

Venous thromboembolism (VTE) is a serious disorder and frequently heralds a fatal outcome. The prevalence of VTE is approximately 2-3 per 1,000 inhabitants of the Western world per year. The clinical implications of deep venous thrombosis (DVT) and pulmonary embolism (PE) will be discussed with a focus on complications, prevention of these complications by rapid (initial) anticoagulant treatment with unfractionated (UFH) or low-molecular-weight heparin (LMWH), and by long-term treatment with coumarins. The various treatment modalities for DVT and PE have changed considerably over the last 10-20 years and will continue to do so in the next century. Major changes in the treatment of VTE have been facilitated by clinical trials comparing entire treatment modalities rather than single components.

Clinical implications of a first VTE event
The long-term clinical course of a first acute DVT has been revealed by carefully conducted, prospective, cohort studies in consecutive outpatients; measurements included recurrent VTE, and death.1,2 Potential risk factors for these outcomes were evaluated, in particular the late discovery of malignancy.

The average cumulative incidence for recurrent VTE after the qualifying event was approximately 15% at the first year and gradually increased to approximately 25% at eight years follow-up. Of the initial cohort of patients, approximately 15% died within the first year and about 70% survived the total of the eight years follow-up. Death was frequently due to cancer which became clinically overt mainly within the first year of follow-up, this being an association particularly found in patients having either idiopathic or recurrent DVT. The elucidation of this association may have an impact on the design of cancer screening and the detection of occult malignancy at an early stage. The frequency of cancer observed in the first year was approximately 7.6-8%. Similarly, incidence of fatal outcomes - also due to malignancy in most cases - was observed in patients with confirmed PE.

Initial treatment UFH versus LMWH
Rapid initiation of optimal anticoagulant treatment is an absolute requirement in patients with confirmed DVT to prevent recurrent VTE at follow-up. A double-blind placebo-controlled study in the Netherlands3 clearly revealed the necessity for initial dose-adjusted heparin treatment.

Twenty percent of patients who presented with proximal DVT and received a placebo infusion, in addition to concurrently commenced coumarin treatment, had a recurrent VTE by three months’ follow-up, while only 6.7% of those who received initial UFH treatment concurrent with coumarins did so.

Once heparin treatment is started, careful laboratory control of coagulation, using an activated partial thromboplastin test (APPT) to assess adequate heparinisation early in the course of therapy, is a matter of some importance. The APPT is used to adjust the dose of heparin at regular intervals, in order to maintain the clotting time prolongation within the prescribed therapeutic range, i.e. an APPT that is increased at least 1.5 times the normal range. Subtherapeutic heparinisation within the first 24 hours is associated with a high rate of recurrent VTE at follow-up (23.3%) versus 4.6% in patients receiving therapeutic doses of heparin.4

These and other studies support the clinical significance of immediate and optimal heparinisation in patients with DVT. However, even in the best centres some patients treated with intravenous heparin will receive sub-optimal therapy, reflecting that LMWH is the likely practical solution to this dilemma. The advantages of LMWH over UFH are longer plasma half-life, an almost complete bio-availability following subcutaneous injection, as well as a reduced inter- and intra-individual variability following subcutaneous injection. These pharmacological characteristics allow for fixed dose treatment which only needs adjustment for specific bodyweight categories without requiring laboratory monitoring.

Initial in-hospital randomised studies comparing the efficacy and safety of fixed bodyweight-adjusted dose LMWH given subcutaneously against a UFH-adjusted dose given intravenously revealed promising results, i.e. at least a comparable efficacy, improved safety and, interestingly, a reduced mortality at follow-up. The results of published studies were assessed by the Cochrane review group on peripher-
al vascular diseases; this report comprises all randomised controlled clinical trials which were identified by electronic (Medline® and Embase®) or manual (relevant journals) searches, and by communication with colleagues and relevant pharmaceutical companies. All studies were independently reviewed by three investigators; discordant results were solved by majority voting. Only those studies which directly compared fixed-dose LMWH with an adjusted dose UFH for treatment of DVT and PE were included, and only if they were truly randomised, precluding prior knowledge of next treatment allocation. Further inclusion criteria were: an unconfounded control group; an objective confirmation of VTE before randomisation by either contrast venography of compression ultrasonography for the diagnosis of DVT, and ventilation-perfusion lung scanning and pulmonary angiography for the diagnosis of PE; a prospective long-term follow-up for objectively confirmed VTE recurrence and for mortality and an independent adjudication of predefined clinical end-points.

Studies that were excluded from the analysis were preliminary reports later duplicated in full reports; dose-ranging studies which made use of doses of LMWH other than those currently in use; studies with hirudinoids. The following relevant clinical outcomes were considered:

1. symptomatic recurrent VTE at long-term follow-up (>3 months); 2. major haemorrhage during initial treatment; 3. death due to any cause and in particular death associated with clinically overt malignancy.

Of the 26 potentially eligible clinical trials, 13 satisfied the inclusion criteria; these studies were published over the years 1988-1997. Pooled analysis for all these clinical trials revealed a reduction of recurrent VTE, at three to six months' follow-up in favour of LMWH (OR 0.75; 95% CI 0.55-1.01; p=0.06). The pooled efficacy analysis in patients with PE revealed no such statistically significant difference (OR 0.91; 95% CI 0.42-1.97; p=0.02).

Important and significant reduction in mortality was observed with LMWH treatment (OR 0.74; 95% CI 0.57-0.98; p=0.03). Mortality reduction was mainly confined to patients with malignancies.

This systematic review thus revealed that LMWH in a fixed dose given subcutaneously and only adjusted for bodyweight, compared to APPT-adjusted therapeutic doses of UFH, was at least as effective and likely to be safer. These, and previous data, led to clinical studies investigating the feasibility and clinical efficacy and safety of home treatment with LMWHs. In these studies a direct comparison was made between different treatment modalities, e.g. in hospital using the established treatment module consisting of i.v. unfractionated heparin continuously by a syringe pump driven system, APPT-monitoring and dose-adjustment when necessary, versus ambulant treatment (at home) with fixed-dose s.c. LMWH. Subcutaneous injections were carried out by the patients, a close relative or a nurse.

### Home treatment with LMWH versus in-hospital treatment with UFH

Two major clinical studies have been reported, i.e. the Dutch Tasman Study and another study from Canada. Both studies - though utilising different brands of LMWH - yielded similar results in terms of efficacy, safety and mortality (Table 1). Moreover, both studies showed that out-of-hospital treatment with LMWH was, as expected, associated with a considerable reduction in days spent in the hospital and hence an important reduction in costs, without a detrimental effect on the quality of life. These data imply that out-of-hospital treatment of patients with proximal DVT is a feasible option, but with the following precautions: 1. patients should be referred to expert centres to receive objective diagnosis of DVT; 2. patients should be analysed for risk factors for DVT; and 3. community facilities for anticoagulant treatment and control should be available.

### Long-term treatment with coumarins: shift in duration of treatment?

Until recently, the duration of oral coagulation following a first or a recurrent episode of DVT has not been submitted to well-designed clinical investigation. Two studies from Sweden have now addressed this issue.

In the first study direct comparison was made between six weeks' versus six months' treatment in patients with a first thromboembolotic event; patients with an inherited risk factor predisposing to VTE (antithrombin, protein C or S deficiency) were excluded from the analysis. In the remaining patients a significant reduction of recurrent VTE was achieved in the six months' treatment group: OR for recurrence in the six weeks group was 2.1 (95% CI 1.4-3.1) without differences in mortality or major haemorrhage. By analysis of subgroups, the OR in patients with temporary risk factors for thrombosis was 1.9 (95% CI

### Table 1. Efficacy and safety outcomes in patients with proximal DVT treated with LMWH at home versus UFH in hospital

<table>
<thead>
<tr>
<th></th>
<th>UFH (n=198)</th>
<th>LMWH (n=202)</th>
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<tbody>
<tr>
<td>Recurrent VTE</td>
<td>17 (8.6%)</td>
<td>14 (6.9%)</td>
</tr>
<tr>
<td>Major bleeding*</td>
<td>4 (2.0%)</td>
<td>1 (0.5%)</td>
</tr>
<tr>
<td>Mortality</td>
<td>16 (8.1%)</td>
<td>14 (6.9%)</td>
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<table>
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<tr>
<th></th>
<th>UFH (n=253)</th>
<th>LMWH (n=247)</th>
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<tbody>
<tr>
<td>Recurrent VTE</td>
<td>17 (6.7%)</td>
<td>13 (5.3%)</td>
</tr>
<tr>
<td>Major bleeding*</td>
<td>3 (1.2%)</td>
<td>5 (2.0%)</td>
</tr>
<tr>
<td>Mortality</td>
<td>17 (6.7%)</td>
<td>11 (4.5%)</td>
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* During the initial treatment phase or within 48 hours thereafter.
0.8-4.5; p=0.24), while in patients with permanent risk factors, the OR was 2.3 (95% CI 1.5-3.6; p<0.001). Hence, the risk reduction for recurrent VTE was observed more prominently in patients with permanent risk factors receiving six months of treatment. A similar VTE reduction was observed in patients with a second episode of DVT or PE, who were receiving continuing oral anticoagulant treatment (48 months’ follow-up) as compared to six months of treatment. As in the first study, patients with documented congenital risk factors were excluded. After four years’ follow-up, the rate of recurrence in the six months group was 20.7% versus 2.6% in the patients receiving continuing coumarin treatment, for a relative risk of 8.0 (95% CI 2.5-25.9). As expected, the risk of bleeding was lower in the six months’ treatment group, i.e. 0.3 (95% CI 0.1-1.1). The following conclusions may be derived from these and other data:

1. patients with a first VTE event and having only a temporary risk factor are better off with 4-12 weeks of treatment (calf vein thrombosis: 4-6 weeks, proximal DVT or PE: 12 weeks);
2. patients with DVT or PE and without a temporary risk factor are better off with six months of treatment;
3. patients with a second episode of VTE deserve longer treatment. Duration should be carefully considered, taking into full account the risk for recurrent VTE and the risk of major bleeding especially with older patients;
4. continuing coumarin treatment for at least four years, e.g. the duration of follow-up in this study should be seriously considered at least on the basis of the data currently available.

The duration of anticoagulation in patients with inherited thrombophilia (excluded in the Swedish studies) is still uncertain and has been challenged recently. Currently, indefinite treatment is the prevailing option, though this is not based on solid evidence.

References

Advances in the detection of genetic abnormalities in chronic lymphocytic leukaemia (CLL) have occurred entirely in conjunction with technical developments. Early cytogenetic studies which used PHA as a mitogen concluded that the karyotype in CLL was normal. With the advent of polyclonal B cell mitogens trisomy 12 and subsequently structural abnormalities of chromosome 13q14 were found in between 10 and 20% of patients with CLL. Abnormalities of chromosomes 11q, 6q, 17p and 14q were found in fewer than 5% of cases. Cytogenetic analysis is a global technique but suffers from two main disadvantages. Firstly it is relatively insensitive in detecting small deletions and secondly the metaphases obtained using standard mitogens such as TPA are frequently derived from T cells rather than the malignant B cell clone. A recent study evaluated the use of different combinations of mitogens including TPA, TNF-α, SAC and IL2 in cases of CLL and showed that by optimising the mitogenic combination for each individual case, the proportion of B cell mitoses rose from 7 to 57% compared to the proportion following TPA stimulation alone and the incidence of clonal aberrations also rose from 50 to 79%.

The use of fluorescent in situ hybridisation with centromeric, YAC, or cosmid probes applied to non dividing peripheral blood cells, without prior culture, has shown a significantly high incidence of abnormalities that are detectable by routine chromosome analysis. In one study which used probes for chromosomes 3q, 6q, 8q, 11q, 12q, 13q and 14q, abnormalities were detected in 82% of cases. Additional abnormalities such as amplification of chromosome 4q were identified in 2 recent studies using comparative genomic hybridisation. In a small study using highly polymorphic microsatellite markers on purified leukaemic cells loss of heterozygosity was found on chromosome 3p and 9p.

There are two important caveats to the interpretation of cytogenetic and genetic data in CLL. Firstly when results differ between studies, this may be partially explainable by the use of samples from patients at different stages of their disease, or from different anatomical sites (blood, lymph node, bone marrow or spleen) but are more likely to reflect either technical differences or the inclusion of patients with related chronic lymphoproliferative disorders such as mantle cell lymphoma or splenic lymphoma with villous lymphocytes.

Secondly, studies in which the same patients have been investigated with a variety of techniques such as cytogenetic analysis, interphase and metaphase FISH and Southern blotting or PCR have shown that the complexity of abnormalities is frequently much greater than might be anticipated if a single technique was performed. For example a patient with a 13q14 deletion on cytogenetic analysis and apparent heterozygous loss of a 13q14 marker on Southern blotting may harbour subclones with homozygous loss only detectable when FISH is also used.

Genetic abnormalities

13q14

Structural abnormalities of 13q14 are found in approximately 20% of CLL patients by karyotyping, in 30-40% by Southern blotting and in 50-60% using interphase FISH. Chromosome analysis shows that two thirds of patients have a deletion of 13q14-q22 or 13q12-q14 while the remainder have a translocation involving 13q14. The translocations are frequently complex involving two or more partner chromosomes and invariably result in the loss of genetic material from the q14 region. Several groups have identified a minimum region of genetic loss containing numerous previously unidentified transcripts, telomeric to the retinoblastoma gene. The smallest region identified is less than 10kb. This includes exons from at least two genes which show little homology to previously identified genes. However no mutations have been discovered in these genes in a large series of patients with CLL.

13q12

One group has reported a high frequency of deletions at 13q12 involving the BRCA 2 gene. While this would be anticipated in cases with deletions of q12 to q14 many patients had loss at q12 and q14 with retention of intervening sequences. Other groups have been unable to duplicate these findings particularly using Southern blotting and the significance of q12 loss in CLL remains uncertain.

Trisomy 12

The incidence of trisomy 12 varies between 10 and 20% evaluated cytogenetically and 15 to 45% using...
interphase FISH. Case selection is an important factor in determining the incidence of this abnormality (see below). Less frequently translocations involving 12p11-12 are found as well as translocations or duplications of 12q12-14. Comparative genomic hybridisation (CGH) also reveals amplification of 12q and detailed genetic analysis on patients with structural abnormalities of chromosome 12 will be important in defining the key genetic defects of importance in the pathogenesis of CLL.

11q23

Although deletions and translocations of 11q23 are found in less than 5% of patients by chromosome analysis, the incidence rises to 20% using interphase FISH. A critical region of loss of 2-3 Mb has been identified containing the ATM gene. Recent studies have suggested that ATM loss may be important in CLL. In one study 8 patients with a cytogenetic abnormality of 11q were also shown to have an ATM mutation in the other allele. A second study found reduced or absent ATM protein in 8 of 20 patients with CLL. Six of the 8 cases had ATM mutations and in two they were germline. There was no loss of heterozygosity at 11q23 in any case.\(^4\)

17p13

Although deletions and translocations involving this region are infrequent in CLL (less than 5%) abnormalities of p53 are found in 10 to 20% of cases of CLL. The incidence varies with the stage of the disease (see below) and the techniques used to study p53 abnormalities. The most frequently used methods are measurement of p53 protein expression using immunocytochemistry or flow cytometry, identification of p53 gene mutations, and detection of p53 loss using FISH. Although there is a strong correlation between p53 gene mutation, loss of the remaining allele and overexpression of p53 protein this is not absolute.\(^4\) For example not all patients with a mutation show allelic loss and approximately 15% of mutations do not lead to p53 protein overexpression.

Immunoglobulin gene translocations

Translocations involving the immunoglobulin heavy chain locus are both frequent and critical to the pathogenesis of many chronic lymphoproliferative disorders but appear to be rare in CLL. The t(14;18) and the light chain variants t(2;18) and t(18;22) are found in 1-2% of cases of CLL and juxtapose the immunoglobulin gene loci with the BCL2 gene. The BCL2 breakpoints are usually 5/ and distinct from those found in follicle centre cell lymphomas. BCL2 protein expression is often higher than typically found in CLL but patients with BCL2 rearrangements appear to have no distinctive clinical features.

The incidence of the t(11;14) in CLL is controversial. In most studies the incidence is extremely low and many would regard its presence as a diagnostic exclusion criterion.

In those studies in which a higher incidence is found the lymphocyte morphology and immunophenotype are atypical and such cases may represent the leukaemic phase of mantle cell lymphoma. The t(14;19) is found in less than 0.5% of cases and involves the BCL3 gene which encodes an i-kB-like protein.

6q

Structural abnormalities of 6q are found in less than 5% of cases on chromosome analysis and in 7% using FISH. There has been less progress in characterising the deletions involving 6q compared to those of 13q and 11q. In NHL two or three separate regions of loss have been found. Preliminary data in CLL shows that deletions of q21 are more common than distal deletions involving q27.

Clinical and laboratory correlations

Lymphocyte morphology

One of the cardinal diagnostic features of CLL is the presence of small lymphocytes with central round nuclei, condensed chromatin and minimal agranular cytoplasm. Morphology is described as atypical if more than 10% of cells have the features of prolymphocytes or if more than 15% of cells have either a cleaved nucleus or lymphoplasmacytoid features. The incidence of chromosome abnormalities is higher in patients with atypical morphology and there is a strong correlation with trisomy 12 and p53 abnormalities.

Clinical features

CLL shows marked clinical heterogeneity not only in the rate of disease progression but also in the distribution of the leukaemic population. Deletions of 11q and 6q are associated with bulky lymphadenopathy\(^6\) and a recent study has shown a correlation between 11q abnormalities and expression of adhesion molecules of the leukaemic cells.

V\(_{H}\) gene status

The variable regions of immunoglobulin heavy and light chains undergo somatic hypermutation during their passage through germinal centres. Early small studies in CLL showed that most cases had unmutated V\(_{H}\) genes and it was hypothesised that CLL arose from a naive B cell, possibly CD5 positive mantle cells. Two large studies have now shown that V\(_{H}\) genes in CLL are mutated in approximately 50% of cases.\(^6\) Preliminary data have shown a strong correlation between trisomy 12 and unmutated V\(_{H}\) genes and between abnormalities of 13q14 and V\(_{H}\) gene mutations. A larger study is required to confirm these findings.

Prognosis

A large multicentre study has clearly shown an association between complex karyotypes and poor prognosis.\(^7\) Among patients with single chromosome abnormalities...
abnormalities, those with trisomy 12 had a shorter survival than those with structural abnormalities of chromosome 13 or those with a normal karyotype. The introduction of interphase FISH has enabled a more sophisticated analysis of the prognostic significance of genetic abnormalities. The results of a recent study are shown in the table, and emphasise the poor prognosis of patients with p53 abnormalities, which are frequently accompanied by drug resistance both to alkylating agents and purine analogues. Another study has yielded similar results but also showed that in patients with abnormalities of 13q14, survival is considerably better for patients with deletions at D13S25 compared to patients with RB1 loss. In a multivariate analysis which included clinical stage, lymphocyte morphology, and response to therapy both D13S25 loss and p53 deletions were independent prognostic variables.

Genetic instability in CLL

The predominant chromosomal abnormalities in CLL involve either the loss or gain of genetic material. Translocations are less frequent and also appear to result in genetic loss rather than the creation of a fusion gene or overexpression of an oncogene. Although genetic abnormalities are more common in advanced disease, 40-50% of patients with stage A CLL have an abnormal karyotype. The combined use of immunophenotyping and interphase FISH has shown that genetic abnormalities such as trisomy 12 and p53 loss only occur in a subclone of leukaemic cells. The initiating event (or events) in CLL remain unknown.

Sequential chromosome analysis shows karyotypic evolution in only 10-15% of cases. However, interpretation of an apparent transition from a normal to an abnormal karyotype is difficult since the original normal karyotype may have derived from T cells rather than a mitotically inactive leukaemic clone. Sequential FISH studies show the occasional acquisition of trisomy 12 and RB1 loss during the course of the disease and also the expansion of the trisomy 12 clone particularly in patients with progressive disease. No acquisition or expansion at the D13S25 locus was found suggesting that loss in this region is an early event in CLL. In contrast p53 mutation or loss is much commoner in advanced disease and may be associated with Richter’s transformation.

The mechanisms responsible for genomic abnormalities and instability in CLL are poorly understood. Microsatellite instability appears to be rare in CLL. Shortening of telomeres is a feature of advanced CLL but it is not known whether this correlates with chromosome abnormalities. Both p53 mutations and ATM loss can be responsible for genetic instability. Since ATM mutations may be germline, they may be linked to the intriguing clinical observation of anticipation in familial CLL in which the disease appears to arise approximately 20 years earlier in the children of a parent with CLL.

There is data both in haematological malignancies and in solid tumours that the site of genetic abnormalities such as chromosomal deletions and gene amplifications correlates with the presence of fragile sites. A recent study identified a polymorphic trinucleotide repeat sequence on chromosome 11q22-q23 within the minimal region commonly deleted in CLL and showed an association between the presence of the larger repeat and poor prognosis.

Conclusions

New techniques have uncovered a multitude of genetic abnormalities in CLL. Much further work is required to understand both the mechanisms whereby CLL cells acquire genetic defects and also their importance in the pathogenesis and clinical course of the disease.

Table 1. Incidence and significance of genetic abnormalities in CLL (adapted from Dohner et al., ASH 1998).

<table>
<thead>
<tr>
<th>Chromosome abnormality</th>
<th>Genetic abnormality</th>
<th>Incidence (%)</th>
<th>Median surv. (years)</th>
<th>Clinical features</th>
</tr>
</thead>
<tbody>
<tr>
<td>all del or +13q14</td>
<td>unknown</td>
<td>54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>single del or -13q14</td>
<td>unknown</td>
<td>36</td>
<td>&gt;15</td>
<td>typical morphology</td>
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<td>ATM</td>
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<td>6.6</td>
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<td>15</td>
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<td>p53</td>
<td>8</td>
<td>3.6</td>
<td>p53 mutations drug resistance</td>
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<tr>
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<td>unknown</td>
<td>7</td>
<td>11</td>
<td>bulky disease</td>
</tr>
</tbody>
</table>

References


Morphology, atypical chronic lymphocytic leukaemia and prognostic features affecting choice of therapy

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Morphological analysis is as important for diagnosis in a patient with lymphocytosis as the histopathological review of lymph nodes is for the diagnosis of lymphoma. As for lymph node biopsies, the immunophenotype of the circulating cells is an essential complement to morphology. Of several thousand cases of lymphocytosis studied in our Department, chronic lymphocytic leukaemia (CLL) remains the most common diagnosis. Only a few cases of CLL are diagnosed by lymph node histology.

A detailed description of the morphological characteristics of the lymphocytes in blood films is important for the differential diagnosis between CLL and other disorders, in particular non-Hodgkin’s lymphomas (NHLs) presenting or evolving with circulating lymphoma cells. The most common low/intermediate grade NHLs which evolve with leukaemia (defined as a lymphocyte count greater than 5×10⁹/L) are follicular lymphoma (FL), mantle cell lymphoma (MCL), splenic lymphoma with villous lymphocytes (SLVL), also known as splenic marginal zone lymphoma, and lymphoplasmacytic lymphoma (LPL).

Another reason why morphology is important in CLL is in order to define cases described broadly as atypical or mixed cell which include those with more than 10% prolymphocytes or CLL/PL; others with a spectrum of small and large lymphocytes, and yet others in which cells with cleft nuclei and/or plasmacytoid features are seen. Cases of atypical CLL may have a different clinical course and are often the ones which present problems of differential diagnosis, in particular with MCL and occasionally with FL or SLVL. Circulating villous lymphocytes are seen not only in SLVL but also in hairy cell leukaemia (HCL) and the rare HCL-variant. Again here, membrane markers are useful to separate CLL from SLVL and the latter from HCL and its variant. Cases of T-cell leukaemia and lymphoma such as T-PLL, large granular lymphocyte (LGL) leukaemia, adult T-cell leukaemia/lymphoma (ATLL) and Sézary syndrome, typically have lymphocytosis and can be distinguished from CLL by a combination of membrane markers and peripheral blood cytology. B-cell prolymphocytic leukaemia (B-PLL) can, in turn, be distinguished from CLL/PL by the immunophenotype which, in CLL/PL, resembles that of CLL and in B-PLL is similar to the B-cell lymphomas.

Immunophenotype

Nowadays it is relatively simple to employ a battery of monoclonal antibodies and identify a patient with lymphocytosis as having predominantly B or T cells. As the B-cell disorders are the more common and the ones which often present diagnostic problems, we have focused our work on developing a scoring system based on the distinct antigenic profile of CLL which is different from all other cases of monoclonal B-cell lymphocytosis. Five antibodies can now define the CLL immunophenotype (Table 1), including a component of the B-cell antigen receptor, the CD79b molecule. According to an earlier proposal we used the intensity of CD22 as one of the five markers. Table 2 depicts the relative incidence of the various B-cell diseases and their score using CLL markers. It is obvious from Table 2 that the combination of immunophenotype and morphology represents a robust and objective methodology for diagnosis of this group of lymphoid malignancies. Minor departures from the score in CLL are seen mainly in cases of atypical morphology and chiefly in CLL/PL. Such cases may occasionally show positivity with FMC7 or CD79b or strong staining for membrane immunoglobulin (SmIg). Preliminary data from the MRC CLL3 trial suggest that positivity with FMC7 is associated with worse outcome than in FMC7 negative patients and that this feature is independent of stage and age.

CLL with more than 10% prolymphocytes (CLL/PL)

CLL/PL has been recognised since the 1980s as a distinct form of the disease. It is not clear whether patients may present as typical CLL and evolve to CLL/PL. In at least half of the cases the percentage of prolymphocyte-like cells (prominent nucleolus, larger size, basophilic cytoplasm) tends to increase at a variable rate. In a minority they may reach values greater than 50% and therefore resemble B-PLL, although they retain the CLL immunophenotype; minor departures are seen in 10-20% of cases (see above).
Our group and others have reported important associations of CLL/PL with genetic changes, namely trisomy 12,3 and p53 abnormalities (deletions, overexpression, mutations).4 These changes are known to be associated with poor response to therapy, high labelling index with the antibody Ki-67, and overall poor prognosis.2,8,9 As both trisomy 12 and p53 abnormalities are genetic changes acquired during the evolution of CLL, it is likely that the morphological expression of CLL/PL is also a secondary event caused by, or closely related to, the above changes.

Prognostic features of CLL and choice of therapy
Stage, age and response to therapy remain the strongest prognostic features of CLL.10 However, the new findings, such as morphology (CLL/PL), immunology (FM C7+) and genetic changes (+12, abnormal p53, del 11q23) are gradually emerging as important for prognosis in individual series. Whether and how this should modify the choice of therapy is not clear. However, the systematic use of such markers in prospective clinical trials will allow such observations to be made. The greater availability of treatment options in CLL, including several nucleoside analogues used alone or in combination and the increasing use of high dose therapy programmes with stem cell transplantation for younger patients will provide the answers if a systematic integration of laboratory markers of prognosis, including morphology, into the analysis of clinical trials is achieved.

References

Chronic lymphocytic leukaemia.
Current strategy and new perspectives of treatment
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Choice of drugs
Until recently, when fludarabine was demonstrated to be superior to chlorambucil, the latter drug was the one more often used for initial treatment of CLL. It had previously been shown that fludarabine is an effective agent as salvage therapy for patients who had failed chlorambucil, or other alkylating agent therapy. Keating et al. in 1991 were the first to report that fludarabine is also an effective drug in the front-line therapy of CLL. Two large multi-institutional randomised trials, one in the U.S. and Canada and the second in Europe have now been reported. Both of these studies prove the superiority of fludarabine as the initial drug in the treatment of CLL. The North American study compared it against chlorambucil and the European study compared fludarabine versus cyclophosphamide, doxorubicin and prednisone (CAP-regimen). Fludarabine induced significantly more frequent complete remissions (CR) and overall remissions (CR plus partial remissions, PR), and fludarabine-induced remissions were significantly more durable than those achieved with chlorambucil or CAP.

Because of the above-noted results, fludarabine is increasingly being used as the drug of choice for the initial treatment of CLL. It is especially recommended when it is desirable to quickly achieve a CR or as good a PR as is achievable without undue toxicity. Fludarabine should be the treatment of choice for a relatively young CLL patient who, after chemotherapy, may wish to consider the option of autologous or allogeneic haematopoietic stem cell transplant therapy. With fludarabine there seems to be a somewhat higher risk of development of autoimmune haemolytic anaemia than is expected in CLL patients, and prolonged exposure to fludarabine may result in significant degrees of immunosuppression. It is best to choose between fludarabine and an alkylating agent on a case-by-case basis, taking into consideration the desired therapeutic end-points and potential toxicities with any of these drugs.

New perspectives of treatment
Need to increase the incidence of CRs
The results from the two large multi-institutional randomised studies mentioned above indicate that with fludarabine as the front-line therapy, approximately 25 to 30 percent of CLL patients achieve a CR...
and an additional 40 percent achieve a PR for an overall (CR+PR) response incidence of about 70%. However, the overall survival curves have shown no significant improvement. In my view, only CRs can lead to a longer life expectancy and PRs do not make a major contribution towards improving survival. The progress recorded in three other examples of haematological malignancies during the past few decades lends support to this view: most recently hairy cell leukaemia, and Hodgkin’s disease and childhood acute lymphocytic leukaemia about twenty years earlier, witnessed a significant increase in survival and even cures as soon as the incidence of CR increased to about 80 or 90%. With fludarabine we have made significant progress, increasing the CR incidence from the 3% achievable with chlorambucil to 27%. New perspectives of CLL therapy will be directed towards further increase of this proportion - to 80 or 90% CRs, we hope.

New therapies

The most attractive agents on the horizon are monoclonal antibodies - Campath 1-H and Rituxan, which are currently undergoing clinical trials in previously untreated patients with CLL. Radiolabelled antibodies such as Bexxar (I-131 labelled anti-CD20) are likely to enter clinical trials in the near future. It is expected that fludarabine and a monoclonal antibody used together, either simultaneously or sequentially, will enable us to increase the incidence of CR significantly from the baseline of 25-30%.

Newer drugs

Interesting new drugs, which are currently in phase I and phase II clinical trials, are listed below:
- protein kinase-C (PKC) inhibitor: UCN-01;
- cyclin-dependent kinase inhibitor: Flavopiridol;
- new nucleotide analogues: Pro-drug for Ara-G (Compound GW 506-U78).

Although bone marrow and haematopoietic stem cell transplantsations have been done both in autologous as well as allogeneic (HLA-matched sibling donor) settings, this method of treatment in CLL will need controlled clinical trials before its true value can be determined. A recent report suggesting benefits with non-myeloablative chemotherapy, allogeneic stem cell transplant followed 2 to 3 months later with donor lymphocyte infusion, especially for its graft-versus-leukaemia effect is worthy of further exploration. In addition to haematopoietic stem cell transplantation, new approaches in the future will also focus on gene therapy in CLL.

In summary, the currently applied risk-adapted approaches and identification of new agents with potential to increase the incidence of complete remissions significantly, have brought us to a point where new perspectives in treatment may soon lead us to curative strategies in chronic lymphocytic leukaemia.

Acknowledgements

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References

Prognostic factors in chronic lymphocytic leukaemia

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The best definition of indolent chronic lymphocytic leukaemia (CLL) is still clinical staging but biological features will improve this prognostic approach.

Clinical staging
Before the staging systems, male sex, young age, anaemia, thrombocytopenia, blood lymphocytosis, lymph nodes, splenomegaly and cytological features were, in univariate analysis, prognostic factors in CLL.

In 1975, Rai et al. described the first staging system with five stages and their respective survival times. The number of clinical stages can be reduced to three: low risk, intermediate risk and high risk. In 1981, we presented a three stage system. Rozman et al. published an evaluation of total tumour mass (TTM) based on the number of peripheral blood lymphocytes, the diameter of the largest palpable lymph node and enlargement of the spleen. Phillips, Rundle, Rozman and Baccarani described other clinical staging systems.

To give a better definition of indolent CLL, Montserrat defines smouldering CLL (Hb ≥ 120 g/L and lymphocytes < 30 × 10⁹/L) and we divide stage A into A’ with lymphocyte count of ≤ 30 × 10⁹/L and A” with lymphocyte count of 30–100 × 10⁹/L and haemoglobin concentration of 120 g/L or both. In patients with stage A’ disease survival is close to that of a sex- and age-matched normal population. In 1996, the National Cancer Institute revised guidelines for treatment of aggressive forms of disease:

1. a minimum of any one of the following disease related symptoms must be present:
   a. weight loss ≥ 10% within the previous 6 months;
   b. extreme fatigue (ie. ECOG PS 2 or worse, cannot work or perform usual activities);
   c. fever of greater than 100.5°F for ≥ 2 weeks without evidence of infection;
   d. night sweats without evidence of infection.

2. Evidence of progressive marrow failure as manifested by the development of, or worsening of, anaemia and/or thrombocytopenia.

3. Autoimmune anaemia and/or thrombocytopenia poorly responsive to corticosteroid therapy.

4. Massive (i.e. > 6 cm below the left costal margin) or progressive splenomegaly.

5. Massive nodes or clusters (i.e. > 10 cm in longest diameter) or progressive lymphadenopathy.

6. Progressive lymphocytosis with an increase of > 50% over a 2-month period, or an anticipated doubling time of less than 6 months.

7. Marked hypogammaglobulinaemia or the development of a monoclonal protein in the absence of any of the above criteria for active disease is not sufficient for protocol therapy.

Patients with CLL may present with a markedly elevated leucocyte count; however, the symptoms referable to leucocyte aggregates that develop in patients with acute leukaemia rarely occur in patients with CLL. Therefore, the absolute lymphocyte count should not be used as the sole indicator for treatment, but should be included as a part of the total clinical picture, which includes the lymphocyte doubling time (see earlier).

Biological staging
Biological features can be studied in four parts: classical, new features, cytogenetic abnormalities and immunoglobulin heavy chain genes.

For Rozman and Montserrat, bone marrow histologic pattern was, in 1984, the best single prognostic parameter in CLL. Retrospective lymphocyte doubling time is very useful. Some patterns of immunophenotype can be used. Serum soluble CD23 level is more sensitive than β2 microglobulin and LDH in our experience.

The overall frequency of p53 protein positivity in CLL is 15% but it is a marker of disease progression and poor prognosis. Cell cycle inhibitor p27kip1 is strongly correlated with both lymphocyte and total tumour mass doubling. High p27 expression may be a valuable kinetic marker by providing instantaneous estimation of the disease doubling time. Cellular expression and serum release of adhesion molecules, degree of abnormal angiogenesis, serum levels of IL-8 and IL-6, and soluble forms of some molecules belonging to the nerve growth factor receptor superfam should be better explored in prognostic studies.

Patients with 13q14 abnormalities characteristically have a benign disease that usually manifests as an isolated, stable or slowly progressive disease.
These patients survive as long as their age-matched controls. In contrast, a significant association between trisomy 12 and CLL with atypical morphologic and/or immunologic features, high proliferative activity, advanced disease and poor prognosis have been reported. Forty-three out of 214 (20%) patients exhibited 11q23 deletions, which affected the prognosis of patients under 55 years of age. Given the conflicting data concerning clinical risk factors in young patients suffering from CLL, this biological finding may be of great relevance in selecting patients for intensive treatment approaches. Döhner et al. studied 338 B-CLL patients by interphase cytogenetics using a disease-specific set of diagnostic DNA probes. Patients with deletion 13q as a single cytogenetic abnormality had the longest median survival (> 15 years), followed by those with 6q deletion and trisomy 12 (median survival 11 and 10.9 years, respectively). In contrast, 17p and 11q deletions were associated with rapid disease-progression and shorter survival (median times, 3.6 and 6.6 years, respectively).

Configuration role of immunoglobulin heavy chain genes in prognostic features has been recently discovered. Hamblin proved that, in CLL, germline configuration of immunoglobulin heavy chain genes is associated with a more aggressive form of the disease. Patients whose tumours showed somatic mutation of VH genes characteristically had stable stage A disease with typical morphology, a normal karyotype or abnormalities at 13q14. Damle identified the same two groups of B-CLL patients who differ in clinical course and in response to therapy. Difference in CD38 expressing cells between the mutated and unmutated group was highly statistically significant (p < 0.001).

Therapeutical staging?

The best prognostic factor in CLL is treatment response but can in vitro tests predict drug sensitivity? This question remains a matter of debate.

In conclusion, biological parameters and chemosensitivity data may be incorporated into clinical prognostic models thus leading to the formulation of clinico-biological and therapeutic systems for CLL.

References

Metaphase FISH, microdissection, and multicolour FISH. Applications in haematology

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Conventional cytogenetics has been greatly enriched by the development of molecular genetics. DNA probes labelled with haptens or fluorochromes and used in experiments of fluorescence in situ hybridisation (FISH) on metaphases allow us to go into molecular details of structural chromosomal rearrangements, namely translocations, deletions, and inversions.

We will review how metaphase FISH, chromosome microdissection, and, more recently, multifish, significantly improve our knowledge of molecular bases of chromosome changes in malignant haematological disorders.

Technical aspects

Probes

In situ hybridization techniques are based on the use of probes complementary to specific DNA regions of the human genome. Centromeric and painting probes give rough information for the assignment of the material to specific chromosomes, while for identification of specific genes or genomic segments, probes are used in which known sequences are inserted in vectors such as plasmids, cosmids, PAC, BAC, and YAC. Vectors differ for the size of insert they bear, from 20-30 kb till 1000 kb. Probes are labelled with haptens or fluorochromes and revealed by fluorescence microscopy. Apart from the SKY system, multicolour karyotyping is obtained by combinations of different chromosomes and adapted filters. Investigations using probes with cDNA are in progress.

FISH

Both interphase and metaphase FISH are largely used in haematological malignancies. FISH can be performed with directly (Texas Red, Fluorescein, Lissamine, Cy3, etc.) or indirectly labelled (biotin or digoxigenin-conjugated) probes. Pretreatment with RNase and pepsin or proteinase K to remove RNA and protein is optional. Simultaneous denaturation of DNA target and probes can be done at high temperature on a hot-plate. Alternatively, they can be denatured separately in formamide containing buffer. This step is not necessary for single strand DNA probes.

After over-night hybridisation at 37°C, post-hybridisation washes of the slides are performed under conditions in which stringency can be modulated to remove the excess of probe and keep stable hybrids. Probes labelled with haptens must be detected with fluorochrome conjugated molecules. Moreover, to detect small sequences, the fluorescence signal can be amplified using a second layer of antibodies. Chromosomes are counterstained with DAPI or propidium iodide and kept in antifade solution after dehydration in an ethanol series (70%-85%-100%) and air dried.

Microdissection

Chromosome microdissection of specific chromosomal regions is obtained under an inverted microscope using glass microneedles controlled by a micromanipulator. The dissected material is further amplified by PCR, namely DOP-PCR, in which partially degenerated oligonucleotides (DOP) are inserted between the 5’ and 3’ ends of the primer. In a second round of PCR, biotin- or digoxigenin-labelled nucleotides are introduced to obtain FISH probes. Furthermore, FISH with microdissected probes (microFISH) is conducted following standard methods. The source of microdissected material is confirmed on original abnormal metaphases, while hybridization on normal metaphases is necessary to identify the chromosomes to which the amplified material belongs.

MultiFISH

This is an interesting technological improvement of karyotyping based on the differential and simultaneous painting of each pair of homologues chromosome. It may help to solve major cytogenetic problems, such as detection of subtle translocations or identification of complex structural rearrangements in one single experiment. This is of particular interest in malignancies in which cytogenetics and molecular cytogenetic investigations are frequently limited by lack of material. There are already a number of proposals from different companies in the market.

Multiple colour FISH uses conventional fluorescence microscopy and multiple exposures. Another system, the so-called SKY (spectral karyotyping), is based on Fourier spectroscopy and fluorescence is detected after a single exposure.

A major limit to applications of this technology are high costs of both equipment and probes. Further-
more, at present, fine changes, such as microdeletions, inversions, telomeric translocations, are not seen by multicolour FISH.

Metaphase FISH and chromosomal deletions

Chromosomal deletions may be found in all types of malignant blood diseases, although they are particularly frequent in myelodysplastic syndromes. Important new insights into comprehension of the molecular side of such structural aberrations have been obtained by FISH investigations in abnormal metaphases.

First of all, FISH may be helpful in identifying deletions hiding translocations. An historical example is represented by the t(6;11)(q27;q23) translocation, one of the variants involving MLL gene at 11q23, which can be missed, despite careful cytogenetic analysis, and which was revealed by painting during a screening of cases classified as 11q- abnormality.6

Even more striking is the discovery of a t(12;21)(p13;q11) translocation in a subgroup of childhood pre-B ALL with low white cell count and a favourable course. This translocation cannot be seen by classical cytogenetics because the small material exchanged between chromosomes 12 and 21 has similar morphology at chromosome banding. Only FISH studies in cases with a 12p deletion revealed the underlying translocation. Cloning of corresponding genes, i.e., ETV6 at 12p13 and AML1 at 21q11, provided us with specific probes to characterise such typical translocation further.7 Interestingly, by using cosmids specific for ETV6 gene, Raynaud et al.8 showed that the t(12;21) is accompanied by deletion of the second allele from the normal chromosome 12. Moreover, deletion of the second allele only involves a portion of malignant cells, as expected for a secondary event in the leukaemogenic process.

More recently, an isolated 5q deletion at karyotypic level in childhood leukaemias has been proven to mask a translocation between chromosomes 5 and 11, i.e., t(5;11)(q35;p15.5).9

In addition to correction of apparent deletions, a most important and new piece of information coming from FISH studies arises from the demonstration of genomic deletions accompanying other structural rearrangements. Studies on balanced and unbalanced 12p translocations in both lymphoid and myeloid malignancies showed that cryptic deletions may happen either in the 12p material adjacent to breakpoints or in more centromeric segments independently of the breakpoints involved in the translocation.6 Similarly, cryptic 13q deletion may accompany complex translocations.7 In some cases the cryptic deletion may involve very short genomic segments, even only part of single genes, such as ETV6 in the 12p.

A cryptic deletion of the MRP gene on the 16p arm may be found in a subgroup of leukaemic cases with the typical inv(16)(p13q22) and M4 leukaemia with eosinophilia. A series of such cases was described as being associated with good prognosis.

In the so-called 17p syndrome in which a myelodysplastic syndrome is associated with a chromosomal change involving 17p13 and typical haematological stigmata, such as dysgranulopoiesis with pseudo-Pelger-Huet anomaly and vacuolated neutrophils, a biallelic involvement of p53 at 17p13 has been shown by molecular cytogenetics with loss of the second allele from the normal appearing chromosome.

In follicular lymphomas characterised by the typical t(14;18) translocation, FISH with YACs containing two suppressor genes, i.e., PTEN/MMAC1 at 10q23.3 and MXI1 at 10q24-25, showed that deletion is a frequent secondary event and pointed to the existence of a third suppressor gene in 10q involved in follicular lymphomas.8

Once a deletion has been established at cytogenetic or molecular level, it is relevant to know whether all affected cases lose the same material. Thus, identification of genomic sequences always being lost (so-called common deleted region, CDR), has been chosen by many laboratories as the privileged approach to discover the critical suppressor gene(s) underlying deletions. Commonly deleted regions have been restricted by chromosome walking with FISH probes in the majority of typical deletions of malignant blood diseases, namely, del(5q) and del(7q) in MDS and AML; del(11q) in lymphoproliferative disorders; del(12p) and del(13q) in both lymphoid and myeloid malignancies; and del(20q) in MDS and myeloproliferative disorders.

Metaphase FISH and translocations

Reciprocal translocations are mainly evidenced by double colour FISH, using painting probes, or, specific probes for the two partner genes, when known. Interestingly, in translocations resulting in a fusion gene, such as BCR-ABL from the t(9;22) of chronic myeloid leukaemia, the fusion emerges as a result of overlapping of the two fluorochromes. This specific reaction is useful not only to increase the specificity of the analysis, but also to understand where a given fusion happens in reciprocal translocations, as well as in complex rearrangements.

FISH on metaphases is one of the most interesting approaches to narrowing and cloning breakpoints of reciprocal novel translocations identified by conventional karyotyping. Breakpoint restriction of reciprocal translocations implies chromosome walking possibly with contigs covering the genomic portion of the chromosomal sub-bands containing the breakpoints. The aim is to obtain the splitting of a probe between the two chromosomes involved in a given translocation, meaning that the insert of the probe contains the genomic sequences where the breakpoint falls. By this approach an impressive variation of partners has been identified to exchange material with BCL6 at 3q27 in B cell diffuse lymphomas; with ETV6 at 12p13 in both
lymphoid and myeloid malignancies; and with MLL at 11q23 in acute myeloid leukaemias. Indeed the number of such so-called promiscuous genes is increasing due to extensive investigations of chromosomal break-points with FISH probes for specific genes.

Metaphase FISH has also been shown to be useful to detect minimal residual disease in leukaemia. Indeed the authors showed that cell culturing with long exposure of cells to colcemid allows recruitment of a high number of metaphases that may be analysed by FISH with specific probes for translocations, even in poor material unusable for conventional karyotyping.

Chromosome microdissection

Chromosome microdissection is an established tool to produce FISH probes for selected chromosomal sites. Further refinements of such technology may also lead to the cloning of translocation breakpoints in malignancies. Microdissection is a direct way of identifying the nature of the amplified genomic material contained in double minute chromosomes and homogeneously staining regions. Cytogenetic classifications of malignant blood diseases contain an undefined group in which complex structural rearrangements may be not identified with chromosome banding. Microdissection is extremely powerful in such cases since the isolation of just one or two copies of a marker chromosome, followed by amplification and labelling by PCR, may be sufficient to generate a probe that, by reverse painting on normal metaphases, may rapidly reveal the chromosomal constitution of markers. Thus, cytogenetically undefined rearrangements may contain typical deletions or translocations. In human B-cell lymphoma a common deletion including 6q11 was shown after microdissection of different types of 6q changes.

Multicolour karyotyping

Hidden chromosomal abnormalities were identified in selected cases of leukaemias and lymphomas with multicolour karyotyping. A major step forward in the knowledge of cytogenetics of multiple myeloma came from multicolour spectral karyotyping. Cytogenetic studies in multiple myeloma were very difficult because of the lack of sufficient metaphases from plasma cells. Some improvements were obtained over the last ten years by dedicated laboratories after the development of cultures with B-mitogens. Thus 13q- emerged as a recurrent structural aberration. A t(11;14), apparently identical to the translocation occurring in mantle cell lymphomas, was also observed. Hyperdiploidy emerged as a non-random event. Moreover cryptic translocations, undetectable by conventional cytogenetics, have already been shown by FISH investigations. New genes have been cloned from the t(4;14) and the t(6;14) in which immunoglobulin genes on 14q32 rearrange with FGFR3 and M MSET on 4p16.3 or with the MUM1 gene on 6p25, respectively. Indeed SKY is not able to pick up the t(6;14) translocation which has been identified by molecular analysis of IgH gene rearrangements. The 6;14 involves telomeric genes escaping resolution of painting analysis which is the basis of the SKY approach. 14q32 appears as the hot site of recombination, as expected in a B cell neoplasm. Partners have been recognised on 12q24, 20q11, 16q22, and 22q11.

Acknowledgements

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References


Interphase FISH in chronic lymphoproliferative disorders and comparative genomic hybridisation in the study of lymphomas

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Interphase FISH in chronic lymphoproliferative disorders

Methods of cytogenetic analysis in chronic lymphoproliferative disorders

Due to the low proliferative capacity of tumour cells, in chronic B-cell lymphocytic leukaemias (B-CLL) conventional chromosome banding analysis is difficult in this disease. Only in 40-50% of cases with this disease can clonal genetic aberrations be identified using this method. In cases without chromosomal aberrations, mitotic cells often do not arise from the malignant cell clone, but rather are derived from non-leukaemic T-cells. This was demonstrated using a technique of sequential immunophenotyping and karyotype analysis.

About ten years ago, the development of fluorescence in situ hybridisation (FISH) using specific DNA probes greatly enhanced the potential to detect genetic aberrations in tumour cells. FISH is based on the specific base pairing of a genomic DNA probe to complementary sequences in cells of the specimen. With this technique, chromosomal aberrations in both metaphase cells and interphase nuclei can be detected (interphase cytogenetics). Numerical and structural aberrations resulting in a change of the copy number in tumour cells are identified by an abnormal signal number per cell. Chromosomal translocations can be detected by an aberrant signal distribution within the interphase cells.

Specific chromosome aberrations in B-CLL

In 1990 and 1991, comprehensive data from chromosomal banding analyses collected within the first and second meeting of the International Working Party on Chromosomes in CLL (IW/CCLL) were published. The second report was based on data from 662 patients from eleven institutions. In this series, clonal chromosomal aberrations were identified in 51% of cases. The most frequent aberrations were trisomy 12 (19% of available cases) and structural aberrations of chromosomes 13 (10%), 14 (8%), 11 (8%), 6 (6%) and 17 (4%) (Table 1).

Sets of specific DNA probes have been developed for the interphase cytogenetic analysis of the most frequent numerical and structural aberrations in B-CLL. Using such a probe set in more than 250 B-CLL cases, we found higher incidences of certain aberrations than those found by banding analysis. In particular, the percentage of cases without clonal aberrations was only 20%. By interphase cytogenetic analysis, the most frequent aberrations were 13q14 deletions, which were present in 53% of the cases. 11q deletions were identified in 19% of cases, followed by trisomies 12 (15%), 6q21-deletions (9%) and 17p13-deletions (8%) (Table 1). Most likely, the striking differences between banding results and interphase cytogenetic data are not due to patient selection but rather reflect different sensitivities of the methods used. Using FISH analysis, the real incidences of chromosomal aberrations are revealed.

Characterisation of specific aberrations using molecular cytogenetics

Recurring chromosomal aberrations pinpoint to regions of the genome containing genes of potential pathogenetic relevance. In case of deletions, such pathogenetically relevant genes are tumour suppressor genes. Many known tumour suppressor genes, such as p53 and p16, have important roles in the control of cell proliferation and cell death. Thus, loss of function of these genes contributes to tumorigenesis by an uncontrolled proliferation or a loss of apoptotic capabilities of the cells. In most cases, such a loss of function requires the inactivation of both alleles of a tumour suppressor gene. Typically, this is achieved by a deletion of one allele, and a mutation of the second allele. Interphase cytogenetics can be used for further characterisation of sub-regions consistently involved in a specific recurring aberration. In the following, this approach is outlined for the characterisation of the commonly deleted region on chromosome arm 11q in B-CLL.

Using chromosomal banding analysis and comparative genomic hybridisation in all B-CLL cases with an 11q-deletion, the smallest commonly deleted region extended from chromosomal bands 11q21 to 11q25. Based on these data, 17 representative DNA clones were selected from a contig map encompassing chromosomal bands 11q14.3-q23.3. With these probes, 43 cases of B-cell neoplasms exhibiting an
11q-deletion (40 B-CLLs and 3 mantle cell lymphomas) were analysed. Thus, for each case, the size and extent of the deletion was characterised. These experiments resulted in a region of minimal overlap of 2-3 Mbp, which was deleted in all 43 cases. This consensus region contains several genes including the ataxia telangiectasia mutated (ATM) gene. Recently, a similar 11q22-q33 deletion cluster was delineated in T-cell prolymphocytic leukaemia.3 In this latter disease, missense mutations or small intragenic deletions were identified in the remaining ATM allele of cases which had an 11q-deletion.3,4 This is strong indication for a tumour suppressor function of ATM in T-cell prolymphocytic leukaemia. Recently, such biallelic inactivations were also described for some cases of B-CLL.

Clinical relevance of chromosomal aberrations detected by interphase cytogenetics

Based on chromosome banding studies, some genetic aberrations were associated with shorter survival times in univariate analysis. These included the presence of clonal aberrations (versus normal karyotypes) and the presence of a trisomy 12. However, an abnormality of chromosome 17 was the only aberration of independent prognostic value.

Interphase cytogenetic analysis provides more reliable information about the real incidence of specific aberrations, independent of the in vitro proliferative activity of the tumour cells. For three aberrations, an association with shortened duration of survival has been published. The respective studies are outlined below.

Trisomy 12. Altogether at least ten studies reporting interphase cytogenetic data for trisomy 12 have been published. Only in one of these was an association with the clinical course described. In this series by Escudier and co-workers, there was no significant difference between survival probabilities in cases with (n=41) and without (n=76) trisomy 12. If, however, banding data were included in the analysis, median survival in patients with trisomy 12 was significantly shorter than in patients with a normal karyotype (7.8 versus 14.4 years).

p53-deletions. In an interphase cytogenetic study, monoallelic TP53-deletions were detected in 17 of 100 cases. Patients with this deletion had a significantly higher probability of shorter survival. In addition, the presence of a TP53-deletion was a strong predictor for non-response to purine analogues. In a multivariate analysis, TP53-deletion was the strongest prognostic factor for poor survival, followed by clinical parameters such as age, Rai stage and haemoglobin level. A study using SSCP analysis in 53 patients also showed the strong predictive value of TP53 gene mutations.

11q-deletions. Strong clinical implications were also identified for 11q-deletions.6 Patients with B-CLL and an 11q-deletion exhibit a characteristic clinical picture: this aberration is associated with a younger age at diagnosis, advanced Rai stages and with extensive lymphadenopathy. The negative prognostic effect of 11q-deletions is dependent on the age at diagnosis: whereas in older patients, there was no significant difference in duration of survival, in the age group <55 years at diagnosis, this difference in survival duration was highly significant (64 months versus 209 months; p<0.001) (Figure 1). The clinical implications of chromosome aberrations in B-CLL are summarised in Table 2.

Comparative genomic hybridisation in the study of lymphomas

In comparison to B-CLL and other leukaemias, in nodal NHL far less is known about the correlation of specific chromosome aberrations with clinical findings. This is mainly due to the lack of appropriate tumour material: in most cases, lymphoma samples are only obtained at the time of diagnosis, when a lymph node biopsy is performed. Usually only paraffin
tissue

Table 1. Frequency of specific chromosomal aberrations in B-CLL as detected by banding analysis and interphase cytogenetics.

<table>
<thead>
<tr>
<th>Chromosomal Aberration</th>
<th>Chromosome Banding</th>
<th>Interphase Cytogenetics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Trisomy 12</td>
<td>112/604</td>
<td>19</td>
</tr>
<tr>
<td>Structural 13q aberrations</td>
<td>62/604</td>
<td>10</td>
</tr>
<tr>
<td>Structural 11q aberrations</td>
<td>49/604</td>
<td>8</td>
</tr>
<tr>
<td>Structural 6q aberrations</td>
<td>36/604</td>
<td>6</td>
</tr>
<tr>
<td>Structural 17p aberrations</td>
<td>22/604</td>
<td>4</td>
</tr>
</tbody>
</table>

*Bandung data were obtained within the Second International Working Party on chromosomes in CLL (IWCCLL; Juliusson et al., 1991). Interphase cytogenetic data are from the Heidelberg interphase cytogenetic study (see also Döhner et al., 1999).

Figure 1. Survival probabilities of 310 B-CLL cases with (n=59) and without (n=251) the presence of an 11q-deletion. Survival times are measured from the time of diagnosis.
Comparative genomic hybridisation - principle of the method

CGH is based on a modified in situ hybridisation. Whole genomic DNA of the tumour of interest (test DNA) is hybridised as probe to well defined (normal) metaphase cells (reverse in situ hybridisation, reverse painting). Hybridisation of genomic DNA results in a more or less homogeneous staining of all chromosomes. Chromosomal regions that are overrepresented (e.g. trisomies or DNA amplifications) or underrepresented (e.g. monosomies) in the test genome, can be detected by a stronger or weaker staining of the respective target regions in the metaphases. Since signal inhomogeneities could also be caused by experimental parameters, an internal standard is introduced by co-hybridisation of normal genomic DNA. Such control DNA is obtained ideally from normal cells of the patient or alternatively from a proband. Signal inhomogeneities of diagnostic relevance are identified by comparison of the differentially visualised signal intensities of the test and control DNAs along the chromosomes. This comparison is performed using digitised image analysis procedures which have been developed for the quantitative evaluation of CGH experiments.

Chromosomal gains and losses detected in NHL

Although by CGH no balanced chromosomal aberrations (e.g. translocations or inversions) can be detected, this technique has considerable relevance in lymphomas. In a compilation of cytogenetic data in NHL, a pattern common to this group of tumours was identified by Johansson and co-workers. The primary aberrations which define the subtype of NHL are balanced aberrations. Examples are the t(14;18) (q32;q21) of follicular lymphomas or the t(11;14) (q13;q32) of mantle cell lymphomas. In contrast, most of the secondary aberrations present in addition to the respective translocation seem to be chromosomal gains and losses. These additional aberrations may be of particular importance for tumour progression and therefore may be associated with a specific clinical presentation. Since 1995, CGH studies focusing on different types of NHL have been performed. These include disease entities for which no (primary mediastinal B-cell lymphoma) or very few cytogenetic data (e.g. aggressive gastrointestinal lymphomas, marginal zone lymphomas) were available. Even in groups which were analysed in large banding studies, novel aberrations were identified by CGH (see e.g. Bentz et al., Table 3).

Identification of target genes for gains and losses of DNA

The types of genes typically associated with chromosomal imbalances are tumour suppressor genes (deletions) and proto-oncogenes (gains). An approach for further molecular cytogenetic analysis of regions containing possible tumour suppressor genes has been outlined above. Due to the limited spatial resolution of CGH, this technique is not suitable for the fine mapping of deleted regions. However, as a genome screening procedure, CGH has contributed to the identification of new deletion regions, such as 11q23 deletions in CLL and mantle cell lymphomas.

In contrast, CGH has proven to be a very efficient method for the characterisation of gains of chromosomal sequences. Such aberrations are supposed to be associated with possible activation of proto-onco-
genes. Due to an increased gene dosage, the level of expression of such genes can be up-regulated resulting in a profound deregulation of cell proliferation and differentiation. In the case of gene amplifications, which are known to play a key role in the pathogenesis of certain epithelial tumours, this increase of the gene dosage is particularly evident. CGH is a very sensitive method for screening DNA amplifications. In addition, the amplified sequences are mapped within the genome by CGH analysis. This has resulted in several new findings in NHL.

Before NHLs were analysed by CGH, gene amplifications were considered rare events in NHL. Using banding techniques, the chromosomal hallmarks of such gene amplifications, i.e. homogeneously staining regions and double minute chromosomes, were reported in only 19 of more than 3,500 NHL cases. By CGH, however, DNA amplifications were identified in 10 to 20% of the cases (Werner et al. 1997). In NHL, amplified target genes were mainly identified based on a candidate gene approach. Using such an approach, amplification of several genes was performed, alterations of which were previously only rarely identified (e.g. CCND2) or not at all (GLI and N-MYC) in NHL before. The CGH findings regarding gene amplification are summarised in Table 3.

Future prospects

Currently, novel molecular cytogenetic methods are being developed that will further extend the application of molecular cytogenetics. Recently, most attention has been attracted by microarray technologies (DNA chips; Matrix-CGH). For matrix-CGH, whole genomewide DNA and a control DNA are co-hybridised to an array of DNA probes immobilised on a glass slide. In the near future this technique may allow a simple and rapid overview of the specific genes and chromosomal loci involved in the pathogenesis of a specific tumour. Furthermore, with further extension of molecular cytogenetic analysis, it can be expected that, as for acute leukaemias and B-CLL, so for nodal NHL, genetic risk factors will be identified that can be used for the development of risk adapted treatment strategies in this group of tumours.

Acknowledgements

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Table 3. Comparative genomic hybridisation in various lymphoma entities.

<table>
<thead>
<tr>
<th>Lymphoma type</th>
<th>Authors, year</th>
<th>number of patients</th>
<th>Most frequent aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>gains</td>
</tr>
<tr>
<td>Chronic B-cell leukaemias</td>
<td>Bentz et al., 1995</td>
<td>28</td>
<td>8q, 12</td>
</tr>
<tr>
<td></td>
<td>Karhu et al., 1997</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>Follicular lymphomas</td>
<td>Bentz et al., 1996</td>
<td>28</td>
<td>X, 7, 12, 8, 18</td>
</tr>
<tr>
<td></td>
<td>Avét-Loiseau et al., 1997</td>
<td>34</td>
<td>18q, X, 7, 2, 6p, 8q</td>
</tr>
<tr>
<td>Primary mediastinal B-cell lymphoma</td>
<td>Joos et al., 1996</td>
<td>26</td>
<td>9p, 12q, Xq</td>
</tr>
<tr>
<td>Diffuse large cell lymphoma</td>
<td>Monni et al., 1996</td>
<td>32</td>
<td>X, 1q, 7, 3, 6p, 11, 12, 18</td>
</tr>
<tr>
<td>Marginal zone B-cell lymphoma</td>
<td>Dierlamm et al., 1997</td>
<td>25</td>
<td>3, 18, X, 1q</td>
</tr>
<tr>
<td>Aggressive G1-lymphomas</td>
<td>Barth et al., 1998</td>
<td>31</td>
<td>11, 12, 1q, 3q</td>
</tr>
<tr>
<td>17p</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mantle Cell lymphoma</td>
<td>Monni et al., 1998</td>
<td>27</td>
<td>3q, 8q, 15q</td>
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</table>

Studies focusing on amplifications

<table>
<thead>
<tr>
<th>amplified genes</th>
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<tbody>
<tr>
<td>Diffuse large cell lymphoma</td>
</tr>
<tr>
<td>B-cell lymphomas</td>
</tr>
<tr>
<td>Diffuse large cell lymphomas</td>
</tr>
<tr>
<td>Diffuse large cell lymphomas</td>
</tr>
</tbody>
</table>

* In these studies, CGH was used for screening a smaller number of cases for amplified chromosomal regions. In a second step, more cases were investigated by Southern Blot analysis using DNA probes for candidate genes mapping to the amplified chromosomal regions.

References


Detection of recurrent translocations using real time PCR; assessment of the technique for diagnosis and detection of minimal residual disease

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Large cytogenetic studies have clearly shown that certain chromosomal abnormalities and in particular some recurrent balanced translocations, represent independent prognostic factors for acute leukaemia patients:
- t(9;22) (q34;q11), t(1;19) (q23;p13) and 11q23 translocations are poor prognostic factors for acute lymphoblastic leukaemias (ALL);
- for acute myeloid leukaemias (AML), inv(16) (p13; q22), t(15;17) (q22;q21) and t(8;21) (q22;q22) identify a good risk group (65% disease free survival (DFS) at 5 years) while 11q23 abnormalities are criteria for the poor prognostic group (less than 10% DFS at 5 years).

At the molecular level, these translocations correspond to the formation of fusion genes that can be detected by reverse transcriptase polymerase chain reaction (RT-PCR) techniques. This molecular screening has some advantages: 1) the analyses can be done on a few cells from either blood or bone marrow without the need of cell culture so the vast majority of the patients can be screened and 2) between 10 to 20% of cryptic translocations (patients with a normal karyotype but bearing the molecular abnormality) have been identified for each translocation.

Besides these diagnostic advantages, PCR analyses allow, because of their high sensitivity, detection of residual leukaemia cells bearing these fusion genes during the course of therapy, the so-called minimal residual disease (MRD). The clinical value of qualitative RT-PCR analyses for MRD molecular studies with chromosomal aberrations as targets has been clearly established for chronic myeloid leukaemia after allogeneic transplant and for acute promyelocytic leukaemia, but their clinical relevance in other diseases remains unclear. Although a role in MDR evaluation has been suggested for CBFβ MYH11 (inv(16)), contradictory results have been reported for AM L1 ETO, the molecular equivalent of t(8;21) for AML and for IgH-Bcl2, the molecular equivalent of t(14;18) for follicular lymphomas, and finally it is still too early to assess the role of TEL AM L1 for MDR evaluation; this fusion gene was discovered in 1995 and represents up to 25% of childhood B-lineage ALL.

Quantitative analyses with competitive PCR on small series of patients have suggested that it is important to determine the level of MRD precisely for assessing the clinical relevance of molecular MRD. However, such competitive techniques are not applicable in large prospective studies because they are very laborious, time consuming and too complicated to be standardised. All these qualitative and quantitative PCR techniques are end-point PCR methods.

A new technique has recently emerged: real time quantitative PCR (RQ-PCR) that allows precise quantification of the target to be amplified which should revolutionise the field of MRD studies.

Principles of real time quantitative PCR

Two methods have been described to detect and quantify specific PCR products in real time, while the PCR reaction proceeds:
- during the extension phase, an internal fluorogenic probe called the Taqman probe, labelled with two dyes: the reporter in 5' and the quencher in 3', is degraded by the 5'-3' exonuclease activity of the Taq polymerase, resulting in emission of a fluorescent signal that accumulates during the reaction (Figure 1a). This reaction is measured by the ABI 7700 or more recently by ABI 5700 ABI prism machines from Perkin Elmer;
- during the annealing step of the PCR reaction, the emission of fluorescence by two hybridisation probes is obtained by energy transfer when the two probes are contiguous (Figure 1b). This reaction is measured by the Light Cycler System from Roche.

In both cases, the fluorescence signal increases with each amplification cycle. During the exponential phase of the PCR reaction the signal is proportional to the starting amount of the target present in the tested sample. The value measured is the Cycle Threshold, i.e. the number of cycles necessary to reach a target-specific fluorescent signal 10 standard deviations above the base line of fluorescence.

When RT-PCR targets are quantified, the amount of fusion-gene target must be normalised against a ubiquitous gene, expressed in all haematopoietic cells, in order to assess the quality of the sample analysed.
Advantages
These RQ-PCR methods have a very large dynamic detection range over five orders of magnitude, thereby eliminating the need to perform serial dilutions of follow-up samples. Quantitative data are available in a brief period of time since post-PCR processing is not necessary, providing a high through-put technique. Furthermore, as the assay is performed in an enclosed tube the risk of cross-contamination, a major problem during PCR MRD studies, is greatly decreased. Finally, standardisation is at last possible with this new technology.

Limitations
These are mainly due to the novelty of the technique and should be resolved in the near future:
1. Current dilution cell lines in limiting dilution assays or modified artificial constructs in competitive assays used to assess the sensitivity of the end point PCR analyses are no longer suitable for RQ-PCR. New standards need to be set and a unit value to express data has yet to be defined and agreed on;
2. For the normalisation with a ubiquitous gene two problems remain: first, the definition of the good control gene(s) (GAPDH, ABL, PBGD, etc.) which needs to be expressed stably under various conditions and to be expressed within the same range as the specific target; second, the target and the control are currently measured in separate tubes in the various reported studies, although the technology allows the use of two different dyes and ideally for the specific target and the internal control to be measured in the same tube;
3. Rules for designing primers and probes and for performing the assay under the best conditions are still to be learned empirically;
4. Prices of the machines and reagents are still high, although a cost-effectiveness analysis was performed suggesting that they are in the range of those of current PCR techniques; details will be presented during the meeting.

Current studies
These all show the feasibility of the technique. Studies with this new technology concerning three recurrent translocations used as MRD targets have been published so far:
1. Chronic myeloid leukaemia patients with t(9;22) or Philadelphia chromosome. The sensitivity of the assay was described: at least 10 copies of a plasmid diluted in water were detectable with a dynamic range of six orders of magnitude. The BCR ABL quantification was normalised by assessment of the amplification of a ubiquitous gene, the PBGD. Cytogenetic analysis was negative below a normalised BCR ABL dose of 3 x 10⁻²;
2. Acute myeloid patients with t(8;21). Again using cDNA dilutions in water, the threefold level of sensitivity of the assay was 5 molecules. Serial samples from six patients at diagnosis and during the course of therapy, were analysed. Normalisation was done with the β actin gene. Each patient showed 10³ or more copies of AML1 ETO fusion transcript at diagnosis and each showed a 2 to 4-log decrease during successful induction therapy;
3. In follicular lymphoma patients with t(14;18) (q32;q21). Here the amplification was done from genomic DNA with a sensitivity estimated at 5 pg of DNA, equivalent to 0.6 to 0.8 genomes per reaction. The authors compared this technique with the classical PCR analyses (one round PCR + Southern blotting); the overall concordance was 98%.

Figure 1. Real time quantitative PCR: principles of detection.
Future developments
An European network has started a standardisation and quality control programme for RQ PCR analyses of fusion transcripts for leukaemia patients with the support of the European Commission DG V “Europe Against Cancer”. This network involves more than 20 expert laboratories from 11 European countries: Austria, Belgium, Denmark, Germany, Great Britain, Italy, Netherlands, Portugal, Spain and Sweden. The aims are to develop a common platform for the main recurrent fusion genes, to assess the reproducibility between laboratories and to perform a quality control programme.

This new technology should allow much better standardisation than that possible with classical PCR analyses allowing comparison of results inter-laboratory and ultimately between therapeutic trials. Studies on large series of patients with a long follow up are mandatory to assess the usefulness of real time quantitative PCR in a clinical setting. It is then reasonable to predict that molecular analyses generated by real time quantitative PCR will be used for monitoring patients bearing a marker at diagnosis in large therapeutic trials. Obviously the interest and applicability of this new technology will be adapted according to the disease and therapy:

a. for CM L patients, MRD after allogeneic transplant in particular, will be definitively assessed using this new technology. Furthermore, even the very well established cytogenetic follow-up of the large majority of patients under interferon therapy may be challenged by this new technology;

b. for AM L patients, the challenge is to identify, within the good cytogenetic sub-group, patients who will eventually relapse under conventional therapy. The best potential application of this technology probably lies in this area;

c. for ALL patients, immunoglobulin and T cell receptor gene rearrangements have been shown to be suitable targets for MRD studies in two large retrospective studies. They have the advantage, compared to fusion genes, of being available for almost every patient, but much simpler techniques need to be developed for prospective studies and clinical application. Real time quantitative PCR is one option;

d. finally, this technology appears as a very useful tool to monitor, quickly and objectively, the efficiency of innovative therapies such as donor lymphocyte infusion or purging of bone marrow or selected peripheral blood stem cells.

Conclusions
Currently, at diagnosis, the RQ PCR technique seems to have a limited application in acute leukaemias or lymphomas because of the heterogeneity of some fusion genes examined such as MLL AF4 [t(4;11)] or CBFp M YH11 [inv(16)]. In contrast, it appears to have great potential for monitoring minimal residual disease during the course of therapy allowing precise quantification with a high through-put. This technique is, however, still in its infancy. Retrospective studies will indicate the clinical significance of such molecular data which current molecular MRD studies already suggest are important. Guidelines need to be established in order to use this new technique efficiently for the benefit of the largest number of patients in the coming years.

References
Neonatal alloimmune thrombocytopenia (NAIT) is the commonest cause of severe neonatal thrombocytopenia. The condition is the platelet equivalent of haemolytic disease of the newborn (HDN). Foetomaternal incompatibility for a foetal platelet alloantigen inherited from the father and absent in the mother may cause maternal alloimmunisation, and foetal and neonatal thrombocytopenia may result from placental transfer of IgG antibodies. Most cases are diagnosed after birth, hence the terminology neonatal alloimmune thrombocytopenia. However, the condition develops in utero, and the foetus may be severely affected, and this is sometimes emphasised through the use of an alternative term for the condition, such as foetomaternal alloimmune thrombocytopenia (FMAIT).

Considerable progress has been made in laboratory aspects of platelet immunology since FMAIT was first recognised, allowing more precise diagnosis of the condition. There have been advances in foetal and transfusion medicine resulting in improvements in its management, particularly the antenatal management of women with a previous history of pregnancies affected by FMAIT. However, current antenatal management strategies all require foetal blood sampling (FBS), which is invasive. Improved non-invasive methods for identifying high risk pregnancies are needed to allow antenatal intervention to be directed at those foetuses at greatest risk of severe thrombocytopenia and haemorrhage. The question of antenatal screening for FMAIT has been raised, but ideally the relationship between the presence of antibodies and clinical disease should first be more closely defined so that those pregnancies in alloimmunised women which might benefit most from the antenatal intervention can be predicted. A better understanding of the immune response to platelet antigens and the mechanisms involved in foetal platelet destruction may allow the design of novel approaches to the prevention and treatment of FMAIT in the future.

Pathophysiology of FMAIT

Platelet alloantigens

There are five well characterised biallelic platelet alloantigen systems, and a number of rare private alloantigens have also been described; most were first discovered during the investigation of cases of FMAIT. Platelet antigens are known to be expressed from 16 weeks’ gestation, and placental transfer of IgG antibodies can occur from 14 weeks, so foetal thrombocytopenia can occur very early in pregnancy.

Knowledge of the genetic basis of platelet-specific antigens makes it possible to carry out molecular genotyping on whatever material is available, for example platelet typing using foetal DNA from amniotic fluid or chorion villous samples.

Platelet alloantibodies implicated in FMAIT

Series of cases of FMAIT with detectable platelet-specific alloantibodies have shown that the commonest antibody in Caucasian women is anti-HPA-1a in 78-89% of cases, followed by anti-HPA-5b in 6-15% and the remainder due to other specificities. In Japan, anti-HPA-5b and anti-HPA-4b are the most frequent antibodies; the HPA-1a/1b polymorphism is very rare in Japan and anti-HPA-1a antibodies have never been found. FMAIT due to anti-HPA-5b has been reported to cause less severe thrombocytopenia than that due to anti-HPA-1a, but there are few data on the severity of FMAIT due to other HPA antibodies.

HLA antibodies are frequently found in pregnant women, but are not thought to cause FMAIT. This is because antibodies against foetal HLA antigens are absorbed by placental HLA antigens; only HLA antibodies against HLA antigens not expressed by the foetus pass into the foetal circulation.

Incidence of FMAIT

The incidence of FMAIT is between 1 in 1,000 to 1 in 5,000 live births. This is surprisingly low considering that 2% of women are HPA-1a negative and that 98% of men are HPA-1a positive. However, only about 10% of HPA-1a negative women develop anti-HPA-1a.

The low incidence of alloimmunisation to HPA-1a in HPA-1a negative women is because the antibody response to HPA-1a is strongly associated with a certain HLA class II type. This association was initially reported with HLA-DR3, which was present in 71-
95% of women developing anti-HPA-1a. It was later found that all alloimmunised women were positive for HLA DRw52, and all responders tested by restriction fragment length polymorphism had the DRw52 allele at the DRB3 locus. Further studies using polymerase chain reaction with sequence-specific probes showed that two cases previously typed as HLADRw52 (HLADR3*0301, one additional case out of seven new ones was found to be HLADRw53 (HLADR4*0101). This means that FM AIT is not always associated with HLADRw52, and that pregnancies in HPA-1a negative women without this HLA type cannot be excluded from the group at risk of FM AIT. There is no similar association between HPA-1b alloimmunisation and HLADRw52a, but a significant association has been reported between HLADRw6 and alloimmunisation to HPA-5b.

The factors influencing the development of foetal thrombocytopenia and its severity in pregnant women with HPA antibodies are poorly understood, and there is no reliable or precise correlation with the titre or isotype of the IgG antibodies. One study found a significant correlation between severe thrombocytopenia and a third trimester titre of anti-HPA-1a ≥ 1:32, but the usefulness of antibody titres as a measure to predict clinical severity is doubtful because there is considerable variability between laboratories in platelet alloantibody detection in quality assessment exercises, particularly in antibody quantification. Alternatively, parameters of the functional activity of HPA antibodies measured by antibody-dependent cell-mediated cytotoxicity or chemiluminescence assays could be used as markers of the severity of FM AIT, as they have been in HDN, but a close correlation with disease severity has not yet been established.

**Diagnosis of FM AIT**

**Clinical aspects**

The diagnosis of FM AIT is usually made on clinical grounds following the observation of bleeding, although occasionally the diagnosis is suspected following the finding of a low platelet count in the absence of bleeding. The usual presentation is that of an otherwise well neonate who is observed to have widespread purpura at the time of delivery or in the first 48 hours after birth. The mother is healthy, and her first child is affected in 50% of cases. Intracranial haemorrhage (ICH), which is the major cause of mortality and long-term morbidity occurs in 15-20% of cases. In one study of 127 cases, death due to severe haemorrhage occurred in 7% and there were neurological sequelae in 21% Although there is a serious risk of severe haemorrhage at the time of delivery, nearly 50% of ICHs occur in utero, usually between 30 and 35 weeks of gestation, but sometimes even before 20 weeks. There may be more unusual presentations such as isolated foetal hydrocephalus, unexplained foetal anaemia, or recurrent miscarriages.

The first step in the diagnosis of FM AIT is confirmation of isolated thrombocytopenia, followed by exclusion of other causes of neonatal thrombocytopenia, such as infection, disseminated intravascular coagulation, maternal autoimmune thrombocytopenia, and conditions causing impaired neonatal megakaryocytopoiesis.

**Laboratory investigations**

A diagnosis of suspected FM AIT on clinical grounds requires laboratory confirmation. Testing is aimed at detection of a maternal platelet-specific alloantibody against the father’s platelets, which are a more convenient source of platelets expressing the relevant antigen than the baby’s own platelets, and determination of the platelet antigen types of the mother and father.

The investigation is best carried out in a reference laboratory, which will be able to carry out the necessary tests speedily and advise on clinical management. Testing usually comprises the combination of a sensitive assay using intact platelets, such as the platelet immunofluorescence test (PIFT), with a glycoprotein-specific antigen capture method, such as the monoclonal antibody-specific immobilisation of platelet antigens (MAIPA) assay, which allows detection of weak antibodies and mixtures of antibodies. Molecular methods, using PCR, are now commonly used for platelet genotyping.
Investigation is more complicated if it is not possible to demonstrate parental incompatibility for platelet-specific alloantigens with detection of the corresponding maternal antibody. Maternal platelet-specific antibodies have been reported to be undetectable in up to 20% of cases, but this is almost certainly less with improved methods for detecting antibodies, and it may be possible to demonstrate parental incompatibility for HPA-1a or another HPA in this situation. Another cause of difficulty in laboratory investigation is if a rare private antigen is involved; the mother has an antibody against the father’s platelets, but it reacts with platelets from very few or no normal donors and its specificity is not obvious.

Management

Unexpected neonatal thrombocytopenia

There is as yet no routine antenatal screening for FMAIT, and so most cases will present without warning. The need for immediate treatment depends on the presence of bleeding and the severity of the thrombocytopenia. Any delays or difficulties in the laboratory confirmation of the diagnosis of FMAIT should not prevent treatment of an infant with bleeding or severe thrombocytopenia. A recent survey identified some of the problems in the delivery of prompt treatment, including a lack of awareness of the potential seriousness of the condition amongst clinical staff, and in the availability of compatible platelets.9

The best way of raising the platelet count rapidly is to transfuse compatible platelets. A strategy for providing compatible platelets before the results of serological testing are available is to transfuse platelets from donors who are either HPA-1a negative, or HPA-1a and HPA-1b negative. Some transfusion centres ensure that HPA-1a negative, or HPA-1a and HPA-5b-negative platelet concentrates are always available. Alternatively, compatible platelets can be provided by platelethpheresis of the mother. Often only one transfusion of compatible platelets is sufficient, as the thrombocytopenia is self-limiting. The clinical condition of the infant should be carefully monitored and the platelet count measured at least once daily until it is obvious that it has reached a ‘safe’ level and is increasing spontaneously. If there has been severe thrombocytopenia, a cerebral ultrasound or nuclear magnetic resonance scan should be performed to detect clinically silent ICH.

The transfusion of platelet concentrates from random donors is unlikely to be effective. There has only been one formal study of the use of intravenous immunoglobulin (IV IgG) for the neonatal management of FMAIT; the response rate was 75% and the increase in platelet count was delayed for 24-48 hours during which time the infant remained at risk of ICH.

Antenatal management

The recurrence of FMAIT in subsequent pregnancies is very high (>85%); the risk obviously depending on whether the partner’s platelet genotype is homozygous (HPA-1a/1a) when the rate of recurrence is essentially 100% or heterozygous (HPA-1a/1b) when the rate of recurrence is 50%. If a previous sibling has had an ICH, the risk of antenatal ICH is high in a subsequent pregnancy, strongly suggesting that antenatal intervention is indicated.

If the previous infant was thrombocytopenic, but did not have a major haemorrhage, the risk of antenatal haemorrhage is more difficult to assess. There are no non-invasive tests for the prediction of which foetuses are at greatest risk of haemorrhage. Levels of maternal platelet antibodies have not been found to be reliably predictive of the severity of thrombocytopenia, and cerebral ultrasound scans will only become abnormal after ICH has already occurred.

The only method available at the present time for assessing the foetal platelet count rapidly is foetal blood sampling (FBS), which allows the diagnosis and severity of FMAIT in utero to be made with certainty.9,10 In addition, this technique provides a means for transfusing compatible platelets to severely affected foetuses. The procedure usually only takes a few minutes, and a few millilitres of pure foetal blood are obtained. Foetal platelet counts are reliable and it is unusual to have problems because of contamination of the sample with maternal blood or amniotic fluid; the normal range is similar to adults and there is no significant variation with gestational age. If the father is heterozygous for the relevant HPA, foetal platelet typing can be performed using a foetal blood sample obtained from 18 weeks’ gestation, or using chorionic villous or amniotic fluid samples obtained earlier in pregnancy.

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of pregnant women studied</th>
<th>HPA-la-negative</th>
<th>HPA-la-positive foetus (if tested)</th>
<th>Maternal anti-HPA-la</th>
<th>Overall incidence of FMAIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mueller-Eckhardt et al. (1985)</td>
<td>1,211</td>
<td>26 (2.2%)</td>
<td>23/25</td>
<td>2/23 (8.7%)</td>
<td>2 (0.16%)</td>
</tr>
<tr>
<td>Blanchette et al. (1990)</td>
<td>5,000</td>
<td>81 (1.6%)</td>
<td>25/29</td>
<td>3/50 (6%)</td>
<td>1 (0.02%)</td>
</tr>
<tr>
<td>Burrows and Kelton (1993)</td>
<td>15,471</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10 (0.06%)</td>
</tr>
<tr>
<td>Dougherty et al. (1995)</td>
<td>3,473</td>
<td>74 (2.2%)</td>
<td>-</td>
<td>2/22 (9.1%)</td>
<td>3* (0.09%)</td>
</tr>
<tr>
<td>Williamson et al. (1998)</td>
<td>24,417</td>
<td>618 (2.5%)</td>
<td>-</td>
<td>46/387 (12%)</td>
<td>22 (0.09%)</td>
</tr>
</tbody>
</table>

*including one twin pregnancy.
The main risks of FBS are severe haemorrhage from the cord puncture site and obstruction of the cord circulation due to a haematoma. The main problems are most likely with the initial FBS at around 20-22 weeks’ gestation when the cord is small and the platelet count may be very low. FBS for FM AIT should be performed at referral centres, where the risk is about 1%. In the authors’ experience of over 200 FBS for FM AIT, there have been two foetal deaths, one due to bleeding and one due to a cord haematoma. Severe bleeding requiring foetal red cell transfusions occurred in two other pregnancies, but there was eventually a successful outcome in these pregnancies. Another group has reported five foetal deaths due to exsanguination.11 Because of the risk of bleeding, it has become routine practice to transfuse platelets to the foetus following FBS for suspected FM AIT.

The aim of antenatal management when there has been a previously affected pregnancy is to minimise the risk of severe haemorrhage such as ICH, but the optimal management remains controversial. The therapeutic options being explored include maternal administration of IV IgG and foetal platelet transfusions; early Caesarean section alone is not considered to be effective in preventing antenatal or perinatal haemorrhage. For both approaches to antenatal management, FBS is required for initial assessment of the foetal platelet count, usually at 20-22 weeks’ gestation, and for the monitoring of the effectiveness of treatment.

Maternal administration of IV IgG has been reported to be successful with no instances of ICH and most, but not all infants, having a platelet count of greater than 30×10⁹/L at the end of pregnancy.9 The addition of steroids does not add to the effect of IV IgG. However, ICH has been found during maternal treatment with IV IgG, and other studies have reported this treatment to be ineffective. A group of European centres treating 27 pregnancies found success with the use of maternal IV IgG in only 4/11 (36%) cases, with steroids in 1/10 (10%), and steroids and IV IgG in 1/6 (16%); the treatment was considered to be successful if there was no ICH, and if there was a significant increase in the foetal platelet count and it was above 30×10⁹/L at the end of therapy.10

It is difficult to understand why there is such a difference in the success of maternal administration of IV IgG between North America and Europe. Relevant factors may include the methods used for assessing the success of treatment and the dose, timing and type of IV IgG used. The selection of cases may also be important, and there is some evidence that therapeutic failures occur more frequently in severe cases. It has been suggested that the direct injection of IgG to the foetus by cordocentesis may be more effective than its indirect administration to the mother. However, this method of administering IgG has all the risks of FBS discussed above, and was not found to be effective by another group.

A number of studies have shown the value of platelet transfusions given by cordocentesis in raising the platelet count, but the platelet count is raised for only a few days. A single pre-delivery transfusion may protect against bleeding at the time of delivery, but the foetus remains at risk of spontaneous ICH earlier in pregnancy. Weekly in utero platelet transfusions have been shown to be effective in preventing ICH in severe cases of FM AIT.12 An example of the use of serial foetal platelet transfusions for FM AIT is shown in Figure 1. If the foetal platelet count is raised to 300-500×10⁹/L after each transfusion, it is usually no lower than 30×10⁹/L one week later; this is thought to be a safe level for the foetal platelet count, and one week to be an acceptable interval between transfusions in high risk pregnancies where serial transfusions are used.

More information is needed about the frequency and time of occurrence of ICH to determine the optimal timing of the initial FBS. As already discussed, there is no reliable non-invasive method for assessing the severity of thrombocytopenia, and because of this uncertainty FBS is usually carried out at 20-22 weeks’ gestation. If the foetal platelet count is less than 50×10⁹/L, antenatal treatment is required to minimise spontaneous antenatal ICH because the foetal platelet count will continue to fall in untreated foetuses. If it is greater than 50×10⁹/L, and the foetus is confirmed to be positive for the relevant HPA, FBS may need to be repeated at regular intervals, possibly every 4 weeks, to detect a significant fall in the foetal platelet count indicating the need for antenatal treatment. Antenatal treatment appears to have improved the outcome of severely affected cases of FM AIT, but there is little information on the long-term development of the children who have been treated in utero.

The main issues in the preparation of platelet concentrates for foetal transfusions are compatibility with maternal antibodies, avoidance of transfusion-transmitted infection and transfusion-associated graft-versus-host disease, and adequate dosage without volume overload. Concentration of the platelet concentrate to a platelet count of 2,500-4,000×10⁹/L is essential to achieve satisfactory post-transfusion platelet counts without an unacceptably high transfusion volume.

Antenatal screening

Progress in the antenatal management of FM AIT in women who have had previously affected pregnancies draws attention to the issue of how to identify and manage the first affected infant. The diagnosis of FM AIT in first affected infants is usually only made after birth, by which time serious haemorrhage might have already occurred. Improvements in methods for large scale platelet antigen typing and platelet antibody detection have raised the possibility of antenatal screening for pregnancies at risk of FM AIT alongside established programmes of screening for haemolytic disease of the newborn. Recent studies
have demonstrated the feasibility of such programmes in determining the frequency of HPA-1a alloimmunisation and its clinical sequelae.\textsuperscript{5,6} Typing for HLA DR3*0101 following initial identification of HPA-1a negative pregnant women would focus on women most likely to develop anti-HPA-1a, but its positive predictive value has been estimated to be only 35%.\textsuperscript{6}

Important obstacles to the introduction of antenatal screening are the lack of reliable non-invasive methods for identifying which foetuses of pregnant women with anti-HPA-1a are severely affected by FMAIT, and uncertainty about the optimal management of FMAIT when there is no previous history of affected pregnancies. FBS has significant risks, which have to be balanced against those of a conservative policy with no antenatal intervention but prompt identification and treatment of affected cases after delivery, and only a large trial will answer this question.

**Future directions**

The current antenatal management of women affected by FMAIT, relying on FBS, is invasive and carries significant risks to the foetus. These dangers, combined with the inability to predict the severity of clinical disease have restricted the development of safe and effective strategies to screen all pregnant women for FMAIT and to treat all affected cases. The aim of current research is, therefore, to develop methods for the non-invasive diagnosis of FMAIT, reliable prediction of disease severity in affected infants and non-invasive, effective treatment of FMAIT.

Work towards achieving these ambitious goals is helped considerably by the detailed understanding we now have of the molecular basis of the disease. In the majority of cases of FMAIT, a strong antibody response to the HPA-1a platelet antigen is only generated in HPA-1a negative women who carry the HLA DR3*0101 allotype. Furthermore, this association permits the identification of the fragments of the HPA-1a antigen that bind to the MHC Class II molecule to prime helper T cells.

This knowledge and other modern molecular methods will be crucial in several areas. First, non-invasive foetal genotyping in the first trimester of pregnancy, using the foetal DNA circulating in the maternal circulation, has been possible for the Rhesus D blood group antigen. Similar non-invasive foetal genotyping for platelet antigens would spare foetal blood sampling of pregnant women with circulating anti-HPA-1a antibodies and a partner who is heterozygous at this locus (HPA-1a/1b).

Prediction of the severity of disease in affected cases would be of considerable value by allowing reliable targeting of foetal platelet transfusion. Elucidation of the maternal foetal factors, both acquired and innate, that determine disease severity will require co-operative large scale longitudinal studies and the application of immunological and genetic tests.

Modulation of the disease process by molecular manipulation of the B or T cell and/or effector mechanisms may be possible. In principle, the currently available detailed knowledge of the molecular components of the immune response would permit attenuation of the maternal B cells making HPA-1a antibody or the maternal T cells that provide help to the B cells. Finally, it may be possible to make designer’ antibodies that inhibit the binding of harmful maternal HPA-1a antibodies to foetal platelets. While it is true that none of these mechanisms of molecular manipulation is yet established in clinical practice the definition of restricted mechanisms to generate a deleterious HPA-1a antibody response in FMAIT suggest that modern molecular treatments may succeed here before more immunologically heterogeneous diseases are tackled.

**References**

Platelets, the smallest cellular components of blood, are critically involved at each step of the haemostatic response, from the initial sealing of damaged endothelium to supporting coagulation reactions and finally, retraction of the fibrin clot which enhances fibrinolysis and wound healing. Consequently, if the platelet concentration is decreased and/or the platelet function is abnormal, the risk of haemorrhage is increased.

Ever since Duke's report in 1910 of the first use of platelet-containing fresh whole blood to treat three bleeding thrombocytopenic patients, platelet transfusions have gained value in medicine and nowadays they are essential for the management of patients with primary thrombocytopenia, or for support of those treated with intensive chemo/radiotherapeutic regimens associated with prolonged periods of bone marrow aplasia. Unfortunately, the supply of platelets for clinical use is hampered by an increasing demand and our limited capacity to preserve this product under 22°C. Platelet transfusions are therefore a major concern in modern transfusion medicine. This overview will briefly discuss the approaches that have been taken over the past few years to develop alternative products to the current 22°C liquid stored platelet concentrates (PCs).

Drawbacks of conventional 22°C liquid stored PCs

Conventional liquid PCs consisting of platelets with a distinct number of contaminating white cells, re-suspended in autologous plasma, are routinely prepared by fractionation of whole blood units by either the platelet-rich-plasma (PRP) or the buffy coat methods, or directly harvested from blood circulation by apheresis. Although each procedure has advantages and disadvantages, there is a tendency in Western countries to increasing use of single donor PCs obtained by apheresis. Currently, all types of liquid PCs are stored in highly permeable plastic bags at a controlled temperature of 22±2°C, and with constant mild agitation to facilitate gas exchange through the plastic container. However, these storage conditions are by no means optimal, and transfusion of conventional 22°C liquid stored PCs has several drawbacks.1

First, the transmission of an infectious agent is a major concern for the transfusion of any blood product, and only recently have approaches been developed to attempt inactivation of infectious pathogens - viruses, bacteria, and protozoa - in PCs. Particularly, septic reactions associated with transfusion of bacterial contaminated PCs are a recognised and still unsolved problem of relevant dimensions (approximately 1 in 1,500 transfusions), with potentially fatal consequences for recipients. This risk is higher for PCs than for other blood components stored under refrigeration, such as red cells, since storage at room temperature facilitates the propagation of a potentially present bacterial load. In fact, the increased risk of bacterial growth associated with the 22°C storage temperature is the primary reason for the current 5-day shelf-life of PCs.

In addition, alloimmunisation against histocompatibility antigens occurs in many patients receiving multiple transfusions of random donor platelets. Alloimmunised patients who, in recent experience, may reach as many as 25-35% of newly diagnosed subjects with acute myeloid leukaemia, become refractory and can be extremely difficult to treat with platelet transfusions. Histocompatible donors are often not available, and as many as 50% of apparently histocompatible platelet transfusions administered to alloimmunised patients do not achieve good post-transfusion recovery. Thus, the cost and difficulty of platelet transfusion therapy in these alloimmunised patients are high, and often they remain at risk of haemorrhagic morbidity and mortality. Fortunately, there is now substantial evidence suggesting that the leukocytes contaminating platelet preparations are the primary stimulus for alloimmunisation. Thus, the use of leukodepleted PCs is becoming a routine in transfusion medicine that could drastically reduce the incidence of alloimmunisation in the near future. Additional benefits of leukodepletion of PCs would be the lower incidence and severity of febrile non-haemolytic transfusion reactions mediated by bioreactive substances released by passenger leukocytes during storage, and the decreased risks of immunomodulatory effects and of graft-versus-host disease.
Finally, it is well established that during storage of PCs at 22°C serial deleterious changes in the platelet properties occur, collectively referred to as the platelet storage lesion. Thus we, and other groups, have demonstrated that storage promotes platelet shape change, surface expression of activation proteins, loss of GP Ib, and impaired response to agonists. Whether these in vitro observed changes are reversible upon transfusion or significantly affect the in vivo viability and function of platelets remains unclear, but the awareness of the platelet storage lesion is an additional reason for the current 5-day restriction of conventional 22°C liquid-stored PCs. It seems clear that the short shelf-life of PCs under current storage methods results in complex inventory management, loss of units due to outdating, difficulty in providing platelets far from the production place, and frequent platelet shortages.

For all the above reasons, much effort has been made in academic and commercial settings to improve the current platelet storage methods, and to develop novel platelet substitutes.

The challenge of designing and evaluating novel platelet products

As mentioned above, platelets have many complex functions (adhesion and spreading on injured vessel, support for coagulant activity, modulation of fibrinolysis, and others). Therefore, an important question in the quest for platelet substitutes is, what function(s) of platelets is needed to be mimicked by the platelet product or substitute? For example, products retaining the platelet procoagulant activity may be sufficient to reduce the risk of venous haemorrhage. By contrast, maintenance of haemostasis in the arterial circulation, where a high shear stress exists, might require most, if not all, of the platelet functions. Thus, we may speculate that in the future it would be possible to tailor and produce specific platelet products for the management of different clinical situations (dilutional thrombocytopenia, immune thrombocytopenic purpura, chemotherapy-induced thrombocytopenia, or a dysfunctional platelet disease).

In the short term, however, we can only hope that alternative platelet products have a few desirable properties. Some of these properties relate to practical aspects such as, need of no specialised storage conditions, prolonged shelf-life compared to that of current PCs, and minimal manipulation before transfusion. Others relate to safety and clinical effects on recipients, such as being sterile or suitable for sterilisation, haemostatically effective during long intervals, and having no major thrombogenic or immunogenic effects. Appropriate demonstration that proposed platelet products accomplish these requirements of safety and efficacy would be a difficult task, because it would need: 1) the performance of in vitro assays valuable for testing platelet function; 2) the carrying out of pre-clinical studies in animal models; and 3) clinical trials in volunteers and/or in patients. In addition, this task would be hampered by 1) the lack of consensus regarding the in vitro tests that best reflect the viability and function of platelets; 2) the controversy about what are valid species for pre-clinical studies regarding generalisation of the data obtained to humans, and about the correct way to induce the animals' thrombocytopenia; and 3) the lack of validated end points or surrogate measurements for demonstration of clinical benefit to the patient population included in trials.

Snapshot of advances in platelet products and substitutes

The research performed in this field over the past few years can be classified in five general areas.

1. Storage of liquid PCs under refrigeration

Early attempts to store platelets were performed, as for whole blood or red cells, at 4°C. However, refrigerated storage was soon abandoned due to the finding that cold temperatures significantly and irreversibly affect platelets. Advances in understanding the cold-induced platelet responses, a process termed cold activation, have shown similarities with physiological agonist-induced platelet activation (shape change, calcium mobilisation, actin filament assembly, aggregation, etc.). This knowledge, and awareness of the risk of bacteraemia associated with transfusion of room temperature-stored PCs, have renewed the interest for development of platelet cold storage.

Several physical methods (increased atmospheric pressures, temperature cycling), and biochemical strategies (cytoskeletal stabilisers, antifreeze glycoproteins, signal transduction inhibitors), for prevention of cold-induced platelet activation have been explored. The microtubule stabiliser agent taxol was used in early attempts to prevent platelet changes induced by chilling, but discoid shape and physiological responses were not fully preserved in treated platelets. More recently, platelets cooled to 4°C in the presence of the drug cytochalasin, an inhibitor of actin filament assembly, in combination with a cytoplasmic calcium chelator (Quin 2) have been shown to remain discoid and responsive to glass and thrombin activation. Whether this treatment preserves other platelet properties is not known.

Another interesting approach to the cold storage of platelets is the use of antifreeze glycoproteins isolated from polar fish. It has been shown that these proteins prevent, in a dose-dependent manner, the cold-induced platelet shape change and surface expression of activation proteins (LAM P, CD63), and preserve the thrombin responsiveness of cold-stored platelets. The underlying mechanism of these actions is unclear, but some evidence suggests that antifreeze glycoproteins could protect membrane phospholipids from a phase transition change during platelet chilling, thus preventing damage to the membrane.
We, and others, have recently evaluated the storage at 4ºC of PCs supplemented with a combination of signal transduction inhibitors.6,7 This cocktail, named ThromboSol, is made up of agents that enhance the platelet cAMP/cGMP concentrations (adenosine, sodium nitroprusside and dipyridamole), amiloride, ticlopidine, and quinacrine. In these studies, ThromboSol protected platelets from morphological changes and spontaneous aggregation during cold storage. Moreover, treated platelets stored-refrigerated for 9 days displayed responses to agonists (ADP, collagen, thrombin) comparable to those of platelets stored at 22ºC for 5 days. The protective effect of this additive solution was related to the ability of its components to sustain high levels of cAMP and to inhibit TxA2 production during the entire storage period at 4ºC. An additional benefit of cold storage of PCs treated with ThromboSol was impaired production of cytokines (IL6, IL8) compared to that found in PCs conventionally stored at 22ºC.

2. Cryopreservation of platelets

As for many cell types, freezing has been long considered an alternative for platelet preservation, with the major advantage of extending the storage period from a few days to years. Unfortunately, current methods for platelet cryopreservation require expertise, are labour-intensive, and involve the use of cryoprotectant agents, potentially harmful for recipients, and thus requiring wash-out before infusion. Thus, frozen storage of platelets is not routinely considered and is scarcely used.

Although it has been shown that cryopreserved platelets display several metabolic and functional changes, they are claimed to exert haemostatic properties when infused in vivo. In fact, several studies have proven the usefulness of frozen platelets in the prophylaxis of bleeding in different clinical settings (cardiopulmonary bypass, onco-haematological patients undergoing high-dose chemotherapy and/or haemopoietic progenitor cell transplantation).4,5

So far, the most widely used cryoprotectant agent for platelet freezing is dimethyl sulphoxide (DM SO) (at 5 to 10%). Other substances sporadically being employed are glycerol based solutions, hydroxyethyl starch, trehalose, or propane-1,2-diol. Since DM SO has well recognised adverse effects, a reduction of its concentration in platelet freezing or substitution by less toxic cryoprotectant regimens is desirable to allow direct infusion of frozen-thawed platelets. In this regard, PCs frozen with a solution containing 20% polyvinyl-pyrrolidone, 10%mannitol, 5%glycerol, and a mixture of salts, appear to function well when infused to thrombocytopenic rabbits without post-thaw washing of the cryoprotectants. Also, a reduced DM SO concentration (2%) combined with a modified ThromboSol mixture (amiloride, sodium nitroprusside and adenosine) is being tested as an alternative cryopreserving solution. Other authors and our group, have observed that platelets frozen with this solution display in vitro properties similar to those cells cryopreserved with the standard 6% concentration of DMSO. Moreover, platelets cryopreserved in this manner retain their haemostatic function in rabbits, and it has recently been shown that their recovery and survival when re-infused to autologous human volunteers is superior to that of 6%frozen platelets, and comparable to that of platelets stored at 22ºC for five days.8

3. Preservation of platelets in a freeze-dried state

Freeze-dried platelets are seen as a potentially durable platelet product, lightweight, and easier to transport and store than liquid or cryopreserved PCs. Early attempts in the 50’s to use lyophilised platelets in animal models and in humans were unsuccessful, and this technology was abandoned for many years. However, the strategy has emerged with great vigour thanks to the work of Read et al.9 These authors developed a lyophilisation procedure in which platelets are first stabilised with 1.8%paraformaldehyde for 1 hour, washed, re-suspended in 5%albumin, and then freeze-dried for 24 hours at –20 to –40ºC. When re-hydrated, the platelets in the lyophilised product appear to have normal morphology, surface expression of receptors, adhere appropriately to thrombogenic surfaces, and have procoagulant activity. Studies in vivo in rats, rabbits, and dogs have shown that lyophilised platelets correct the bleeding time. In addition, the platelet treatment with 1.8%paraformaldehyde is virucidal and bactericidaly effective, providing a 5-7 log reduction in infectious material (including HIV).4,5

Other recent works have also shown the presence of GP receptors Ib and Ila in lyophilised platelets, and have demonstrated that these freeze-dried cells retain their ability to interact with exposed subendothelium in a perfusion model. The safety and efficacy of these lyophilised platelet products in humans remain to be elucidated in clinical trials.

4. Use of platelet fragments or microparticles

Activation of platelets is known to induce shedding of platelet membranes or microvesicles with procoagulant properties that support the haemostatic function of intact platelets. This microparticle formation occurs during conventional storage of PCs, and increases with platelet chilling. Recently, human infusible microvesicles have been developed and studied.4,5 The manufacturing process involves repeated freezing/thawing of platelets, high speed centrifugation to isolate particulate material, wet heat viral inactivation, and lyophilisation. The resulting product has a phospholipid content similar to that of platelets, and retains varying quantities of platelet membrane receptors and procoagulant activity. It appears to have reduced class I HLA expression, and has a shelf life of three years. These platelet microparticles were first found to shorten the bleeding time with no evidence of toxicity in a rabbit model of busulfan-induced
thrombocytopenia. In phase I studies, human volunteers tolerated the treatment well with platelet membranes, which were not associated with adverse changes in biochemical or coagulation indices. In addition, no signs of immunogenicity (antibodies reactive to normal platelets or lymphocytotoxicty HLA antibodies) were observed in fifteen normal subjects injected twice with microparticles for up to two months after the first exposure. There is also a phase II clinical study of microparticles in thrombocytopenic patients with non-life threatening active mucosal bleeding. Sixty-five percent of patients infused with microparticles achieved improvement or cessation of bleeding. More importantly, 58% of patients refractory to normal platelets did respond to microparticle treatment. No patients experienced serious adverse events attributable to microparticle infusion. These are promising data, but routine bleeding prophylaxis with platelet microparticles awaits definitive confirmation of safety and efficacy in prospective randomised controlled trials.

5. Synthetic or semi-synthetic platelet substitutes

There have been several investigative approaches to preparing synthetic or semi-synthetic products that could sustain some of the properties of functional platelets. Agam and Livne explored the haemostatic properties of red cells coated with fibrinogen. They found that these coated erythrocytes enhance platelet aggregation induced by agonists, and shorten by 4-fold the tail bleeding times in thrombocytopenic rats. Dr. Barry Coller and co-workers have developed and evaluated thromboerythrocytes, which are red cells that are covalently coupled with RGD-containing peptides. This Arg-Gly-Asp (RGD) sequence is present in fibrinogen and other ligands that bind to the GP IIb/IIIa receptor. Thromboerythrocytes bind to platelets adhered to collagen under static or low shear conditions. They also co-aggregate with platelets stimulated with ADP. However, discrepant results have been found concerning the capacity of thromboerythrocytes to reduce prolonged bleeding time in animal models.

More artificial products are thrombospheres and liposome-based agents. The former are spheres of cross-linked human albumin with fibrinogen covalently bound to the surface. They are small (1.2 µm), thus circulate freely through small vessels with appropriate haemorrhagic properties. These microspheres do not spontaneously clump, but co-aggregate with platelets in the presence of a platelet agonist. When infused as a single bolus to thrombocytopenic rabbits, thrombospheres shorten ear bleeding time and reduce 51Cr-blood loss, while they do not seem to have apparent thrombogenicity. Their safety and efficacy in humans have not yet been investigated.

The haemostatic efficacy of a liposome-like product, namely Plateletsome, has been suggested. This product consists of a deoxycholate extract of platelet membranes, including major glycoprotein receptors, incorporated into unilamellar lipid vesicles. Plateletsomes have no in vitro effect on platelet aggregation, but decrease the tail bleeding time in thrombocytopenic rats by 67%. No evidence of intravascular coagulation is observed upon intravenous infusion of Plateletsomes to rabbits, and no pathologic thrombi were detected on post-mortem examination of treated rats. Procoagulant liposomes have also been explored. In one study, infusion of a combination of phosphatidylcholine: phosphatidylserine (80:20) (PC/PS) vesicles and factor Xa normalised the bleeding time in haemophilic dogs. However, this preparation was toxic in dogs and baboons, inducing a decrease in factor V and VIII as well as in the platelet count and haematorcrit. Recently, Galán et al. described liposomal preparations that under in vitro conditions of flow, fulfil the procoagulant function of platelets. By using the Baumgartner perfusion system, these authors demonstrated that addition of vesicles made of PC, phosphatidylinositol (PI), phosphatidylethanolamine (PE)/PC (1:1), and (PS/PC) (3:1) to platelet and white cell depleted blood, significantly increased fibrin formation on exposed subendothelium, and increased post-perfusion levels of F1+2, suggesting thrombin generation. These in vitro results further support the value of phospholipid preparations as potential platelet substitutes for treatment of thrombocytopenic patients. However, issues such as toxicity, optimal lipid composition, concentration, size, and stability of these procoagulant liposomes need to be determined.

Concluding remarks

The technologies to produce many kinds of platelet products are in place, and should be encouraged in order to avoid shortages of this blood component, and the problems associated with transfusion of conventional PCs. The major goal now is to develop appropriate ways to assess the safety and efficacy of these products before they are used in the clinical setting.

References

4. Alving BM, Reid TJ, Fratantoni JC, Finlayson JS. Frozen platelets and platelet substitutes in transfusion med-
icine. Transfusion 1997; 37: 866-76.
Non-transfusional haemostatic agents are indicated in the treatment of bleeding resulting from multiple or unknown defects of haemostasis. These drugs may also be indicated in patients who refuse blood transfusion or in those who undergo surgical procedures or suffer from mucosal lesions associated with large blood losses necessitating many transfusions of donor blood. Non-transfusional haemostatic drugs of proven clinical efficacy include antifibrinolytic amino acids (epsilon-aminocaproic acid and tranexamic acid), aprotinin, desmopressin and conjugated oestrogens.

Antifibrinolytic amino acids
Two synthetic derivatives of the amino acid lysine, epsilon-aminocaproic acid (EACA) and tranexamic acid, bind reversibly to plasminogen thereby blocking its binding to fibrin and its activation to plasmin. EACA and tranexamic acid (which is about 10 times more potent and has a longer half-life) are haemostatically effective even when bleeding is not associated with laboratory signs of hyperfibrinolysis. Since both drugs enter the extravascular space and accumulate in tissues, the basis for their efficacy is thought to be inhibition of tissue fibrinolysis and consequent clot stabilisation.

Primary menorrhagia
Tranexamic acid reduces menstrual bleeding by 40 to 50 percent. It is recommended only when organic lesions in the uterus have been excluded. Recommended oral doses are 10 to 15 mg per kilogram of body weight every 8 hours, from the onset until the arrest of menstrual bleeding.

Gastrointestinal bleeding
A meta-analysis, based on results from 1,267 patients with peptic ulcers, mucosal erosions or other causes of bleeding demonstrated that use of antifibrinolytic drugs is associated with a 20 to 30 percent reduction of re-bleeding, 30 to 40 percent reduction in the need for surgery, and 40 percent reduction in mortality. Despite these results tranexamic acid is not widely used to treat patients with upper digestive tract bleeding because of the efficacy of other medical and endoscopic treatments.

Urinary tract bleeding
After prostatectomy, the urine, which is rich in plasminogen activators, dissolves clots in the prostatic cavity, resulting in haematuria and sometimes anaemia. In clinical trials EACA or tranexamic acid reduced blood loss by approximately 50 percent, as compared with placebo. The drugs are contraindicated in patients with upper urinary tract bleeding because of the risk of retention of clots in the ureter and the bladder. Recommended dosage is: tranexamic acid, 10 to 15 mg per kilogram every 8 hours intravenously, starting immediately after surgery, followed by 20 mg per kilogram orally every 8 hours daily until macroscopic haematuria stops; EACA, 50 to 60 mg per kilogram intravenously 6 times daily followed by oral administration of the same dose.

Oral bleeding in congenital and acquired coagulation disorders
Antifibrinolytic drugs are useful adjuvants for the control of bleeding after dental extractions in patients with haemophilia, reducing the amount of clotting-factor replacement therapy needed. Mouthwashes containing tranexamic acid (1 g every 6 hours) are effective for prevention of oral bleeding in haemophiliacs and in patients who need dental extractions while receiving long-term oral anticoagulant therapy. Extractions can be performed without stopping or reducing the anticoagulant, which may increase risk of thrombosis in patients with atrial fibrillation or artificial heart valves. Recommended oral doses: in adults are of 50 to 60 mg EACA per kilogram every 4 hours or 20 to 25 mg tranexamic acid per kilogram every 8 hours until healing of the sockets is complete.

Bleeding in patients with thrombocytopenia
EACA may be efficacious to stop mucosal (nasal, uterine, gastrointestinal) bleeding and bleeding associated with dental extractions in patients with thrombocytopenia.

Bleeding after thrombolytic treatment
Antifibrinolytic drugs are of potential efficacy in controlling bleeding complicating thrombolytic therapy. There is, however, little evidence that they are useful when bleeding complications develop during or shortly after thrombolytic treatment.

Surgical blood loss
Cardiac surgery is the prototype operation warranting adoption of blood saving measures. The
results of clinical trials involving at least 1,000 patients treated with tranexamic acid or EACA consistently demonstrated that either drug reduced blood loss by 30 to 40 percent, as compared with placebo. However, the amount of blood products given was either not measured or not reduced by antifibrinolytic drugs, and none of the studies was of sufficient size to determine whether the incidence of serious side-effects was increased by the treatment. A recent meta-analysis, however, showed that EACA is as effective as aprotinin in reducing blood loss and transfusion requirements and had no effects on risks of post-operative myocardial infarction or overall mortality.

Recommended dosage: a bolus intravenous dose of 150 mg EACA per kilogram given before the operation, followed by an infusion of 15 mg per kilogram per hour during the operation; 10 mg tranexamic acid per kilogram intravenously before the operation, followed by 1 mg per kilogram per hour during the operation.

Although there are demonstrations that antifibrinolytic drugs reduce blood loss and transfusion requirements in patients undergoing knee replacement surgery, their use should be considered only for patients in whom large blood losses are predicted, such as those undergoing double joint replacements and re-operations.

Patients undergoing orthotopic liver transplantation lose large amounts of blood, due in part to pre-existing coagulopathy and intraoperative fibrinolysis. In a clinical trial of 45 patients given high-dose tranexamic acid (20 to 30 mg per kilogram) or placebo during surgery, treated patients had about 50 percent less intraoperative blood loss and lesser transfusion requirements. These preliminary results need confirmation.

**Side effects and contraindications**

The drugs are contraindicated in patients with subarachnoid bleeding, because they induce vasospasm and ischaemic stroke. The side effects are dose-dependent and usually involve the gastrointestinal tract (nausea, vomiting, abdominal pain and diarrhoea). The main risk of these drugs is thrombotic complications, through inhibition of fibrinolysis, a naturally-occurring defence mechanism against thrombus formation. There are at least ten case reports of formation of thrombi in abnormal amounts or at abnormal locations associated with the use of these drugs. On the other hand, no strikingly increased risk of thrombosis has emerged when the drugs were used during operations often complicated by venous and arterial thromboembolism, such as cardiac surgery and knee replacement. However, these studies were not designed to evaluate thrombotic complications and usually were too small to detect differences in relatively low-incidence outcomes such as stroke, myocardial infarction or coronary bypass graft occlusion.

**Aprotinin**

Aprotinin, a polypeptide with a molecular weight of 6,512 daltons, inhibits several serine proteases (trypsin, chymotrypsin, plasmin and tissue and plasma kallikrein) through the formation of reversible enzyme-inhibitor complexes. By inhibiting kallikrein, aprotinin indirectly inhibits the formation of activated factor XII. Hence, aprotinin inhibits the initiation of fibrinolysis induced by the contact of blood with a foreign surface. The enzymatic activity of the compound is expressed in kallikrein inactivator units (KIU). Plasma concentrations of 125 KIU per milliliter are necessary to inhibit plasmin and concentrations of 300 to 500 KIU per milliliter are needed to inhibit kallikrein.

**Surgical blood loss**

The broad antiproteolytic action of aprotinin prompted its use to reduce blood loss in patients undergoing cardiac surgery, during which there is increased plasma proteolysis. Several double-blind studies demonstrated that aprotinin is effective in reducing blood loss and transfusion requirements in patients at high bleeding risk, such as those undergoing repeat surgery or cardiac transplantation, taking acetylsalicylic acid, or suffering from endocarditis, and also in those undergoing more common operations at lower bleeding risk, such as valve replacement and coronary artery bypass grafting.

Aprotinin is effective only when infused intravenously. The first clinical studies of aprotinin in cardiac surgery used a loading dose of 2 million KIU followed by a continuous infusion of 500,000 KIU per hour during surgery, with 2 million KIU added to the priming solution. Lower-dose regimens have been subsequently proposed, which, with few exceptions, are as effective haemostatically as the full-dose aprotinin regimen. Aprotinin is less effective when given post-operatively than when given prophylactically.

Results of non-randomised, uncontrolled trials suggest that aprotinin may be effective in reducing blood loss and transfusion requirements in patients undergoing orthotopic liver transplantation. However, a small randomised study of 20 patients given aprotinin showed that the drug was not effective as compared with placebo.

**Side effects**

Aprotinin is an heterologous polypeptide, and therefore it could cause hypersensitivity reactions, particularly after repeated exposure. Theoretically, aprotinin could cause venous and arterial thrombosis and therefore occlusion of coronary bypass and other vascular grafts. However, in controlled studies, aprotinin did not lead to an increased rate of mortality, acute myocardial infarction or early occlusion of saphenous vein or internal mammary artery grafts after coronary bypass grafting, or to an increased risk of venous thromboembolism after hip replacement.
Desmopressin

Plasma concentrations of factor VIII, the clotting factor deficient or defective in haemophilia A, and von Willebrand factor, the adhesive protein deficient or defective in von Willebrand disease, can be increased for a short time by 1-deamino-8-D-arginine vasopressin (desmopressin), an analogue of arginine vasopressin. These effects, mimicking replacement therapy with blood products, are the rationale for the use of desmopressin in the treatment of patients with these congenital bleeding disorders. Subsequently, desmopressin has also been used in patients with other congenital and acquired bleeding disorders. In these, the effect of desmopressin is mediated by mechanisms that are both dependent and independent of the attainment of high plasma concentrations of von Willebrand factor with ultralarge multimers, which support platelet aggregation and adhesion to the vascular subendothelium more than multimers of normal size. Other mediators of increased haemostasis might be high plasma concentrations of factor VIII, a rate accelerating factor in the process of fibrin formation.

Congenital bleeding disorders

Desmopressin is the treatment of choice for patients with mild haemophilia A or type 1 von Willebrand disease who bleed spontaneously or who are to undergo any operation. Plasma concentrations of factor VIII and von Willebrand factor increase approximately 2 to 4 times, with a peak 30 to 60 minutes after intravenous infusion and 60 to 90 minutes after subcutaneous and intranasal administration. These doses can be repeated as necessary at intervals of 12 to 24 hours, but tachyphylaxis may occur after 3 to 4 doses.

Effects on the bleeding time. Desmopressin shortens the prolonged bleeding time in most patients with type 1 von Willebrand disease and also in some patients with congenital defects of platelet function. However, the bleeding time in patients with type 3 or type 2 von Willebrand disease is usually not shortened. The optimal intravenous or subcutaneous doses of desmopressin are 0.3 µg per kg and the optimal intranasal dose is 300 µg in adults and 150 µg in children.

Acquired bleeding disorders

Desmopressin has also been used in patients with uraemia, who have complex abnormalities of haemostasis reflected in part by a prolonged bleeding time. In a group of these patients given an intravenous infusion of desmopressin, the prolonged bleeding time became normal for 4 to 6 hours in about 75 percent. Desmopressin given before invasive procedures (biopsies and major surgery) seems to prevent bleeding, but controlled studies are lacking.

In spite of the fact that patients with cirrhosis have high plasma concentrations of factor VIII and von Willebrand factor, they have a prolonged bleeding time that is shortened by intravenous desmopressin. Hence the drug is a possible prophylactic treatment for patients who need invasive diagnostic procedures and have a prolonged bleeding time.

Surgical blood loss

In a single study of 70 patients undergoing complex cardiac surgery, desmopressin given at the time of chest closure reduced blood loss and transfusion requirements by about 30 percent. However, subsequent studies carried out in patients undergoing simpler cardiac operations, showed that there were no significant differences between desmopressin and placebo. In a meta-analysis of 17 clinical trials which included 1,171 patients desmopressin significantly reduced post-operative blood loss by 9 percent, a value of little clinical importance. However, a sub-analysis of the data revealed that desmopressin reduced blood loss by more than 30 percent under conditions causing excessive blood loss.

Side effects

Frequent side effects include mild facial flushing and headache. Because of its potent anti-diuretic effect desmopressin can cause water retention and hyponatraemia. In patients given more than one dose, plasma sodium and body weight should be measured daily and excessive administration of fluids avoided. Arterial thrombosis (sometimes fatal stroke or myocardial infarction) has occurred in a few patients after treatment. In patients at high risk for thrombosis (such as those undergoing coronary artery bypass grafting) there was no excess in thrombotic complications in those given desmopressin.

Prophylactic value of haemostatic drugs in cardiac surgery

EACA, tranexamic acid, desmopressin and aprotinin have been evaluated in patients undergoing cardiac surgery. Based on direct comparison studies and a meta-analysis all four reduce operative blood loss. The order of efficacy in terms of reduction in blood loss (greatest to least) is aprotinin, tranexamic acid, EACA and desmopressin; the order of cost at the most commonly recommended doses is the same. In terms of reduction in blood transfusion requirements, the most important efficacy criterion, the results favour aprotinin. In terms of safety, firm data from clinical trials demonstrating no increased frequency of graft occlusion are available for aprotinin only.

The cumulative evidence leads to the choice of aprotinin, but it should be reserved for those patients who are likely to need transfusion of donor blood. They are those undergoing re-operation, those with pre-existing haemostatic defects or taking antiplatelet drugs and those with sepsis.

Conjugated oestrogens

Conjugated oestrogens shorten the prolonged bleeding times and improve or stop bleeding in patients with uraemia. The mechanism whereby con-
Conjugated oestrogens affect the bleeding time in these patients is unknown. Recommended dosage in patients with uraemia is a single i.v. daily infusion of 0.6 mg per kilogram repeated daily for 4 to 5 days which shortens the bleeding time by approximately 50 percent for at least two weeks. A daily oral dose of 50 mg shortened the bleeding time after an average of 7 days of treatment.

The clinical value of conjugated oestrogens in patients with uraemia rests on data indicating that the bleeding tendency of these patients is directly related to degree of prolongation of the bleeding time. The chief advantage of conjugated oestrogens over desmopressin is the longer duration of the effect on the bleeding time (10 to 15 days vs 6 to 8 hours). Hence, conjugated estrogens should be used when long-lasting haemostatic competence is required, such as during elective surgical procedures or recurrent episodes of gastrointestinal or nose bleeding. On the other hand, desmopressin should be given when an immediate effect on haemostasis is required (for instance, to stop acute bleeding or to prevent bleeding at the time of emergency surgery). The two products can be given concurrently, exploiting the different timing of their maximal effects.

In patients with chronic renal insufficiency, recombinant erythropoietin causes a dose-dependent rise in the haematocrit and eliminates the need for blood transfusions. The progressive increase in the haematocrit is paralleled by a pronounced shortening of the bleeding time and improvement of platelet adhesion. Since most patients with chronic renal insufficiency are now regularly treated with erythropoietin, there is less need for short-acting haemostatic drugs such as desmopressin and conjugated oestrogens.

Conjugated oestrogens are well tolerated and side effects are negligible or absent.

Conclusions

The antifibrinolytic drugs EACA and tranexamic acid are useful in patients with a broad range of haemorrhagic conditions, particularly when there is excessive bleeding from mucosal sites. Desmopressin is the treatment of choice for patients with mild haemophilia and type 1 von Willebrand disease. It has also been used successfully to treat or prevent bleeding in patients with other haemorrhagic disorders, including congenital defects of platelet function, chronic liver disease and haemostatic defects induced by the therapeutic use of antithrombotic drugs such as aspirin and ticlopidine, but there is still no well conducted clinical trial that demonstrates efficacy. In cardiac surgery, antifibrinolytic drugs (lysine derivatives and aprotinin) are more effective than desmopressin, aprotinin being preferable because its efficacy and safety have been more extensively evaluated.

References