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CD34-positive cells: biology and clinical relevance

The CD34-positive cell: definition and morphology

Cellular expression of the CD34 antigen identifies a morphologically and immunologically heterogeneous cell population that is functionally characterized by the in vitro capability to generate clonal aggregates derived from early and late progenitors and the in vivo capacity to reconstitute the myelo-lymphopoietic system in a supralethally irradiated host.1–3

Immunohistochemical studies have demonstrated that the CD34 antigen is stage but not lineage specific. In fact, independently of the differentiative lineage, it is expressed only by ontogenetically early cells.4 For years a major obstacle to the morphological identification of putative hematopoietic stem cell has been the difficulty in separating them from their direct progeny. The use of CD34 and other suitable cell surface markers (i.e. CD33, CD38, HLA-DR antigens) in fluorescence-activated cell-sorting techniques or other cell separation methods has allowed considerable progress in this field.

Positively selected, lineage committed CD34+ cells and more immature, lineage negative CD34− CD33− HLA-DR− cells are shown in Figure 1 and Figure 2, respectively. On May–Grünewald–Giems stained preparations, CD34+ cells are medium sized cells having large nuclei, eccentrically surrounded by narrow rim of deep blue cytoplasm occasionally containing cytoplasmic granules. Some normal CD34+ cell nuclei show one or more pale blue nucleoli. Taken together, these findings reflect the heterogeneous proliferative status and protein synthesis of this cell population. Conversely, earlier hematopoietic progenitors, identified as CD34− CD33− HLA-DR−, seem to be more homogeneous in size (small lymphocyte-like cells) and lack cytoplasmic granules and prominent nucleoli. Again, the morphology of this cellular population appears to reflect the functional characteristics of these cells (e.g. low protein synthesis, very low proliferative activity with predominantly G0 phase).

Several monoclonal antibodies (MY10, 12.8, B1–3C5, 115.2, ICH3, TUK3, etc.) raised against the leukemic cell lines KG1 or KG1a and an anti-endothelial cell antibody (QBEND10) assigned to the CD34 cluster have been shown to identify a transmembrane glycoproteic antigen of 105–120 kD expressed on 1–3% of normal bone marrow cells, 0.01–0.1% peripheral blood cells and 0.1–0.4% cord blood cells.5 Different antibodies recognize distinct epitopes of the same antigen. CD34 antigen expression is associated with concomitant expression of several other markers that can be classified as lineage non-specific markers (Thy1, CD38, HLA-DR, CD45RA, CD71) and lineage specific markers, including T-lymphoid (TdT, CD10, CD7, CD2, B-lymphoid (TdT, CD10, CD19), myeloid (CD33, CD13) and megakaryocytic (CD61, CD41, CD42b) markers.5 The expression of lineage non-specific markers allows the heterogeneous CD34+ population to be divided into two distinct subpopulations characterized, respectively, by low or high expression of Thy1, CD38, HLA-DR, CD45RA, CD71. These two cell subpopulations contain early and late hematopoietic progenitor cells, respectively.6–8

In addition to conventional immunological markers classified on the basis of their assignation to specific clusters of differentiation, CD34 cells express receptors for a number of growth factors. Two distinct families of
related receptors have been identified: (i) tyrosine kinase receptors, including the stem cell factor receptor (SCF-R, CD117) and the macrophage colony-stimulating factor receptor (M-CSF-R, CD115); (ii) hematopoietic receptors not containing a tyrosine kinase domain, such as the granulocyte-macrophage colony-stimulating factor receptor (GM-CSF-R, CDw116).5,9

The identification of new markers selectively expressed on primitive lymphohematopoietic cells (CD34+ CD38–) represents a stimulating research field. In this context, stem cell tyrosine kinase receptors (STK), such as STK-1, a human homologue of the murine Flk-2/Flt-3, are of particular relevance.10-12 The ligands for these receptors might represent new factors able to selectively control stem cell self-renewal, proliferation and differentiation.13-15

Clonogenic and biologic activity

The structural and functional integrity of the hematopoietic system is maintained by a relatively small population of stem cells, located mainly in the bone marrow, that can (i) undergo self-renewal to produce more stem cells or (ii) differentiate to produce progeny which are progressively unable to self-renew, irreversibly committed to one or another of the various hematopoietic lineages, and able to generate clones of up to 10^5 lineage-restricted cells that mature into specialized cells.16-18

The decision of a stem cell to either self-renew or differentiate and the selection of a specific differentiation lineage by a multipotent progenitor during commitment are intrinsic properties of stem cell progenitor cells and are regulated by stochastic mechanisms.19 Survival and amplification of hematopoietic progenitors are controlled by a number of regulatory molecules (hematopoietic growth factors) interacting according to complex modalities (synergism, recruitment, antagonism).19 A further level of hematopoietic control is exerted by nuclear transcription factors that activate lineage-specific genes regulating growth factor responsiveness and/or the proliferative capacity of hematopoietic cells.20

Detection of the most primitive hematopoietic cell types is now possible due to the technique of long-term bone marrow culture. In the case of human bone marrow, a 5- to 8-week time period between initiating cultures and assessing clonogenic progenitor numbers allows quantification of a very primitive cell in the starting population, the so-called long-term culture-initiating cell (LTC-IC).21 Committed progenitors of the various hematopoietic cell classes can be quantitated by a number of short-term culture clonogenic assays.19 CD34 antigen expression associated with low CD38 and CD45RA expression and variable HLA-DR expression is a typical feature of LTC-IC, CFU-Bl, CFU-T, CFU-B. In contrast, CD34 antigen expression associated with CD38 and HLA-DR expression is a typical feature of multipotent (CFU-GEMM) and lineage-restricted (CFU-GM, BFU-E, BFU-Meg, CFU-Meg) hematopoietic progenitor cells8 (Figure 3). Recently reported data have shown that low or absent expression of the Thy1 or SCF receptor can be efficiently used to enrich primitive hematopoietic progenitors from the heterogeneous CD34+ cell population.7,9 Although the CD34 antigen is virtually expressed by all progenitor cells, the percentage of CD34+ cells with assayable in vitro clonogenic activity ranges from 10 to 30%. The problem of non-clonogenic CD34+ cells is still open and not adequately explained by the presence of lymphoid progenitors which are not assayable with current in vitro systems. Non-proliferating CD34+ subset might represent a subpopulation that is not responsive to conventional myeloid hematopoietic growth factors. The non-proliferating CD34+ subset might require the presence of co-factors, such as the ligand of STK-1 or the hepatocyte growth factor, able to activate stem cell-specific genes whose expression is a prerequisite for acquiring responsiveness to conventional growth factors.14,22,23

In lethally irradiated non-human primates, both autologous and allogeneic CD34+ cells have been shown to have the capacity to reconstitute the myelo-lymphopoietic system, thus suggesting that the stem cell responsible for hematopoietic reconstitution is CD34+.24,25 It
has also been shown that human CD34+ HLA-DR- cells transplanted in utero in the fetus of sheep initiate and sustain a chimeric hematopoiesis producing human progenitor cells of all differentiative lineages. Autologous CD34+ cells enriched by avidin-biotin columns have been shown to be able to reconstitute myelo-lymphopoiesis in patients receiving high-dose chemoradiotherapy. The results of studies using CD34+ bone marrow cells in the allogeneic setting in patients receiving both related as well as unrelated allogeneic marrow transplants will soon be available. In addition, trials are planned that will use allogeneic peripheral blood CD34+ cells either alone or with marrow.

**Characterization and function of the CD34 cell surface molecule**

The CD34 cell surface molecule has been biochemically characterized and both the human cDNA and gene have been cloned and sequenced in the last few years. CD34 is a one-pass type I transmembrane glycoprotein with a molecular weight of 105-120 kD in either the reduced or unreduced form (Figure 4). CD34 protein is not homologous to any other known protein. The minimum size of the CD34 protein is 354-amino acids and contains nine sites for N-glycosylation and a several for O-glycosylation that are essential constituents of the three epitopes of the molecule; this molecule is also rich in sialic acid. Its biochemical composition suggests a mucin-like structure and in some respects resembles leucosialin (CD43). Sequence comparisons between human and mouse CD34 show a very low level of identity in the glycosylated region, 70% identity in the globular domain, and 92% in the transmembrane and cytoplasmic regions.

Using a KG1 cell line library, it has been shown that the human CD34 gene is located on chromosome 1, and recent studies with in situ hybridization have assigned its localization to band 1q32, in close proximity to other genes that encode growth factors or function molecules such as CD1, CD45, TGF2, laminin, LAM/GMP, etc.

Seven CD34 monoclonal antibodies (MoAbs) were clustered at the 4th Workshop on Leukocyte Differentiation Antigens (Vienna, 1988) and another 15 MoAbs were verified as recognizing the CD34 molecule during the 5th International Workshop on Leukocyte Differentiation Antigens (Boston, 1983), the most direct evidence being reactivity with cells transfected with CD34 cDNA and binding to CD34 protein. The epitope specificity of the CD34 antibodies was classified into three distinct groups according to the sensitivity of the epitopes to enzymatic cleavage (which was performed using neuraminidase, chymopapain and glycoprotease from *Pasteurella haemolytica*), reactivity with fibroblasts and high endothelial venules, and cross blocking experiments (Table 1). We know, in fact, that glycoprotease from *Pasteurella haemolytica* specifically cleaves only proteins containing sialylated O-linked glycans. Based on these data, it can be further postulated that class III epitopes are more proximal to the extracellular side of the cell membrane than class I and class III epitopes.

Furthermore, for most CD34 MoAbs (with few exceptions) cross blocking experiments are in agreement with the classification based on enzymatic cleavage of the CD34 protein. In other words, using a cocktail of CD34 MoAbs, CD34 reactivity is blocked only in the case that MoAbs belonging to the same CD34 epitope are simultaneously employed. On the contrary, the combined use of MoAbs recognizing class II and III or class I and II or class III epitopes does not affect cell reactivity. Moreover, CD34 MoAbs defining class III epitopes are unable to react with CD34 glycoprotein in Western blots because this epitope is sensitive to denaturation.

The pattern of expression of CD34 antibodies exhibited by CD34+ acute leukemias is partially in accordance with that derived from epitope mapping based on the differential sensitivity of CD34 to enzymatic treatment. However, about one third of CD34 MoAbs do not seem to belong to any of these subgroups and for this peculiar pattern of expression are referred to as atypical CD34 reagents. The widest variation in CD34 MoAb reactivity has been demonstrated in acute myeloid leukemia (AML) samples, allowing us to postulate the occurrence of aberrant antigens or of distinct epitopes in subgroups of leukemias. Alternatively, it can be hypothesized that the expression of different antibod-
ies could reflect the degree of maturation of leukemic cells. The presence of new, distinct non overlapping epitopes could be proposed on the basis of the data published so far in the literature. As far as the expression of CD34 in normal and leukemic cells is concerned, it has been calculated by flow cytometry that the number of molecular equivalents of soluble fluorochrome (MESF) expressed by leukemic and normal progenitors ranges from 18,200 to 322,000 and from 8,000 to 124,000, respectively.

Recent data collected by Lanza et al.\textsuperscript{36} seem to indicate that class I-type MoAbs are more sensitive to freezing procedures than class II and III MoAbs, since the epitope is not identifiable following a frozen/thawed methodology.

The function of CD34 surface glycoprotein in hematopoietic stem and progenitor cells is still the object of debate. In light of recent findings, it would seem to play a relevant role in modulating cell adhesion.\textsuperscript{36} Furthermore, it has been demonstrated that CD34 probably acts as an adhesive ligand for L-selectin. It has been further postulated that the CD34 molecule could play a protective role against proteolytic enzyme-mediated damage due to its high number of O-glycosylation sites. The cytoplasmic domain contains two sites for protein kinase C phosphorylation and one for tyrosine phosphorylation.\textsuperscript{28}

The type of CD34 MoAb used to enumerate progenitor cells is probably relevant in the peripheral blood stem cell autograft setting as well, since both early and late engraftment following transplantation are, to some extent, related to the number of hemopoietic stem cells collected at the time of blood or bone marrow harvests, and to the degree of progenitor cell maturation related to the expression of lineage markers such as HLA-DR, CD71, CD38, CD33, and myeloperoxidase.

**Techniques for CD34\textsuperscript{+} cell separation**

A number of different techniques have been proposed for separating CD34\textsuperscript{+} cells. The common aim is to produce a cell population with optimal purity and viability by means of a low cost, rapid and simple separation technique. The first separation techniques exploited parameters such as size and cell density and were represented by Ficoll–Hypaque and Percoll density gradi-

<table>
<thead>
<tr>
<th>Epitope class</th>
<th>Clones</th>
<th>CD34 reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Sensitive to neuraminidase (from Vibrio cholerae), chymopapain, and glycoprotease (from Pasteurella haemolytica)</td>
<td>14D3, B. B05, My10, 12.8, ICH3, Immu-133, Immu-409</td>
<td>positive, negative, positive</td>
</tr>
<tr>
<td>II. Resistant to neuraminidase, glycoproteinase, and chymopapain sensitive</td>
<td>434.1, M/034.3, M/034.1, M/034.2, OBend10, 441, 5044, 5049</td>
<td>positive, positive, positive</td>
</tr>
<tr>
<td>III. Resistant to neuraminidase, chymopapain, and glycoproteinase</td>
<td>CD34 9F2, HPCA2, 581, 553.963, 903, 1152</td>
<td>negative, positive, negative</td>
</tr>
</tbody>
</table>

\*Incomplete digestion by neuraminidase.

![Figure 4. Schematic representation of the CD34 cell surface molecule.](image)
ents. In the last two decades, the development of monoclonal antibodies has allowed a more specific and careful cell selection by identifying surface antigens used as targets for cell separation (Table 2).

**FACS (Fluorescence Activated Cell Sorter)**

Flow cytometry is able to physically separate different populations after incubation of cells with fluorochrome-conjugated monoclonal antibodies. This cell sorting technique can yield a highly purified (> 98%) cell population. In addition, the use of electronic gates allows selection and recovery of several subpopulations according to antigenic expression and different characteristics such as size and cytoplasmic granularity. This technique has been very useful for studying CD34+ subpopulations but cannot be employed to select large numbers of cells due to its complexity and low recovery. The recent development of high-speed cell sorting, however, might allow clinical utilization of this technique.

**Panning**

Anti-CD34 monoclonal antibodies bound to one of the surfaces of cell culture flasks were recently used to select CD34+ cells. When a cell suspension is introduced into the flask, the positive population is blocked on the plastic surface, while CD34 negative cells remain in suspension and can be easily eliminated. Adherent cells should contain the CD34+ population with a viability > 90%.

**Immunomagnetic systems**

Immunomagnetic beads are uniform, super-paramagnetic, polystyrene beads with affinity purified anti-mouse Ig covalently bound to the surface. They are equally suited for negative and positive cell separation; the rosetted target cell can easily be isolated by applying a magnet on the outer wall of the test tube for 1-2 minutes. Immunomagnetic beads coupled with CD34 monoclonal antibodies can be utilized for positive selection of CD34+ cells to obtain a population with > 80% viable CD34+ cells. Similarly, immunomagnetic beads can be employed for negative depletion with monoclonal antibodies binding lineage-specific antigens.

**High-affinity chromatography based on biotin-avidin interaction**

This method is based on an immunoadsorption technique that relies on the high affinity interaction between the protein avidin and the vitamin biotin. Avidin-biotin immunoadsorption has been employed for both positive selection and depletion of specific cell populations. The instrument includes a set of non-reusable products including biotinylated anti CD34 antibody, plastic bags, filters and a column of avidin-biotin beads. An automated version controlled by a computer which guarantees reproducibility of operation and reduces risks of operator errors has been developed for clinical use. Its capacity has recently been increased so that a single column can process more than 50×10^6 bone marrow or peripheral blood cells in 1-2 hours and sustain bone marrow engraftment in patients submitted to autograft.

**CD34-positive subpopulations: phenotypic and functional analysis**

The normal CD34+ cell population likely contains progenitors of all human lympho-hematopoietic lineages, including stem cells capable of hematopoietic reconstitution after bone marrow transplantation. Levels of CD34 expression decline with differentiation; consequently, the earliest clonogenic cells (CFU-blast, LTC-IC, etc.) express the highest levels of CD34, while the most differentiated (CFU-G, CFU-M, CFU-E, CFU-Meg) express only low levels of CD34 (Figure 5). The CD34 antigen has been used to identify, enumerate and isolate cells from different lympho-hematopoietic lineages, as well as develop in vitro tests for indirect evaluation of cells with different functional and clonogenic capacity.

**Pre-clinical studies**

Several animal-human systems have been created to utilize chimeras for the study of lympho-hematopoiesis in vivo. The first experiments demonstrated the feasibility of transplanting human fetal stem cells to sheep fetuses; the postnatal presence of human cells in the sheep was documented at several points in time. Furthermore, some early CD34+ subpopulations were able to repopulate sheep bone marrow; animals were transplanted in utero with CD34+DR- cells and the presence of a chimeric population with the functional characteristics of hematopoietic progenitors was demonstrated in the marrow and peripheral blood in a percentage of cases. Berenson et al. also showed how hematopoietic progenitors (positive for the la antigen and subsequently for the CD34 antigen) could reconstitute the marrow of lethally irradiated dogs. Of the seven animals treated, all showed complete marrow engraftment after reinfection of la-positive cells; only one dog died from infection. Similarly, marrow cells from 5 primates (baboons) were treated in vitro with a biotinylated anti-CD34 antibody and then passed through a column of avidin; after autograft, all the animals showed marrow engraftment followed by hematologic reconstitution comparable to that of control animals. Furthermore, the demonstration that allogeneic CD34+ cells can reconstitute the hematopoietic system in lethally irradiated baboons confirmed that this cell population includes pluripotent stem cells.

**Lymphoid precursor cells**

The CD34+ cell compartment contains all the cells expressing terminal deoxynucleotidyltransferase (TdT), which is an intranuclear enzyme expressed in early lymphoid cells undergoing immunoglobulin or T-cell receptor gene rearrangement. Flow cytometry has shown that the great majority of TdT+ cells in the marrow coexpress CD34, CD19 and CD10 (B-cell precursors), as well as T-cell differentiation antigens such as CD7, CD5 and CD2.
A small proportion of CD34+/TD T cells coexpress CD10, which might represent a common lymphoid progenitor for both B and T lineages. Recently, Miller et al. reported that CD34+ cells may also generate NK cells in vitro.

**Granulocyte-macrophage precursors**

Marrow erythroid progenitors lack specific markers and therefore are difficult to identify. Glycophorin A-directed monoclonal antibodies recognize all hemoglobinized cells, but this molecule is expressed in only a small proportion of CD34+ cells and is absent in clonogenic cells.

High levels of CD45 are present on BFU-E, but this antigen is lost by the CFU-E stage; however, the CD45R0 isoform is well represented on earlier erythroid progenitors, while the CD45RA isoform is present on committed myeloid progenitors. The expression of CD71 (transferrin receptor) is currently considered to be the specific antigen for the CD34+ erythroid population. CD71 is present at high levels on erythroid progenitor cells and at very low levels in all the other progenitor cells. Expression of CD71 increases from stem cells to BFU-E, then declines during erythroid maturation. In addition, marrow CD34+ erythroid cells might express IL-3, GM-CSF (CD116) and erythropoietin receptors, based on the action of these growth factors on CFU-erythroid cells.

Myeloid precursor clonogenic cells (CFU-GM, CFU-G, CFU-M, CFU-MK) coexpress CD34, HLA-DR, CD117 (c-kit), CD45RA, CD33 and CD13; CD15 is present at low levels on CFU-G, while CFU-M specifically express CD115 (M-CSF receptor). CFU-MK are the only CD34+ cells which express the platelet glycoproteins identified by the CD61, CD42 and CD41 monoclonal antibodies. Dendritic cells also originate from bone marrow, but the conditions that direct their growth and differentiation are still poorly characterized. GM-CSF stimulates the growth of dendritic cells from mouse peripheral blood; however, it was recently reported that CD34+ cells may give rise to dendritic/Langerhans cells after stimulation with GM-CSF and tumor necrosis factor-α.

**Multilineage progenitors and stem cells**

CFU-GEMM contain precursor clonogenic cells of both myeloid and erythroid lineages and express CD34, HLA-DR, CD38, CD117 and CD45RA. They also express low amounts of CD33, but not CD13. In a hypothetical differentiation scheme involving pluripotent stem cells, the lympho-hemopoietic compartment is the next cell type and can be identified in vivo with CFU-Blast and LTC-IC.

The lack of expression of CD38 is the most important characteristic of these early progenitors, which represent 1% of CD34 and less than 0.01% of mononuclear cells. The lack of CD38 allows separation of committed progenitors (CD34+/CD38-) from earlier compartments (CD34+/CD38+) by a single marker combination.

Furthermore, the earliest CD34+ cells coexpress low levels of CD45RO and are negative for staining with the fluorescent dye rhodamine 123. The role of HLA-DR in defining earlier cell types is still controversial; a series of evidence indicates that the stem cell compartment has the CD34+/CD38+/HLA-DR+ phenotype. Recent works, however, have not found HLA-DR in the earliest cells. These data were confirmed by the possibility that HLA-DR expression may discriminate the Ph-positive leukemic compartment (HLA-DR+) from normal residual cells (HLA-DR-+) in chronic myeloid leukemia.

Table 2. Recovery of CD34-positive cells after different separation techniques.

<table>
<thead>
<tr>
<th>Enrichment</th>
<th>Recovery</th>
<th>Large-scale separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative depletion by immunomagnetic beads</td>
<td>20-60</td>
<td>30-60</td>
</tr>
<tr>
<td>Positive selection by immunomagnetic beads</td>
<td>60-80</td>
<td>30-60</td>
</tr>
<tr>
<td>Fluorescence activated cell sorter (FACS)</td>
<td>&gt; 95</td>
<td>30-60</td>
</tr>
<tr>
<td>Panning</td>
<td>50-80</td>
<td>30-60</td>
</tr>
<tr>
<td>Ceprate SC</td>
<td>50-80</td>
<td>40-70</td>
</tr>
</tbody>
</table>

**Adhesion molecules and cytokine receptors**

A number of molecules within the integrin family have been shown to mediate interactions between early CD34-positive cells and bone marrow stromal cells. These include VLA-4/VCAM-1, VLA-5/fibronectin, LFA-1 or ICAM, and several others. Each adhesion molecule appears to mediate a specific cell interaction. Hematopoietic growth factors may be active in a soluble form or in a membrane-anchored form; adhesion molecules may be crucial for allowing anchored growth factors to bind the target cell. Table 3 lists the main adhesion molecule and growth factor receptors expressed on CD34+ cells.

**CD34 expression on normal and neoplastic cells**

It has been known that CD34 monoclonal antibodies bind specifically to vascular endothelium ever since a 110 kd protein extracted from freshly isolated umbilical cord blood was identified with CD34 antibodies in Western blots and in Northern blot analysis. CD34 molecules have a striking ultrastructural localization on endothelial cells: they are concentrated primarily on the luminal side, in particular on membrane processes, many of which interdigitate between adjacent endothelial cells. Since this region is an important site for leukocyte adhesion and transendothelial...
traffic, in contrast to previous experiences,\textsuperscript{33} it has been hypothesized that CD34 may be antagonistic or inhibitory to the adhesive functions of vascular endothelium. This was supported by the demonstration that CD34 gene expression at the mRNA level is reciprocally down-regulated when adhesion molecules ICAM-1 and ELAM-1 are up-regulated by IL-1 during inflammatory skin lesions associated with leukocyte infiltration.\textsuperscript{33,59} Furthermore, additional studies conducted on both paraffin embedded and cryopreserved sections demonstrated that fibroblasts also react with anti-CD34 MoAbs.\textsuperscript{60} However, it should be noted that while CD34 MoAbs reacted with all classes of epitopes on cryopreserved sections, class III epitopes were not recognized by specific anti-CD34 MoAbs on paraffin embedded sections. Levels of CD34 expression, highest in immature hematopoietic precursor cells, decrease progressively with cell maturation.

Regarding hematologic malignancies, CD34 is expressed in a large percentage of acute leukemias.\textsuperscript{61} The fluorescence intensity of CD34 expression is variable and higher in acute lymphoblastic (ALL) than in acute myeloblastic leukemia (AML). In these latter patients, the CD34 antigen is found on 40–60% of leukemic blasts and is most frequently associated with M0, M1 and M4 FAB cytotype, secondary leukemias, karyotypic abnormalities involving chromosome 5 or 7, P170 expression and poor prognosis.\textsuperscript{61–64} Thus, CD34 expression may be considered the most predictive negative factor in AML patients strictly correlated with intensity of expression.\textsuperscript{65,66} In ALL, CD34 is expressed in 70% of patients, particularly in those with a B-lineage phenotype. In these patients, unlike AML cases, the clinical relevance of CD34 expression is controversial; however, according to the findings of a Pediatric Oncology Group,\textsuperscript{67} its expression in B-lineage cases was associated with hyperdiploidy, lower frequency of initial central nervous system (CNS) leukemia and a favorable prognosis. In T-cell ALL cases, on the other hand, CD34 expression showed a positive correlation with initial CNS leukemia and CD10 negativity, but not with any presenting favorable-risk characteristics.\textsuperscript{67,68}

Lastly, CD34 and HLA-DR expression may be very useful in discriminating between the very few benign primitive hematopoietic progenitors and their malignant counterparts in patients with chronic myeloid leukemia (CML). In fact, it has been demonstrated that normal progenitor cells are CD34\textsuperscript{+} and HLA-DR\textsuperscript{−}, while malignant progenitor cells, which exhibit Ph and bcr/abl rearrangement, express HLA-DR antigens.\textsuperscript{56}

**Positive and negative regulators of hematopoietic progenitor cells**

The hematopoietic stem cell is defined by its extensive self-renewal capacity, multilineage differentiation potential and capacity for of long-term reconstitution of normal marrow function in lethally irradiated animals.\textsuperscript{68} Transplantation of retrovirally marked murine

![Diagram](image-url)  

Figure 5. Functional differentiation and antigenic expression of hematopoietic cells.
Table 3. Adhesion molecule and growth factor receptors expressed on CD34-positive cells.

<table>
<thead>
<tr>
<th>Antigen name</th>
<th>CD</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlyCAM-L-selectin</td>
<td>none</td>
<td>adhesion molecule</td>
</tr>
<tr>
<td>ICAM1,2,3</td>
<td>CD11a/CD18</td>
<td>adhesion molecule</td>
</tr>
<tr>
<td>H-CAM</td>
<td>CD44</td>
<td>adhesion molecule</td>
</tr>
<tr>
<td>VLA-4</td>
<td>CD49d/CD29</td>
<td>adhesion molecule</td>
</tr>
<tr>
<td>VLA-5</td>
<td>CD49d/CD29</td>
<td>adhesion molecule</td>
</tr>
<tr>
<td>FGF-R</td>
<td>none</td>
<td>growth factor</td>
</tr>
<tr>
<td>M-CSF</td>
<td>CD115</td>
<td>growth factor</td>
</tr>
<tr>
<td>GM-CSF-R</td>
<td>CD116</td>
<td>growth factor</td>
</tr>
<tr>
<td>SCF (c-kit)</td>
<td>CD117</td>
<td>growth factor</td>
</tr>
<tr>
<td>Interferon γ-R</td>
<td>CD119</td>
<td>growth factor</td>
</tr>
<tr>
<td>IL 7-R</td>
<td>CD127</td>
<td>growth factor</td>
</tr>
</tbody>
</table>

stem cells has shown that only a few multilineage progenitor cells induce the repopulation of engrafted hematopoietic tissue, suggesting that hematopoiesis may be supported by a succession of short-lived clones. Moreover, experimental evidence indicates that the processes of self-renewal, differentiation and selection of lineage potentials are intrinsic properties of the stem cells and occur according to a stochastic (random) model. In the steady state, most of the stem cells are quiescent (G0 phase) and begin active cycling randomly. Conversely, survival and proliferation of hematopoietic progenitors are regulated by cytokines, which also act in preventing apoptosis. According to this model, the induction of differentiation by a cytokine may be considered as the consequence of the proliferation of a specific population stimulated by that factor. The broad term cytokines includes growth factors such as fibroblast growth factor, colony stimulating factors (CSFs) (e.g. granulocyte-CSF, granulocyte-macrophage-CSF), and interleukins (ILs). Depending on their target cells and different proliferative potential, cytokines can be divided into three categories: 1) late-acting, lineage-specific factors; 2) intermediate-acting, lineage-nonspecific factors; 3) early-acting growth factors inducing the recruitment of dormant early progenitors in the cell cycle (Figure 6). The majority of late-acting factors support the proliferation and maturation of lineage-committed progenitors and the functional properties of terminally differentiated cells. Erythropoietin (Epo) is the physiologic regulator of erythropoiesis and thrombopoietin has the same role in thrombopoiesis, while M-CSF and IL-5 are considered specific for macrophages and eosinophils, respectively. G-CSF exerts its activity on committed neutrophil precursors, although it has been shown to be a synergistic factor for primitive hematopoietic cells.

Intermediate-acting, lineage-nonspecific factors include IL-3, IL-4 and GM-CSF. Their activity is mainly directed toward progenitor cells in the intermediate stages of hematopoietic development. However, they interact with later-acting growth factors for the production of more mature cells, as well as with the cytokines capable of triggering stem cell cycling. On their own, they act as survival factors for stem cells and appear to stimulate early hematopoietic precursors only after their exit from G0. Several cytokines have recently been identified for their capacity to stimulate the proliferation of the earliest hematopoietic cells. Mapping studies of normal blast cell colony formation from single progenitors have shown that IL-6, G-CSF, IL-11, stem cell factor (SCF), IL-12 and leukemia inhibitory factor (LIF) recruit dormant stem cells into the cell cycle that are then able to respond to additional growth factors. Whereas the permissive factors retain limited proliferative potential by themselves, they strongly enhance the stimulatory activity of IL-3, GM-CSF, G-CSF and EPO on CD34+ and more immature CD34+ lineage-progenitors. In addition to positive interaction with intermediate- and late-acting growth factors, SCF synergistically or additively augments the colony-forming ability of other early-acting growth factors, such as IL-11, IL-12 and IL-6, on myeloid and bilineage (i.e. lymphomyeloid) primitive cells. Recently, the ligands for the STK-1 or FLT3 receptor, and the hepatocyte growth factor were shown to be able to stimulate very primitive hematopoietic progenitor cells. The main sources of both positive and negative regulatory proteins are accessory myeloid cells and the stromal component of the bone marrow. In general, microenvironment cells do not constitutively produce cytokines, rather transcription and/or translation processes are rapidly induced by cytokines such as IL-1, TNF. The extracellular matrix also participates in the regulation of hematopoiesis by binding growth factors and presenting them in a biologically active form to bone marrow progenitor cells.

Most data suggest that stem cells express low levels of growth factor receptors and require multiple proliferative stimuli to enter into the cell cycle, while committed progenitor cells can be effectively stimulated by individual cytokines. Combinations of two or more growth factors can stimulate hematopoietic cells either by amplifying the progeny cell production of single precursors (synergy) or by inducing additional clonogenic cells to proliferate (recruitment). Examples of these two types of enhancement are given in Figures 7 and 8, where CD34+ CD33+ DR+ cells are simultaneously stimulated by two or three growth factors. The molecular basis regulating the complex interplay between cytokines is still largely unknown. However, one proposed mechanism for growth factor synergism is the...
induction of CSF receptors on early hematopoietic progenitor cells. This appears to be a coordinate cascade transactivation via up-modulation of growth factor receptors that leads to proliferation and differentiation of human marrow cells. Conversely, the structural homology between some growth factors, the presence of shared receptor subunits on the cell membrane and common signal-transduction proteins provide a potential explanation for their functional similarities and the apparent redundancy of their activity.

The growth of hematopoietic progenitor cells is also regulated by soluble negative regulators such as macrophage inflammatory protein-1a (MIP-1a), tumor necrosis factor-α (TNF-α), interferons (IFNs), prostaglandins and transforming growth factor-β (TGF-β). The TGF-β family of proteins includes at least five isoforms (TGF-β1-5) which are encoded by different genes and produced by stromal cells, platelets and bone cells. Moreover, a subset of very primitive murine hematopoietic cells has been shown to secrete active TGF-β1 by an autocrine mechanism. TGF-β1 and 2 isoforms are bimodal regulators of murine and human hematopoietic progenitor cells, and their activity is based upon the differentiation state of the target cells and the presence of growth factors. In the human system, for instance, CFU-GEMM derived from purified CD34+ /CD33- cells are inhibited by TGF-β1, whereas more committed progenitors such as CFU-G or CFU-GM are not affected. In addition, high proliferative potential-CFC (HPP-CFC) responsive to a combination of CSFs are markedly inhibited by TGF-β1, while more mature CFU-GM are actually enhanced when GM-CSF is used as colony forming factor. TGF-β1 and 2-induced myelosuppression is partially counteracted by early-acting growth factors such as G-CSF, IL-6 and fibroblast growth factor. On the other hand, TGF-β3 has been shown to be a more potent suppressor of human BM precursors and its activity on hematopoiesis is only inhibitory, although the synergistic growth factors IL-11 and IL-9 seem to be able to oppose the negative regulation of TGF-β3 on human CD34+ cells. Several potential modes of action of the TGF-β family have been suggested, including down-modulation of cytokine receptors, interaction with the underphosphorylated form of the retinoblastoma gene product in late G1 phase, and alteration of gene expression. Early studies have shown that TGF-β1 and 3 exert their activity on normal and leukemic cells by lengthening or arresting the G1 phase of the cell cycle, and this effect is functional to protect normal CD34 positive cells from the toxicity of alkylating agents in vitro. More recent investigations have demonstrated that TGF-β regulates the responsiveness of mice hematopoietic cells to SCF, which is known to be the main synergistic factor for both murine and human stem cells, through a decrease in c-kit mRNA stability that leads to decreased cell-surface expression.

Similarly to TGF-β, TNF-α has been reported to have both inhibitory and stimulatory effects on hematopoietic progenitor cells. TNF-α potentiated the IL-3 and GM-CSF-mediated growth of human CD34+ cells in short-term liquid culture assay. However, it inhibited

Figure 6. Cytokines exert their activity at different levels of the hematopoietic differentiation pathway. Each progenitor cell is concurrently affected by multiple regulators.
the growth promoting activity of G-CSF. Two TNF receptors with molecular weights of 55 and 75 kDa, respectively, have recently been identified. Whereas the p55 receptor mediates TNF-α effects on committed progenitor cells, the p75 receptor is involved in signaling the inhibition of murine primitive cells.

Furthermore, it was recently shown that TNF-α is capable of inhibiting the multi-growth factor (GM-CSF, IL-3, G-CSF, SCF, IL-1)-dependent growth of human HPP-CFC derived from CD34+ cells through interaction with both p55 and p75 receptors, while the p55 receptor exclusively mediates the bifunctional activity of TNF-α on more mature BM precursors responsive to single cytokines.100

MIP-1α is a peptide of 69-amino acids with a molecular weight of 7.8 kDa produced by activated macrophages, T-cells and fibroblasts.101 It belongs to a large family of putative cytokines that includes MIP-1β, MIP-2 and IL-8 (chemokines). Biologic characterization has shown that MIP-1α enhances the M-CSF- and GM-CSF-dependent growth of CFU-GM, while it inhibits the colony-forming ability of hematopoietic precursors present in a cell population enriched for CD34+ DR+ cells stimulated with erythropoietin, IL-3 and GM-CSF.102

Taken together, these results indicate that a complex interplay between positive and negative regulatory proteins determines the proliferation or inhibition of early hematopoietic progenitor cells (Figure 9). In general, the activity of inhibitors of hemopoiesis appears to be reversible, lineage-nonspecific and directed at the early stages of differentiation. In addition, TGF-β has shown some degree of differential activity between normal and neoplastic lymphoid cells.103 Thus, negative regulators may be clinically relevant to the protection of the hematopoietic stem cell compartment from the dose-limiting toxicity of neoplastic disease therapy.96,103,104

**Collection of CD34+ cells**

Bone marrow and peripheral blood are the only sources of immature hematopoietic precursors identified as CD34+ cells. A diagnostic marrow sample contains only a few CD34+ cells, while even fewer of them are present in peripheral blood samples taken under steady state conditions. Large quantities of CD34+ cells can be collected with massive marrow harvests, such as for transplantation purposes. Nevertheless, marrow CD34+ cells are somewhat elusive due to their scattering among the predominant CD34+ hematopoietic population.105 Recently developed cell separation procedures allow collection of highly enriched CD34+ cell populations. However, this is generally accomplished through aspecific and often unacceptable cell loss.106 So far the limited number of immature precursors commonly obtained from both bone marrow and peripheral blood has been the major obstacle to a simple identification and analysis of marrow CD34+ cells. Indeed, the growing interest in CD34+ cells is primarily the result of the development of new therapeutic modalities that allow easy access to large quantities of hematopoietic precursors through the peripheral blood. The key role in these innovative approaches is represented by the introduction of hematopoietic growth factors for clinical use.107

At present GM-CSF and G-CSF are the most extensively employed and studied hematopoietic growth factors in the clinical setting. From the very beginning, it was observed that GM-CSF or G-CSF administration was associated with an increase in circulating hemopoietic precursors.108,109 Later on, it was demonstrated that this phenomenon could be extensively and reproducibly amplified by combining growth factor administration with high-dose chemotherapy.110-112

Indeed hematopoietic progenitors are massively, though transiently, mobilized into peripheral blood during hemopoietic recovery following high-dose chemotherapy given with growth factor support. Such an abundance of immature hematopoietic cells makes them easily recognizable by cell sorting techniques that employ anti-CD34 MoAbs.113 Under optimal conditions, the proportion of CD34+ cells may reach values as high as 20-30% of the total leukocyte count. In steady state conditions CD34+ cells do not exceed 4% of the total marrow population, while they are undetectable by cell sort-
ing techniques in the peripheral blood. Chemotherapy and growth factors do not merely induce a relative increase of immature hemopoietic cells; their absolute number is amplified several times over basal conditions. This allows collection of sufficient amounts of hemopoietic progenitors for autografting purposes by means of a few leukapheresis procedures.\textsuperscript{110,113,114} Values of $10^{-20} \times 10^4 \text{CFU-GM/kg}$ represent the minimal required dose for marrow engraftment with peripheral blood progenitors.\textsuperscript{115-117} In fact, much higher quantities of circulating progenitors can be collected using appropriate mobilization procedures. Under optimal conditions, circulating CD34\textsuperscript{+} cell peak values may range between 150 and 700/µL on days of maximal mobilization. As a rule, at least $8 \times 10^6 \text{CD34}^+ \text{cells/kg}$ or more can thus be collected with 1 to 3 leukapheresis procedures.\textsuperscript{114} These huge quantities, approximately corresponding to $50 \times 10^6 \text{CFU-GM/kg}$, must be considered the ideal threshold dose of peripheral blood progenitors for autografting purposes. Indeed values of $8 \times 10^6 \text{CD34}^+ \text{cells/kg}$ or more guarantee a rapid and durable hemopoietic recovery when circulating progenitors are used as the sole source for marrow reconstitution following submyeloablative treatment.\textsuperscript{118-121}

Thus far, massive CD34\textsuperscript{+} cell mobilization has been most commonly observed when growth factor is administered following high-dose cyclophosphamide, given at 7 g/m\textsuperscript{2}. Indeed chemotherapy that induces profound leukocytopenia seems to be crucial for optimal mobilization; for instance, cyclophosphamide at doses lower than 7 g/m\textsuperscript{2} produces a reduced mobilizing stimulus.\textsuperscript{111,122} Several other chemotherapy schedules have also been successfully employed for mobilization purposes. The principal chemotherapy protocols reported to be highly effective in inducing CD34\textsuperscript{+} cell mobilization are summarized in Table 4.\textsuperscript{110, 111, 115, 117, 122-127}

As stressed earlier, hemopoietic growth factors play a key role in mobilization. This has been clearly documented with cyclophosphamide. A median peak value of 75 CD34\textsuperscript{+} cells/µL has been recorded following high-dose cyclophosphamide alone, whereas 420 and 500/µL are the median values recorded when GM-CSF or G-CSF, respectively, are added to cyclophosphamide.\textsuperscript{112,113,128,129} Extensive growth factor-induced mobilization is further substantiated by the possibility of collecting sufficient CD34\textsuperscript{+} cells using growth factor alone.\textsuperscript{130,131} In this setting the most promising experiences have been produced with G-CSF. The results reported indicate new opportunities for the utilization of mobilized progenitors in allogeneic transplantation procedures.\textsuperscript{132,133} Combined chemotherapy and growth
Further improvement might derive from the clinical availability of new cytokines, such as IL-3, SCF and others, to be employed alone or in combination with G- or GM-CSF. However, the potentially high mobilizing activity of such cytokine combinations must be accompanied by no or very few side effects in order to be considered for a wide clinical applicability.

Mobilizing protocols generally include daily delivery of growth factors, starting 1 to 3 days after chemotherapy administration and continuing until harvesting procedures are completed. Growth factor is usually administered for a total of 7–12 consecutive days. The most convenient route of delivery is subcutaneous, with 1 to 2 doses per day. Progenitor cell harvests are performed during hemopoietic recovery, provided that progenitor cell mobilization is documented. Indeed various parameters have been considered as an indirect indication of progenitor mobilization, including an increase in WBC or, alternatively, in monocytes, basophils or platelets. However, CD34+ cell evaluation remains mandatory for an accurate definition of the extent of progenitor mobilization.

CD34+ cells should be monitored daily from the early stages of hemopoietic recovery. Detection of circulating CD34+ cells is not sufficient reason for starting leukapheresis procedures; an adequate number of CD34+ cells (>20–50×10^9/L) and WBC > 1.0×10^9/L and platelet count > 30×10^9/L are required for safe and effective progenitor cell harvesting. Leukaphereses are performed using continuous-flow blood cell separators. A complete leukapheresis procedure generally takes 2–3 hours and the total blood volume processed ranges from 6 to 10 liters. The harvested cells are resuspended in freezing medium and then cryopreserved for subsequent transplantation. One to 3 leukaphereses repeated on consecutive days usually provide large amounts of progenitor cells capable of rapid engraftment after submyeloablative treatments.

Factors and procedures favoring CD34+ cell mobilization have been well established. However, other conditions adversely influencing the mobilization phenomenon should be considered. A major limitation is represented by impaired marrow function, as can occur in previously treated patients. In fact, patients at first relapse following a single treatment protocol maintain an adequate mobilization capacity; however, few if any mobilized CD34+ cells can be obtained from heavily treated patients previously exposed to multiple chemotherapy courses. Mobilization is also profoundly reduced and often totally abolished in patients previously exposed to radiotherapy, especially if it was delivered to the pelvis or to extended vertebral areas. Lastly, marrow invasion by tumor cells may negatively affect mobilization capacity. This is typically reported in myeloma patients in whom the extent of CD34+ cells often correlates with the degree of marrow involvement by tumoral plasma cells.

In conclusion, several new findings have dramatically improved the procedures for collection of large amounts of CD34+ cells. However, optimal marrow function is a prerequisite for exploiting fully the activity of all those factors known for their mobilization-inducing capacity.
make cord blood CD34+ cells more suitable candidates than bone marrow cells for ex vivo expansion.

It is clear that in vitro expansion and maturation of hematopoietic progenitor cells might be of particular relevance for transplantation of cord blood hematopoietic cells; however, information about the effects of such an expansion on the cells required for long-term hematopoietic reconstitution is highly desirable.

The dual role of peripheral blood hematopoietic progenitor cells in onco-hematology

CD34+ progenitor cells circulating in the peripheral blood represent an enriched and easily accessible source of two distinct cell populations: committed progenitor cells and hematopoietic stem cells.

Although circulating progenitors are commonly called stem cells, the presence of circulating stem cells has been formally proved only in mice.148 In humans the presence of hematopoietic stem cells in the peripheral blood is almost certain (see below), but to call reinfusion of circulating progenitors transplantation of peripheral blood stem cells (PBSC or similar acronyms) is hardly appropriate. This terminology overlooks the fact that the tremendous interest in peripheral blood autografting is not (at least so far) a consequence of its being a simple surrogate for autologous bone marrow transplantation (as the term PBSC would suggest), but rather derives from the unique property of this procedure to reduce the duration of the severe pancytopenia that follows submyeloablative treatments from two–three weeks (when bone marrow cells are used) to a few days only.110 The reason for the rapid recovery which occurs after circulating progenitor autotransfusion (CPAT) has not been formally proved, but it is most likely a consequence of the much larger (10–100 fold higher) amount of committed progenitors reinfused when a patient is autografted with blood–derived (as opposed to marrow–derived) cells. The most likely hypothesis is that these late progenitors (post–progenitors) of granulocytes and platelets are capable of giving rise to mature progeny within a few days, thus allowing submyeloablative patients to survive the initial aplastic phase.

The fundamental role of committed progenitor cells was elegantly proved in mice by Jones et al.150 None of the lethally irradiated animals transplanted with a pure population of stem cells free of more mature progenitors (CFU-GM, CFU-S) survived the initial aplasia. A more recent paper151 challenged this ‘conventional wisdom’ model, and maintained that peripheral blood stem cells per se are capable of rapidly maturing in vivo.

Other authors, using a very similar approach, reached an opposing conclusion.152 In humans the most convincing, albeit indirect, evidence in favor of the role of committed progenitors in accelerating post–transplant recovery was provided by Robertson et al.,132 who documented a significantly prolonged hematopoietic recovery for patients undergoing autologous bone marrow transplantation purged ex vivo with anti-CD33 monoclonal antibodies. This result, which occurred after a treatment that selectively kills the most mature progenitor cells (expressing the CD33 surface antigen), represents convincing indirect proof of the role of committed progenitors in early engraftment.

The clinical role of committed progenitors in reducing the morbidity and mortality of high-dose therapy has expanded since hemopoietic growth factors have become clinically available. In fact, infusion of rhGM-CSF or rhG-CSF, in particular after administration of myelotoxic doses of certain stem cell–sparking agents, allows easy collection of an amount of CFU-GM/kg body weight 10 to 100 times higher than that contained in a bone marrow harvest.110

As already documented by a large number of papers, the use of circulating progenitors has brought about a dramatic change in the perspectives of high–dose submyeloablative regimens. In fact, these once specialized, expensive and highly toxic treatments are now well tolerated, easy to administer, and clinically useful (cost effective). As an example, it is worth mentioning the initial Milan Cancer Institute experience in a group of over 50 poor–risk breast cancer patients who received high–dose melphalan plus an optimal amount of circulating progenitors (> 5x10^6 CFU-GM/kg body weight). These patients required a median of 10.5, 11 and 12.5 days to score > 0.5, 1.0 and 2.0x10^9/L neutrophils/L, respectively. Moreover, more than half of them did not require platelet transfusions, while the remaining ones needed only one or two transfusions during the first week after autografting. Such mild to moderate toxicity was never described before the clinical availability of committed progenitor cells.

In conclusion, born as a compassionate surrogate of bone marrow autografting, today CPAT is rapidly replacing ABMT. In fact, today it is the latter that should be considered a compassionate need procedure, useful in those few patients unable to mobilize a sufficient number of circulating progenitor cells.

The second, distinct role of circulating progenitors is related to the presence of stem cells, i.e. totipotent precursors responsible for durable reconstitution of all lympho-hematopoietic lineages following marrow ablative therapy. Since virtually no high–dose treatment that can be safely administered to humans is genuinely myeloablative, formal proof of the existence of circulating stem cells must await either stable transduction of DNA markers into autografted cells or the use of this cell population for allografting.

These experiments are presently underway in several laboratories,154,155 and preliminary data do confirm the presence of stem cells in the peripheral blood of humans. Their utilization is expected to revolutionize fields like allogeneic bone marrow transplantation and somatic gene therapy, whenever the target of genetic manipulations is the hematopoietic cell.156
References


myeloid and progenitor cells from chronic myeloid leukemia. Leukemia 1993; 7:1570-5.


106. Gabbianelli M, Sargiacomo M, Pelosi E, Testa U, Isacci G, Peschle C. Pure human hematopoietic progenitors: permissive action of basic fibroblast growth fac-


136. D’Hoindt V, Guillaume T, Humbert Y, et al. Tolerance of sequential or simultaneous administration of IL-3 and G-CSF in improving peripheral blood stem cell


140. Jansen WE. Peripheral blood and bone marrow hematopoietic stem cells: are they the same? Semin Oncol 1993; 20:19-27.


Peripheral blood stem cells in acute myeloid leukemia: biology and clinical applications

Clinical application of circulating stem cells for autologous transplantation is steadily expanding. It has become increasingly clear that mobilized peripheral blood progenitor cells (PBSC) induce faster hematopoietic recovery, fewer febrile days, lower transfusion requirement and shorter hospitalization than bone marrow (BM)-derived cells. More recently, rapid and sustained engraftment has also been reported using granulocyte colony-stimulating factor (G-CSF)-mobilized allogeneic PBSC following myeloablative therapy.

In contrast to solid tumors and many hematological malignancies, PBSC transplantation is not widely used for acute myeloblastic leukemia (AML) patients. In this setting there are still unanswered questions such as the role of autologous stem cell transplantation in post-remission therapy, as well as major issues concerning PBSC mobilization and collection: the expression of CD34 antigen on leukemic stem cells as compared to their normal counterparts, the biologic significance of CD34+ AML, the response of leukemic cells to CSFs used to optimize PBSC harvest, the potential contamination of PBSC grafts by residual AML cells and the role of ex-vivo purging of leukemic cells.

This review analyzes the most recent advances in this field, addressing clinical and biological issues relevant to the use of autologous PBSC for AML patients.

Growth factor receptor expression and response of leukemic cells to human CSFs

The CD34 antigen is a 105-120 KD glycoprotein expressed on the cell surface of hematopoietic progenitors and stem cells, but it is not expressed on late hematopoietic cells or on many tumor cells. CD34+ cells are responsible for the self-renewal and the expansion of the large majority of AML. It has recently been shown that most of the clonogenic cells in AML derive from the CD34+ cell fraction as opposed to CD34- cells. Moreover, CD34+ cells co-expressing differentiation markers (CD33, CD38) have a reduced proliferative potential since in vitro they give rise to small colonies unable to originate secondary clones. This phenomenon is likely the expression of a limited self-renewal potential.

Lapidot et al. provided the most convincing evidence of the stem cell role of CD34+ cells in AML by showing that only the CD34+/CD38- cell fraction was capable of generating acute leukemia when transplanted into SCID mice.

These observations indicate the relevance of defining the growth and receptor expression pattern of leukemic CD34+ cells, their response to CSFs as well as their kinetic status compared to their normal counterparts. Among the different cytokines involved in the regulation of hemopoiesis, a key role in the pathogenesis of the leukemic growth is probably played by stem cell factor (SCF), interleukin 3 (IL-3), granulocyte-macrophage CSF (GM-CSF) and G-CSF.

SCF receptor (c-kit) is expressed by the vast majority of AML. Both high and low affinity receptors have been demonstrated (Table 1). c-kit shares structural similarities with the receptors for M-CSF and PDGF. A linear correlation between the percentage of CD34+ cells and c-kit expression has been documented, thus indicating that CD34+ AML express high levels of c-kit. In adult patients, the presence of a high number of CD34+ cells has been shown to correlate with a bad prognosis.

c-kit activation plays a fundamental role in the regulation of the early phases of CD34+ cell stimulation. The interaction of SCF with its ligand exerts a modest proliferative stimulus on immature quiescent cells and up-regulates the expression of receptors for other growth factors. While in normal hemopoiesis this triggers myeloid differentiation, in AML it may activate self-renewal and expansion of the leukemic population.

High affinity receptors for GM-CSF and IL-3 (Table 1) are expressed by nearly all AML, irrespective of the FAB subtype. IL-3, GM-CSF (and IL-5) receptors consist of an α subunit (ligand specific) and a shared β subunit. While the α subunit has a low affinity for the ligand and alone is incapable of transducing the signal, the association of the two subunits gives rise to a functioning

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high affinity receptor which is a type I receptor devoid of endogenous tyrosine-kinase activity. β chain activation induces several tyrosine-kinases like Fyn, Lyn, Fps, Jak, which transduce a signal common to IL-3 and GM-CSF, whereas a subunit activation induces ligand specific pathways. In the majority of AML cases IL-3 and GM-CSF induce the proliferation of CD34+ cells, although to variable extents. Correlations have been observed between responses to different factors but no significant additive effects have been noted.

Exposure of leukemic cells to GM-CSF or IL-3 in vitro can give rise to a generation of mature cells. However, the persistence of blast cells capable of secondary leukemic colony formation indicates that the differentiation potential of IL-3 and GM-CSF is negligible and that they are unable to abolish the self-renewal of the leukemic population. SCF synergizes with IL-3 and GM-CSF in inducing large clones primarily composed of undifferentiated cells.

G-CSF receptor is expressed by nearly all AML (Table 1). However, M2 and M3 AML appear to express the highest number of receptors. In vitro growth stimulation is not consistent except for M2 and M3 AML; G-CSF action is additive or synergic with that of IL-3, SCF and, to a lesser extent, with that of GM-CSF. G-CSF also induces some degree of differentiation of leukemic CD34+ cells, and the presence of the growth factor affects the formation of secondary colonies. In addition, CSF treatment seems to prevent cell death in AML.

The in vivo use of growth factors in AML patients derives from contrasting hypotheses:

a) use of growth factors before and during cytostatic treatment to induce the proliferation of quiescent leukemic progenitors. The increased proliferative rate and, possibly, the intracellular accumulation of some cytotoxic drugs (i.e. Ara-CTP) should increase the fraction of cells killed. This approach has never been tested in randomized trials specifically addressing this issue. It seems, however, to be of modest value with G-CSF or GM-CSF. It remains to be seen if this approach would be more useful with molecules such as SCF that are particularly active on leukemic CD34 cells;

b) use of growth factors as differentiating agents with the aim of exhausting the self-renewal potential of the leukemic progenitors. On the basis of in vitro and preliminary (although still to be confirmed) in vivo data, G-CSF seems the most promising molecule;

c) use of growth factors for accelerating the recovery of residual normal progenitors after induction chemotherapy. This approach has been pursued with G-CSF and GM-CSF in AML patients > 60 years of age, for whom the pancytopenia following cytotoxic treatment is particularly profound and long-lasting and carries a relevant risk of life-threatening infections. In this setting, both G-CSF and GM-CSF given after induction chemotherapy reduce the duration of neutropenia without affecting the rate of severe infections. Moreover, G-CSF, but not GM-CSF, appears to increase the complete remission rate. Both cytokines, however, have no impact on the survival rate. Of interest, no evidence of accelerated growth of residual leukemic cells has been observed.

All these data demonstrate how controversial the use of hemopoietic growth factors in the treatment of AML is, although the most recent results suggest the safety of G-CSF administration following induction-consolidation treatment.

**Stem cell kinetics in AML**

The hematopoietic cell renewal process is supported by a small population of bone marrow cells termed hematopoietic stem cells. They are defined as cells capable of long-term hematopoietic reconstitution and differentiation into multiple hematopoietic lineages. It is generally held that, in the steady state, the majority of normal stem cells are dormant in the cell cycle and only a few of them supply all the hematopoietic cells at a given time. More than thirty years ago, stem cell kinetic studies proposed the concept of a true resting state and coined the term G0 as the state from which stem cells randomly move to the active cell cycle. Subsequent studies confirmed Lajtha’s observations by showing that brief in vitro exposure of bone marrow cells to highly specific radioactive thymidine does not reduce the number of multipotential progenitors. As shown in Figure 1, most normal bone marrow CD34+ progenitor cells are indeed quiescent in G0. Culture of enriched human progenitors documented that they remain as single cells for as long as 2 weeks in culture and begin proliferation upon stimulation with combinations of cytokines. Based on mathematical studies, stem cell function was seen as a model in which the decision to self-renew and differentiate followed a stochastic process. By replating individual blast cell colonies, Till and coworkers showed that the production of secondary blast cell colonies is a self-renewal process and that the generation of secondary multilineage colonies is differentiation. Thus the self-renewal process is associated with renewed dormancy in the cell cycle while the differentiation process is characterized by continuous cell doubling.

**Table 1. High affinity receptors for hematopoietic growth factors in AML.**

<table>
<thead>
<tr>
<th>Affinity kD</th>
<th>No. of receptors per cell</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCF</td>
<td>16-150 pmol/L</td>
<td>200-8000</td>
</tr>
<tr>
<td>G-CSF</td>
<td>36-130 pmol/L</td>
<td>55-1200</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>64-404 pmol/L</td>
<td>40-1263</td>
</tr>
<tr>
<td>IL-3</td>
<td>26-467 pmol/L</td>
<td>21-145</td>
</tr>
</tbody>
</table>
Peripheral blood stem cells in acute myeloid leukemia

Similar work was performed on leukemic stem cells by several authors and two fundamental antithetical models were proposed, based on the presence of quiescent progenitor cells in human leukemia.

It was postulated that leukemic progenitors were predominantly involved in rapid cell cycling as judged by measuring the proportion of S-phase cells using H3-TdR or hydroxyurea. However, this was in contrast with previous observations obtained in vivo by continuous infusions of 3H-thymidine for 8–10 days. In those experiments, 88 to 93% of the leukemic cells were labeled at the end of infusion, whereas almost all the smallest leukemic cells were not, suggesting that they were in an extended G0. Using in vivo pulse labeling with tritiated thymidine in AML patients, it was further shown that blasts with a high proliferative rate do not behave as a pool of normal self-maintaining cells, but rather as a normal multiplication–maturation compartment. Data obtained with different techniques (labeling and cell culture methods) and the heterogeneity of study cell populations may represent the reason for these discordant results. The hypothesis that AML progenitors are characterized by a substantial number of nonproliferating or very slowly proliferating blast cells (lower RNA content) was the rationale for different approaches to AML treatment. For instance, the combined use of cytokines and chemotherapy to recruit quiescent cells into the cell cycle, enhancing the cytotoxicity of cycle-specific agents, may help.

Raymakers et al. have studied the proliferative capacity of the bone marrow fraction double stained for CD34 and CD33 in AML patients. The cloning efficacy was highly variable in different AML samples, with predominant cluster growth. Cluster and colony growth was similar between CD34+/CD33+ and CD34+/CD33−, in contrast to what is observed in normal bone marrow. The most primitive CD34+/CD33− fraction was found in highly proliferative colony growth. When this analysis was extended to AML with a more mature phenotype (small fraction of CD34+/33−), the highly proliferative colonies deriving from the CD34+/33− fraction were found to be disomic by in situ hybridization in all patients who were characterized by chromosomal abnormalities. Nevertheless, the authors could not exclude the presence of leukemic stem cells kinetically characterized by low or no proliferation under their experimental culture conditions.

A further study aimed at evaluating the specific activity of SCF on enriched CD34+ in suspension culture by measuring Ki67 expression and flow cytometric DNA content showed no difference in cell cycle distribution among progenitors obtained from normal bone marrow, umbilical cord blood and chronic myeloid leukemia CD34+ peripheral blood stem cells.

Further investigations on the role of a family of proteins recently identified as cell cycle regulators, such as cyclin A, B, D, E and of their catalytic subunits, the cyclin-dependent kinases cdk2, cdk4, cdk6 and cdc2, may help to identify kinetic features and fine differences between normal and leukemic hemopoietic stem cells, as well as events involved in neoplastic transformation.

In conclusion, the kinetic characteristics of leukemic stem cells have still not been defined, mainly because different experimental conditions allow evaluation of progenitors with different degrees of maturation and therefore with different proliferative characteristics. The heterogeneity among different leukemia subtypes should also be taken into account.

Expression of CD34 antigen in AML and CD34+ leukemias: clinical and biological significance

Based on current information, there is no doubt that a substantial number of acute leukemias express the CD34 antigen on the cell membrane of blast cells. However, the incidence of such expression in AML has been found to be highly variable (25–64% of the patients examined), depending on a number of factors, as shown in Table 2.

The variability in the reported incidence of CD34+ AML has also influenced the prognostic relevance of CD34 expression in AML. Most authors found a clear association between CD34+ AML and a lower incidence of complete remission following induction therapy. In addition, the relapse rate was higher in AML showing positivity for the CD34 antigen compared to that of the CD34− group. However, other authors did not confirm these results and found no significant difference in the complete remission rate or overall survival of CD34+ and CD34− AML patients.

Expression of the CD34 antigen in AML and its association with different survival rates could be due to a

![Figure 1. DNA/RNA cellular content (acridine orange) of normal enriched CD34+ cells. Cell cycle measurements confirm that the majority of progenitors are quiescent (G0) with only few going into cycle (G1).](image-url)
number of factors. First of all, the cut-off point for CD34 positivity that should be used to decided whether an AML sample is carrying this antigen. Since the proportion of CD34+ cells is around 1% of normal bone marrow mononuclear cells and 0.01-0.1% of peripheral blood leukocytes, many authors have considered 5% as the optimal cutoff level for classifying CD34+ AML. Nowadays, most authors agree that the cutoff point for CD34 should be 20% in order to avoid misinterpretation of the data coming from surface marker analysis. However, there is no scientific basis for considering a sample with 20% positive cells as positive, while another specimen with 19% positivity as negative, since the level of CD34 expression in a substantial number of patients is characterized by a continuous spectrum. Furthermore, it must be kept in mind that the choice of a cutoff level of 5% could give rise to erroneous results, since it can be influenced by the methods used to detect antigen expression, which are characterized by different levels of sensitivity and specificity. Indirect immunofluorescence staining is more sensitive, although less specific, while the opposite is true for the direct technique. As far as the instrumentation is concerned, it must be underlined that modern flow cytometers are highly sensitive in detecting surface marker positivity with respect to microscope analysis and immunoenzymatic techniques such as APAAP, PAP, etc. In addition, whenever theoretically performed on fresh, not cryopreserved bone marrow samples, and if this is not possible the number of blasts present in the specimen analyzed should be carefully evaluated. A recent report showed that CD34 antigen expression in AML samples having a marked heterogeneity in cell size was found preferentially on small leukemic cells with little or no side scatter. This feature was also associated with shorter remission duration and survival, suggesting that this morphological heterogeneity could reflect a peculiar biological behavior of AML.

Moreover, discrimination of blast cells from residual normal nucleated cells is less likely to be obtained in AML cells by looking at light scattering properties (forward and side scatter) and expression of the CD45 antigen. For this reason, a multiparametric approach using two-three-colour analysis is strongly recommended in order to define the predominant leukemic population as well as minor pathological clones or subclones. In addition, CD34 positivity has to be evaluated solely on the blast population in order to avoid misinterpretation of the data. In fact, the percentage of blasts could vary from 30% to 99% in the bone marrow, and from 1 to 99% in the peripheral blood.

Another point which deserves careful discussion is represented by the level of expression for CD34 in AML. In normal hemopoiesis, the CD34 antigen is expressed on virtually all colony forming cells (CFU) and lymphocyte progenitors of either T or B lineage. However, within the progenitor cell compartment the degree of positivity for CD34 decreases with cell differentiation (maximum for multipotent cells and minimum for unipotent cells), and disappears in morphologically identifiable bone marrow precursors. Studies performed at the V International Workshop on Leukocyte Differentiation Antigens (Boston, 1993) recognized three main subsets of CD34+ normal bone marrow cells with CD34 antigen densities: low (2,000-5,000 binding sites per cell-ABC), medium (10,000-20,000 ABC), high intensities (25,000-40,000 ABC). This heterogeneity in CD34 antigen expression in normal progenitors makes it difficult to use this molecule for the monitoring of minimal residual disease (MRD) in AML patients treated with chemotherapy and/or bone marrow transplantation. Flow cytometry allows the recognition of a subset of CD34+ AML characterized by bright expression for CD34 (> 50,000 ABC), which could therefore be easily recognized even when present in a very low percentage (< 0.1%) of nucleated cells. This subset represents about 20-30% of CD34+ AML, so the remaining AML patients should be checked for MRD by using alternative ways (strategical double or triple staining: CD34/CD56; CD34/CD65/TdT; cytogenetics, molecular biology, etc.).

Another source of variability in detecting CD34+ AML is represented by the type of CD34 monoclonal antibody used for immunophenotypic analysis. It has been demonstrated that at least three distinct CD34 epitopes exist, based on their differential sensitivity to enzymatic cleavage (using neuraminidase, chymopapain and glycoproteinase). Western blotting analysis, cell reactivity studies, and cross blocking experiments. So far

Table 2. Possible explanations for the differences reported in the literature concerning the incidence of CD34+ AML.

1. Cut-off levels for the discrimination of positive and negative cases
2. Detection systems employed (flow cytometry, type of flow cytometer, immunoenzymatic technique-APAAP, immunogold, PAP-immunofluorescence microscope)
3. Specimen analyzed (bone marrow, peripheral blood)
4. Percentage of leukemic cells present in the sample examined
5. Use of cryopreserved rather than fresh cells
6. Use of different CD34 antibodies recognizing distinct CD34 epitopes
7. Percentage value and level of intensity for CD34
8. Light scattering properties of CD34+ cells
9. Patients analyzed (de novo AML or secondary AML)
10. Biologic characteristics of AML cells (chromosome alterations, gene abnormalities, immunophenotypic profile of CD34+ AML blasts)
11. Type of chemotherapy regimen employed

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at least twenty-two CD34 monoclonal antibodies (McAbs) have been shown to recognize the CD34 molecule, the most direct evidence being reactivity with cells transfected with CD34 cDNA and binding to CD34 glycoprotein. Recently, it has been reported that a number of AML cases are positive for some CD34 McAbs and negative for others (especially if they belong to a different epitope class), confirming the necessity of using the same CD34 McAbs in order to achieve comparable results between different centers.

Another point which needs to be considered when evaluating the incidence of CD34-positive AML is represented by patient characteristics at diagnosis. The number of CD34-positive cases is higher in secondary than in newly diagnosed AML. If we consider that both the biology and the clinical pattern of secondary AML are quite different from those of de novo AML, one may argue that including of both types of leukemias in a clinical setting could interfere significantly with the prognostic relevance of CD34 expression on AML blast cells. In this context, the type of treatment utilized by various authors (chemotherapy regimen, allogeneic and autologous bone marrow transplantation), which can influence the outcome of the disease, is also of some relevance.

CD34-positive AML are characterized by a higher incidence of chromosomal abnormalities involving chromosomes 5 (–5, 5q–), 7 (–7, 7q–), and to a lesser extent chromosomes 16 (16q), 17 (17p), 11 (trisomy 11), or multiple chromosomes at the same time, generating the so-called major karyotypic abnormalities. Recent studies have found a close relationship between CD34 expression in AML and previous exposure to chemotherapy, radiotherapy and/or pesticides. CD34-positive AML are also associated with trilineage myelodysplasia, dysgranulopoiesis, and/or abnormalities of the p53 tumor suppressor gene.

The correlation between CD34-positive AML and FAB subtypes is illustrated in Table 3. On the other hand, most biphenotypic acute leukemias (BAL) show positivity for the CD34 antigen.

Finally, the antigenic profile of CD34-positive AML is rather heterogeneous, depending essentially on the morphological subtype and to a lesser extent on the differentiation stage of the leukemic clone. The large majority of CD34-positive AML coexpress a number of antigens which are not associated with cell commitment, such as HLA-DR, CD3, CD45, CD45RA, CD71, and IL3 receptor. In addition, some surface and cytoplasmic glycoproteins expressed by committed myeloid cells were found to be positive in CD34-positive AML, i.e. CD33, CD13, CD117 (stem cell factor receptor), CDw116 (GM-CSF receptor), G-CSF receptor, myeloperoxidase, lysozyme (Figures 2 and 3). The stem cell-associated antigen Thy-1 (CD90) is negative in this AML subtype, while nuclear TdT is sometimes positive. CD34-positive cells also express high levels of P-glycoprotein, which is the product of the multiple drug resistance (MDR) gene.

### Post-remission therapy of acute myeloid leukemia and potential role of autologous stem cell transplantation

About two thirds of previously untreated adults with primary acute myeloid leukemia enter complete remission (CR) after induction therapy based on cytarabine and an anthracycline. However, long-term disease-free survival occurs in a minority of cases since most subjects relapse from proliferation of occult residual leukemic cells. Following conventional consolidation treatment less than 25% of patients remain in complete remission at four years.

In order to eradicate residual AML cells and improve disease-free survival, three approaches have been employed in the last ten years: (a) intensive postremission chemotherapy, (b) allogeneic bone marrow transplantation (BMT), and (c) myeloablative conditioning regimens followed by autologous BMT as supportive therapy.

Intensive postremission chemotherapy is essentially based on the use of high-dose cytarabine (1 to 3 g/m²3 6 to 12 doses), either alone or in combination with other agents. Results of uncontrolled studies performed in the late ’80s and early ’90s (reviewed by Cassileth et al.83) indicated that intensive postremission therapy achieves long-term disease-free survival in 25–30% of patients in first CR. Mayer et al.84 recently reported a prospective study aimed at evaluating the effect of the intensity of postremission chemotherapy on survival of leukemic patients. Acute leukemia individuals in first CR were randomly treated with four courses of cytarabine at one of three doses: 100 mg/m² per day for 5 days by continuous infusion; 400 mg/m² per day for 5 days by continuous infusion, or 3 g/m² in a 3-hour infusion every

### Table 3. Clinical and biological characteristics of CD34+ AML.

| Incidence: 30-50% (de novo AML); 50-70% (secondary AML) |
| History: previous exposure to chemotherapy or pesticides |
| Correlation with FAB subtypes: M0, M1/M5 (70-90%); M2, M4 (20-60%); M3 1-5%, M6, M7: 20-50% |
| Prognosis: poor (mean survival rates less than 12-18 months) |
| Cellular density for CD34: variable from case to case (range 3,000-100,000 per blast cell) |
| Immunophenotypic profile: in most cases CD15+, HLA-DR+, CD38+, CD33+, CD117+, Thy-1+ |
| Chromosome abnormalities: -5, -7, 5q-, 7q-, 16q, 17p, major karyotype alterations |
| Therapy to be defined (more aggressive chemotherapy regimen?) |
12 hours on days 1, 3 and 5. In patients 60 years of age or younger the probability of remaining disease-free after four years correlated with the postremission cytarabine dose: 24% for the 100-mg group, 29% for the 400-mg group, and 44% for the 3-g group (p = 0.002). In patients older than 60 the probability of remaining disease-free after four years was 16% or less in each of the three postremission cytarabine groups, with no significant difference between groups. It should be noted that a significant proportion of AML patients achieving CR cannot proceed to intensive chemotherapy due to persistent bone marrow aplasia.

Allogeneic bone marrow transplantation offers many advantages, including the graft-versus-leukemia effect; however, the availability of a histocompatible sibling donor is restricted to approximately 25% of potential candidates. Published studies report disease-free survival rates at four years ranging from 45 to 58%. These figures should be considered with caution since they are biased by the exclusion of patients who relapsed before allogeneic BMT. The new approach recently described by Aversa et al., i.e. a strong immunosuppressive and myeloablative conditioning regimen followed by transplantation of a large number of haploidentical stem cells depleted of T lymphocytes, may open new perspectives for allogeneic bone marrow transplantation in AML patients. In this setting, the mobilization and collection of allogeneic PBSC is crucial to overcoming HLA-disparity.

Pilot studies on the use of autologous BMT as postremission therapy indicate that disease-free survival at four years is on the order of 50% (i.e. comparable to that of allogeneic BMT). Autologous BMT has the advantage of lower procedure-related mortality than allogeneic BMT (approximately 10–15%), but involves a high risk of leukemic relapse (about one half treatment failures). A recent trial by Zittoun et al. showed that both autologous and allogeneic BMT performed in first CR resulted in a significantly better disease-free survival than intensive consolidation chemotherapy. The projected rate at four years was 55% for allogeneic BMT, 48% for autologous BMT and 30% for intensive chemotherapy with no differences between allogeneic and autologous BMT.

Taken together, these results demonstrate the potential benefit of autologous stem cell transplantation for leukemic patients. However, the high incidence of leukemic relapse and delayed hematological recovery after ABMT have prompted several authors to investigate the use of mobilized PBSC.

**CD34** mobilized hematopoietic cells for support of intensive postremission chemotherapy of AML

As stated above, autologous transplantation of mobilized hematopoietic progenitor cells has been shown to reconstitute hematopoiesis more efficiently than BM-derived grafts. Moreover, early studies have failed to detect neoplastic cells in the peripheral blood of AML patients during the early recovery phase following...
remission induction/consolidation chemotherapy. Based on these reports, several investigators have addressed the question of whether the use of PBSC might result in a more rapid engraftment and a lower risk of relapse in AML patients. Relevant issues include the level of malignant cell contamination, the threshold dose of hematopoietic precursors (i.e. CFU-GM and CD34+ cells), the optimal timing for stem cell collection and the potential benefit derived from the use of selected cytokines to improve PBSC harvest.

To et al. studied leukemia-associated cytogenetic abnormalities in myeloid colonies derived from early remission PBSC. At a sensitivity level of 2:100 cells, they were not able to detect the t(8;21) in 293 samples examined. Recently, the more sensitive nested reverse transcriptase polymerase chain reaction (RT-PCR) was used to monitor minimal residual disease in the BM and peripheral blood of leukemic patients considered in remission by morphologic analysis. In that study, the authors found no differences between PBSC collections and simultaneous BM harvests. However, the degree of leukemic contamination may have been different among the PCR-positive groups because the two-step PCR is highly sensitive but not quantitative.

The issue of leukemia-free autograft was recently underscored by gene-marking studies showing that residual contaminating AML cells contribute to relapse when reinfused into patients. In this regard, leukemic recurrence remains the most frequent cause of treatment failure in AML patients and preliminary non-randomized clinical studies have not reported any advantage for ABMT over PBSC in terms of disease-free survival and overall survival rate (Table 4). Moreover, it could be argued that the interval between complete remission and myeloablative therapy may be shorter for PBSC patients, since the exclusion rate is higher for ABMT patients. Thus, randomized studies are warranted to rule out selection bias. Reinfusion of PBSC markedly shortened the period of marrow aplasia compared to purged and unpurged ABMT and reduced morbidity and resource utilization (Table 4). In particular, To et al. demonstrated an advantage of 11 and 19 days in the median time to achieve $0.5 \times 10^9$ neutrophils/L and $50 \times 10^9$ platelets/L, respectively, in favor of PBSC, whereas two other studies showed a more rapid neutrophil engraftment (28 days and 12 days, respectively) but not a highly significant faster platelet recovery. Most likely the acceleration of hematopoietic reconstitution derives from the reinfusion of a higher number of early pluripotent precursors and larger amounts of committed progenitor cells which require less time to reach maturation.

Early studies in acute leukemia indicated that an optimal CFU-GM dose of $50 \times 10^5$/kg body weight is required for complete and sustained reconstitution of BM function. Other reports and our own preliminary experience (Tables 4 and 5) have demonstrated similar results with lower CFU-GM numbers. These differences are probably related to different assay methods, where-
case of stem cell mobilization to take advantage of in vivo purging, coupled with a low number of apheresis procedures. To this end, it has been shown\textsuperscript{108} that administration of G-CSF during the recovery phase of consolidation chemotherapy in acute leukemia patients increased the peak level of CFU-GM and CFU-MIX by 5.8 and 4.3 times, respectively, compared to cycles were G-CSF was not used, and significantly prolonged the period of mobilization of stem cells. Although the role of cytokine treatment in AML patients is still under evaluation, preliminary data from a large cohort of leukemic patients suggest that G-CSF administration does not affect either the remission or relapse rate,\textsuperscript{109,110} whereas as a protective effect regarding relapse was shown in a randomized study.\textsuperscript{29}

In practice, leukapheresis sessions should be started after a careful evaluation of CD34\textsuperscript{+} cells in the peripheral blood by flow cytometry, at time of hematopoietic recovery from transient myelosuppression.

When adequate mobilization of progenitors (CD34\textsuperscript{+} cells > 10–15(\mu\text{L}) occurs, daily leukaphereses should be performed until the collection of a minimum number of \(2\times10^{10} /\text{Kg}\) CD34\textsuperscript{+} cells. The leukapheresis products should be evaluated for the presence of residual contaminating leukemic cells by immunophenotyping, karyotypic analysis and RT-PCR-based molecular analysis in those samples deriving from patients who had shown a specific phenotypic and/or molecular marker at diagnosis.

Moreover, because the content of circulating progenitors is generally low (< 1% of the mononuclear cell fraction), blood cell separator efficiency must be optimized. Collection efficiency (CE) is the percentage of cells entering the system that are eventually collected:

\[
\text{CE} (%) = \frac{\text{No. harvested cells}}{\text{No. of cells in preapheresis blood unit volume}} \times \text{processed blood volume}
\]

Acceptable CE should not be lower than 50%. CE is a useful parameter for evaluating blood cell separator effectiveness in harvesting PBSC, independently of the patient’s clinical condition.

**Purging in AML**

Considering the possibility of relapse from minimal residual disease (MRD) derived from autologous graft, several investigators addressed the issue of ex vivo purging of leukemic cells prior to stem cell reinfusion. Using the Brown Norway myelocytic leukemia rat model, it has been shown that injection of 25 leukemic cells induces leukemia in 50% of recipients.\textsuperscript{111} By applying the same mathematical model to humans, it has been suggested that reinfusion of 10,000 residual leukemic cells may result in a relapse rate as high as 50%.\textsuperscript{111} More recently, the role of residual tumor cells in clinical relapse after autograft was indicated by a clinical study involving 114 B-cell lymphoma patients with t(14;18) who received autologous marrow treated with a combination of monoclonal antibodies directed against B-cell associated antigens plus complement.\textsuperscript{112} Following purging, no lymphoma cells could be detected by PCR amplification of the bcl-2 gene in the marrow of 57 patients. Disease-free survival was increased in these individuals with respect to that of patients whose marrow contained detectable tumor cells. Moreover, the ability to remove lymphoma cells was the most important prognostic factor for predicting relapse (39% versus 5% of purged patients after a median follow-up of 23 months).\textsuperscript{113} Lastly, genetic marking of marrow cells with the neomycin-resistance gene has provided the formal proof that reinfusion of residual leukemic cells in AML patients contributes to a recurrence of the disease.\textsuperscript{113} Taken together, these findings demonstrate the need for effective ex vivo treatments to improve the outcome of autologous transplantation.

### Table 5. Experience of the Institute of Hematology “Seragnoli”, Bologna on autologous stem cell transplantation in AML patients. The results are expressed as median (range) and refer to AML (n=7) and RAEB-T (n=2) patients in I CR. PBSC collections were carried out following consolidation treatment. PMN and PLT recovery was recorded as such when the PB count was > 0.5 and 20 x 10\(^{9}\)/L, respectively.

<table>
<thead>
<tr>
<th>Apheresis products</th>
<th>Pts</th>
<th>PBSC collections</th>
<th>MNC (10(^{6})/Kg)</th>
<th>CFU-GM (10(^{5})/Kg)</th>
<th>CD34+ (10(^{6})/Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9</td>
<td>3(2-3)</td>
<td>7(2.8-11.6)</td>
<td>11.8(28-78.2)</td>
<td>7(1.1-17.5)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hematological reconstitution</th>
<th>Pts</th>
<th>day to PMN</th>
<th>day to PLT</th>
<th>PLT</th>
<th>RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8</td>
<td>14 (11-34)</td>
<td>18 (16-29F)</td>
<td>2.5 (3-10)</td>
<td>4 (2-5) 18 (14-38)</td>
</tr>
</tbody>
</table>

Abbreviations: MNC; mononuclear cells; RBC; red blood cells.
Peripheral blood stem cells in acute myeloid leukemia

recognize tumor-associated or cell-differentiation antigens not expressed by primitive cells responsible for hematopoietic engraftment have been selected for clinical trials after in vitro studies demonstrated a high purging efficacy with the use of complement, toxins and radioactive molecules. However, the heterogeneity of antigen expression on neoplastic cells and the lack of tumor-specific determinants may greatly affect the efficiency of antibody-based strategies for depletion of leukemic cells. In this context, the combination of two purging techniques has been explored with promising results. Other approaches include the use of phototoxic compounds which sensitize leukemic cells and specifically damage their cell membranes upon exposure to light, and biological methods based on the different proliferative patterns of leukemic cells and their normal counterparts when cultured ex vivo for several days in the presence of stromal cells.

Clinical trials

Clinical retrospective data supporting the beneficial effect of purging have been progressively accumulating. The Baltimore team provided indirect evidence in favor of purging by correlating effective CFU-GM colony elimination with a significant decrease in relapse. Furthermore, the same authors associated the sensitivity to 4-HC of CFU-L grown in remission with the posttransplant outcome. The 3 most recent surveys of the Leukemia Working Party of the EBMT group have consistently reported lower relapse rates following reinfusion of BM purged with mafosfamide, especially in patients transplanted within 6 months of CR and in slow remitters (> 40 days to achievement of CR), two patient populations considered at high risk of disease recurrence. In fact, the proportion of patients relapsing in the purged and unpurged groups was 29±5% vs 50±4%, respectively, following a conditioning regimen that included total body irradiation (p < 0.0001). More striking differences were found when considering only those patients autografted early after CR (16±6% vs 60±6%) and patients with an interval from diagnosis to CR greater than 40 days (20±8% vs 61±6%). Gulati et al. and Laporte et al. reported disease free survival which approximated 60% in AML patients in CR reinfused with autologous marrow treated with 4-HC and V-P16 and mafosfamide. In the same paper by the Paris group, it was suggested that the higher the initial content of BM CFU-GM, the lower the risk of transplant related mortality and the higher the chance of curing the disease.

Despite these results in favor of ex vivo elimination of contaminating leukemic cells, this procedure is not routinely performed in the majority of transplant centers. The main reasons might be: 1) the delay in hematological recovery after reinfusion of purged autografts; 2) the increase in the cost of ABMT; 3) the need for technical training and, most of all, 4) the lack of results derived from prospective clinical studies demonstrating the effects of purging. The feasibility of such trials is rather limited due to the high number of patients needed to obtain adequate statistical power.

So far, no data are available on purging protocols for PBSC collections; however, there are several reasons for proposing purging strategies for autograft of circulating autologous stem cells. Unlike solid tumors and malignant lymphomas, acute leukemias easily involve PB. Moreover, the number of hematopoietic progenitor cells (and possibly leukemic precursors) in PB autotransplants is usually at least 10 times higher than that of ABMT. Finally, as discussed above, the interval between CR and autotransplant may be shorter for PBSC patients, who may be thus considered high risk patients for relapse. Critical issues for designing experimental studies in this setting would include the proper assessment of MRD before and after purging, the establishment of reproducible technical protocols (cell concentration, RBC contamination, etc.), and careful evaluation of the toxicity of purging agents on PB progenitors, since the kinetic status of circulating stem cells following mobilization protocols (especially if CSFs are used) may be different from BM stem cells.

Conclusions

Autologous BMT has been widely used as consolidation therapy in AML patients in first or second remission; however, delayed hematopoietic engraftment occurs in a substantial proportion of patients resulting in significant morbidity and mortality. This is mainly due to the adverse effects of prior intensive chemotherapy on BM harvest, a decrease in the normal stem cell pool in leukemic patients and, perhaps, toxic damage to the marrow microenvironment. Thus, several groups have investigated the use of circulating progenitor cells with the twofold aim of reducing transplant-related toxicity and widening the number of potential candidates for myeloablative therapy with the support of autologous stem cells.

As for hematopoietic reconstitution, previous studies have provided evidence that PBSC transplantation may offer some advantages over BM autografting. However, crucial issues such as asynchronous mobilization of normal vs leukemic cells and potential contamination of PBSC collections, timing of PBSC harvest, detection of minimal residual disease, and the role of growth factors to accelerate hematological recovery and optimize stem cell collection have not been fully addressed.

In the present paper, the latest advances in this field have been reviewed with special focus on the biology of putative leukemic stem cells; operative guidelines have also been provided for those investigators who wish to design proper clinical trials on PBSC autotransplantation in acute leukemia.

Definitive answers regarding the role of PBSC will be coming from a large European randomized trial which is currently comparing peripheral blood stem cell and BM-derived graft.
References


Peripheral blood stem cells in acute myeloid leukemia


Peripheral blood stem cell transplantation for the treatment of multiple myeloma: biological and clinical implications

The aim of this review is to define the role of peripheral blood stem cell transplantation for the treatment of multiple myeloma. Therefore, we first review our present knowledge of this disease and then analyze the clinical trials based on the use of autologous bone marrow or peripheral stem cell transplantation. Optimal methods for peripheral blood stem cell transplantation will also be discussed.

Myelomagenesis

Multiple myeloma (MM), the prototype plasma cell malignancy, is characterized by the uncontrolled accumulation of plasma cells that replace normal bone marrow (BM) and by the overproduction of monoclonal immunoglobulins (Ig) and cytokines. A number of observations provided both by basic sciences and by clinical investigation allow us to place the disease and its unusual features in a more coherent perspective and to discuss new therapeutic options properly.

Epidemiology

The reported incidence of MM is available for the years up to 1982 and varies substantially in different countries. A striking increase in the incidence of MM has been noticed in the last thirty years and is only partially explained by amelioration of diagnostic capabilities. Between 1973 and 1990 an increase of 40% among people over 65 and of almost 15% among people under 65 has been recorded in US Cancer Death rates. Ethnic differences are apparent: the incidence is twice as high and the mortality rate has quadrupled in blacks, while doubling in whites. By contrast, rates among Asians are lower than those of whites living in the same geographic area.

Both genetic and environmental factors can be invoked to explain these ethnic differences. A significant increase has been detected in first-degree relatives of patients. Moreover, an increased risk has been observed to be associated with occupational and environmental elements that include farming exposure to pesticides, exposure to ionizing radiations, petroleum and rubber processing, as well as persistent (viral) infections. The main conclusion that can be drawn from a large body of observations is the necessity of discriminating the genetic roots from the environmental links of the disease. As a corollary, it may be asked which elements (genetic vs. environmental) are associated with the development of monoclonal gammopathy of undetermined significance (MGUS) and how they relate to the progression of MGUS to overt MM.

Cytogenetics and molecular biology

Two major pieces of information have emerged from cytogenetic studies. The first is that no consistent (yet not random) chromosome abnormalities have been detected in MM. The second is that numeric chromosome abnormalities are shared by MGUS and MM. Both facts lead us to ask what the prerequisite is and what the additional events are in the development of plasma cell malignancies. We still do not know the prerequisite events that lead to MGUS, to MM or to the evolution of MGUS into MM, or how they differ from collateral elements that simply favor the malignant process. Along the same vein, it is interesting that no known specific oncogene has yet been related to the development of MM or to the transition from MGUS to overt MM. The genes most commonly implicated in MM, like N-RAS, PS3 and retinoblastoma gene (RB), are all involved in the late stages of the disease.

If the same cytogenetic abnormalities are shared by two clinical situations as different as MGUS and overt MM, a patrolling role for the immune system can be envisaged in the natural history of plasma cell disorders. It is not unreasonable to suspect that if the immune system is able to keep a malignant clone...
target of the primary transforming event or where, when and how the unknown cellular target was hit by the transforming event. By contrast, the information available on the B cell population that feeds the downstream compartment of plasma cells and disseminates the disease indicates that this population has been generated in peripheral lymphoid organs during secondary T-cell-dependent Ab response, is programmed to home to the BM, and is committed to differentiate in close association with the BM microenvironment (Figure 2).\textsuperscript{21,22} On the basis of existing data, the most likely candidate for the physiological B lymphocyte equivalent of the MM plasma cell precursor is either a B memory cell or a plasma blast (Figure 1).\textsuperscript{14,15,23,24}

**Microenvironment and cytokines**

It is assumed that BM-seeking plasma cell precursors receive a differentiation signal after contact with the BM stromal microenvironment (Figure 2).\textsuperscript{25,26} Microenvironmental stromal cells play an essential role in the growth of plasma cell tumors both in mice\textsuperscript{27} and in humans.\textsuperscript{28} MM BM stromal cells are well equipped with a large series of adhesion and extracellular matrix molecules that mediate homotypic and heterotypic interactions and provide anchorage sites to cells selectively exposed to locally released growth factors.\textsuperscript{22,29,30} MM BM stromal cells produce cytokines like IL-6 known to play a crucial role in the evolution of the disease both in experimental systems, including IL-6 transgenic mice,
The role of autologous transplantation in the treatment of multiple myeloma

Investigations into the use of myeloablative therapy for the management of MM were pioneered in the mid-1980s and were stimulated by a persistent lack of progress in prognosis with conventional chemotherapy.\(^\text{38,39}\) As is the case with any experimental approach, initial trials were restricted to the treatment of patients with advanced refractory or relapsing disease and were focused mainly on defining the feasibility and toxicity of the procedure. These preliminary experiences were performed without the support of hemopoietic stem cells and demonstrated that high-dose melphalan (HDM), given intravenously (i.v.) at doses ranging between 100 and 140 mg/m\(^2\), yielded an increase in the complete remission (CR) rate, albeit at the expense of prolonged marrow aplasia and an unacceptably high early mortality rate.\(^\text{40-42}\) On the basis of these observations later studies with chemotherapeutic agents administered at myeloablative doses, and possibly added total body irradiation (TBI), were carried out with the support of autologous BM and/or peripheral blood hemopoietic stem cells (PBSC).\(^\text{43}\) Demonstration of the safety and relative efficacy of autotransplants in refractory MM\(^\text{41,44-46}\) encouraged subsequent application of this procedure in earlier phases of the disease\(^\text{45,46}\) and, more recently, in newly diagnosed patients as well.\(^\text{47,48}\) Over the past decade interest in this new treatment strategy has progressively grown, and the number of reported patients receiving autologous hemopoietic stem cell–supported myeloablative therapy is now approximately one thousand worldwide.

What lessons have we learned from this collective experience? It is difficult to draw firm conclusions from published trials since none of them were controlled and patient populations were different, as were the preparative treatments and the criteria used for evaluating tumor response. In addition, the bias introduced by patient selection and, in most of the cases, the lack of an adequate follow-up also helped complicate correct interpretation of the data. As a consequence, the exact role of autotransplantation in the management of MM still remains poorly defined and could be properly addressed only in controlled clinical studies comparing autografting and conventional chemotherapy. There are at least several such trials in progress at the moment in Europe and the United States. Data reported at the last ASH meeting in Seattle (1995) by the \textit{Intergroupe Français du Myelome} is promising and suggest an advantage for autografted patients in terms of increased CR rate and extended survival duration.\(^\text{49}\)

Obviously, these results warrant confirmation in larger independent series. For this reason, similar investigations are currently being conducted in the United States under the auspices of the National Cancer Institute. While the conclusions of these studies are being awaited, analyses of available transplant data have provided the following important information.
Transplant-related mortality

Transplantation of autologous hematopoietic stem cells following myeloablative therapy has greatly improved the tolerance to this modality of treatment and reduced the frequency of procedure-related mortality to less than 5–10% (Tables 1, 2). More recently, with the combined support of BM and PBSC followed by post-transplant administration of hematopoietic growth factors, early mortality was further decreased to approximately 1–2%.

Tumor response and overall survival

Increased tumor response, as recognized by an increase in the CR rate, has been reported by many groups following myeloablative treatments (Table 1). Basically, criteria for CR included both the disappearance of monoclonal plasma cells in the bone marrow, as evaluated on cytological smear examination or on flow cytometric analysis of DNA and cytoplasmic immunoglobulins, and no detectable M component by routine electrophoresis (later immunofixation was added). As would be logically expected, the CR rate varied in different studies, with a range between 20% and 80%, mainly depending on the use of more or less stringent definition criteria and the status of the disease at transplant (Tables 1 and 2). Moreover, the length of survival was generally extended after autotransplant, up to a median of approximately 3 to 5 years (Tables 1 and 2).

Choice of myeloablative therapy

Historically, the autotransplant experience in MM can be divided into two groups of studies: the ones using and those not using TBI as part of the conditioning regimen. With few exceptions, HDM, administered at doses ranging between 140 and 200 mg/m² has been the mainstay of both chemo-radiotherapy and radiation-free regimens for the following reasons: it shows a close dose relationship, is not cross-resistant with other alkylating agents and compared to cyclophosphamide, seems to offer a better chance of overcoming chemotherapy resistance. In the absence of controlled clinical studies comparing different preparative treatments in specific subgroups of patients, it is hard to draw any meaningful conclusion concerning the best conditioning treatment. The impression from the data available in the literature is that no particular regimen demonstrated clear-cut superiority over the others. Therefore the choice of treatment to be used as preparation for autotransplant should ultimately take into account the ability to perform TBI, patient eligibility for TBI (those previously irradiated on the spine cannot, in fact, be candidates for radiation), and the expected toxicity. HDM at 200 mg/m² probably has less acute extrahematological toxicity than regimens including TBI, a finding that formed the basis for exploring repeated administrations of this drug with tandem (or double) autotransplant programs.
**Remission duration**

As previously emphasized, myeloablative therapy requiring autologous hemopoietic stem cell support provides substantial antitumor response, especially in patients with good prognosis (see below). However, even in this favorable condition, a considerable relapse rate, approaching 60% at 3 years, is reported after autotransplant and no plateau is yet apparent on relapse-free survival curves.50-52 These results contrast with the 30% probability of long-term unmaintained remissions (and possible cures) reported by several groups for patients receiving allogeneic transplantation.60 It has been suggested that the lack of an immunological effect by the donor’s marrow T lymphocytes on the residual myeloma cells (i.e. graft-versus myeloma)51,52 and/or possible tumor reseeding may account for the apparently less durable duration of disease control following autologous as opposed to allogeneic transplantation. For this reason, important issues currently under clinical investigation in the autografting setting include further increases in the cytotoxic dose intensity level and depletion of tumor cells from the graft (see below).

**Prognostic variables**

Several important variables affecting the outcome of autologous transplantation have been identified (Table 3), including $\beta_2$-microglobulin ($\beta_2$-M) levels,45,47,50-52,56 pre-transplant disease status,45,51,52 age,45,51,52 performance status,45 Ig isotype45,51,52 and response to myeloablative therapy (e.g. attainment or non-attainment of CR).47,48 In particular, at multivariate regression analysis early mortality was reported to be highest among resistant relapsing patients, who also had the poorest response to myeloablative therapy and the shortest relapse-free survival duration.45 In contrast, low serum $\beta_2$-M levels, both at diagnosis and before autografting, and prior responsiveness to conventional chemotherapy conferred the highest CR rate, as well as prolonged relapse-free and overall survival durations.45,47,50-52,56 In addition, the timing of autotransplant also emerged as an important and independent prognostic parameter.56,64 This observation, on the one hand, was related to the generally reported improved outcome of patients transplanted earlier and, on the other hand, reflected the acquisition of multiple biological abnormalities in advanced phases of the disease53 that ultimately led to refractoriness even to high-dose therapy.64 Conversely, retaining sensitivity to high-dose therapy in earlier phases of MM assured better results, even in patients with primary refractory disease.45,47,65

**New perspectives under clinical investigation**

Based on the assumption that the failure of the conditioning regimen to eradicate the myeloma clone contributes most to post-transplant relapse, attempts to increase the intensity, and possibly the efficacy, of treatment by means of repeated courses of myeloablative therapy have recently been undertaken.46,53 The more rapid recovery of hemopoiesis assured by the combined use of PBSC and post-transplant administration of hemopoietic growth factors53 made the double transplant strategy feasible for approximately 60% of patients within one year.53 Results of pilot trials in primary refractory MM indicated that such an approach provided superior antitumor effect with improved event-free and overall survival durations with respect to a single transplant.53

A controlled clinical study comparing in a randomized fashion single vs. double autografting in newly diagnosed patients is currently underway in France. A similar trial is already in the early accrual stage in Italy. These studies will clarify in the next several years whether double transplant is associated with better prognosis. Alternatively, efforts to improve the clinical impact of autotransplant have been carried out by several groups and have included depletion of tumor cells from autografts by both negative selection of myeloma cells and positive selection of CD34+ hemopoietic stem cells,66,67 as well as post-transplant immunomodulation with interferon-α (IFN-α).47,49,68

In summary, hemopoietic stem cell-supported myeloablative therapy holds the promise of being a safe and effective treatment modality for MM. It yields better overall response and CR rates than conventional chemotherapy and may prolong the duration of survival.49

These conclusions, while encouraging, have been drawn mainly from uncontrolled studies carried out in select groups of patients and obviously warrant confirmation in controlled clinical trials which are currently under way. Therefore the next several years will clarify whether newly diagnosed patients with symptomatic MM can be routinely offered a single or double autotransplant as first-line or early salvage therapy for their disease.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Disease status</th>
<th>Refractory</th>
<th>Refractory + Responsive</th>
<th>CR</th>
<th>RFS</th>
<th>Surv.</th>
<th>CR</th>
<th>RFS</th>
<th>Surv.</th>
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<td>Low $\beta_2$-M</td>
<td>-</td>
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<td>Double transplant</td>
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<tr>
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</table>

*In multivariate analyses. Abbreviations: CT, conventional chemotherapy; RFS, relapse-free survival. *Ref.: 45,47,48,50,51,52,56,63,64,65.
While the results of these studies are being awaited, wider application of myeloablative therapy should probably be encouraged. Less heavily pretreated patients who did not respond to prior conventional chemotherapy are more likely to benefit primarily from autotransplant. In addition, data available from the literature do suggest that a superior outcome of this procedure can be anticipated in patients with chemosensitive disease and low tumor burden at diagnosis. Hence, ongoing clinical trials aimed at comparing conventional versus myeloablative therapy will also address the important issue of the role of autotransplant as early consolidation therapy in patients with intrinsically good prognosis. However, even in this favorable situation, recurrence of the underlying malignant disease remains a major problem and is the most common cause of treatment failure. For this reason, attempts to improve the clinical impact of autografting are under active clinical investigation.

In addition, many other problems regarding autologous transplantation for MM are still unresolved and should be formally addressed in future clinical trials. The most important of these issues include the choice of the best conditioning regimen, the optimal source of hematopoietic stem cells, the nature of relapse after autografting, the benefit from purging techniques and, finally, the likelihood of long-term disease control, especially for patients with molecularly defined CR.

Advantages offered by the use of PBSCs in the treatment of multiple myeloma

The use of PBSC in support of high-dose chemotherapy (peripheral blood stem cell transplantation) (PBSCT) is a valid alternative to autologous bone marrow transplantation (ABMT) in the treatment of both hematologic and non-hematologic neoplastic disorders. The growing interest in this procedure can be explained by: i) the possibility of mobilizing and collecting large amounts of hematopoietic progenitors, and ii) the rapid hematopoietic recovery observed following PBSCT.

Progenitor collection represents the critical step in the procedure. Daily monitoring of circulating CD34+ cells is an essential assay in predicting the number and timing of leukaphereses. Under proper conditions, only a few leukapheresis procedures are required to collect enough progenitor cells for marrow reconstitution after myeloablative treatments. Indeed, when circulating CD34+ cells rise to >50/µL, 1-2 leukaphereses may yield more than 50×10^6/CFU-GM/kg or 8×10^6/CD34+ cells/kg, which are considered the ideal values for optimal engraftment. In addition, it has been shown that large quantities of very immature elements, identified as long-term culture-initiating cells (LTC-IC), are mobilized as well.

Inclusion in the harvested material of very immature elements is responsible for the stable and durable marrow reconstitution observed in patients autografted with circulating progenitors. Thus the term PBSC, now commonly employed to identify mobilized hematopoietic progenitors, relies on both biological and clinical observations. As previously emphasized, the rapidity of engraftment is the major advantage offered by PBSC. Nevertheless, some authors argued that BM cells stimulated by growth factor administration might be at least as efficient as mobilized progenitors in ensuring rapid engraftment after myeloablative treatment. However, it has recently been shown that both committed and early progenitors are by far more frequent in PB than in BM during maximal mobilization. This conclusive observation points toward the preferential use of PBSC as the hematopoietic cell source for grafting purposes.

Since its introduction into clinical practice, PBSCT has been considered a promising approach for MM patients. Several studies have been designed in the last few years.

Reported results have shown a significant decrease in hemopoietic toxicity following this procedure as compared to ABMT, with recovery of granulocytes >0.5×10^9/L and platelets >25-30×10^9/L within approximately 2 weeks after autograft (Table 4). This was paralleled by rather good tolerability with rare early fatal events.

In addition, hematopoietic reconstitution by PBSC seems to be long lasting. MM patients may require repeated exposure to high-dose cytotoxic therapy. Reducing hemopoietic toxicity might be critical for the ultimate treatment outcome. Therefore, also for its long-term effect, PBSCT may have a positive impact on the life expectancy of those patients who are suitable for intensified chemo-radiotherapy treatments.

PBSC mobilization and collection in multiple myeloma

PBSC mobilization in myeloma patients

PBSC collection presents specific problems in patients with MM, where a decrease of progenitors in the bone marrow is due in part to a defect of the monocyte/macrophage activation pathway. In fact, CD34+ cells from MM patients grow normal numbers of colonies when stimulated by normal monocytes, while normal CD34+ cells have a reduced growth rate with MM monocytes. Another aspect is prior treatment. Repeated courses of chemo-radiotherapy are able to exhaust the pool of pluripotent stem cells, resulting in insufficient progenitor cell harvests. Studies specifically addressed at MM patients show that melphalan and treatment-free interval prior to PBSCT mobilization also have an influence on the release of progenitors into the peripheral blood, while the value of BM plasmacytosis as an independent factor is more questionable. As a consequence of these and other unknown factors, progenitor yields in MM are often unpredictable and lower than those observed in other malignant disorders.
Noretheless, cell harvests sufficient for one or two subsequent autografts are usually obtained,10,96,97,108,111-113 even in patients with markedly infiltrated marrow or primary resistant disease.108 To avoid the adverse influence of pre-mobilization treatment, PBSC collection in MM patients should be planned as early as possible in the course of disease, and alkylating drugs should be omitted in the primary treatment. It should also be kept in mind that heavily pre-treated patients require more leukaphereses and show slower platelet recovery than those with lower resistance.

PBSC also may be collected from patients with malignancies in steady state conditions;114 however, multiple aphereses are required with this method. Mobilization of progenitors with cytotoxic chemotherapy, hemopoietic growth factors, or a combination of the two is therefore generally preferred. The hematopoietic recovery that occurs after cytotoxic chemotherapy is accompanied by a PBSC rise that is proportional to the intensity of myelosuppression.102,108

In MM, chemotherapy alone with either HDM,115 or CHOP-like regimens112,113,116 or intermediate- to high-dose cyclophosphamide (Cy)117,118 has been used to mobilize PBSC. However, the failure rate, defined as the percentage of patients with a low progenitor cell peak in the blood or poor collections at the end of the apheresis program, was relatively high, ranging from 20 to 30%. Moreover, when using high-dose therapy protocols without growth factor support, one should consider that this implies an undue risk of severe toxicity.118

G-CSF17,119-121 and GM-CSF,75,112 as well as other cytokines are able to promote a dramatic rise of progenitors in the circulation. In a study of MM patients, administration of G-CSF at 10 μg/kg alone for six days induced a considerable increase in CFU-GM and CD34+ cells,111 with rapid recovery of counts after autograft. However, the use of growth factors alone in patients with neoplastic disorders produces little enthusiasm among hematologists. In fact, the spike of progenitor cells can be further amplified by combining growth factors with chemotherapy.71 Together with the demonstration that tumor cells are also mobilized by growth factors,122 this fact makes the combination of chemotherapy with G-CSF or GM-CSF the most reliable approach.86,109,112,113

In MM as in other diseases,74,77 the use of growth factors following cytotoxic treatment proves to be superior to chemotherapy alone in terms of progenitor cell yield,108,112 and significantly contributes to minimizing treatment toxicity.112,124 High progenitor peak levels are reported108 with high-dose chemotherapy, namely Cy at 7 g/m2 and etoposide (VP16) at 2 g/m2 followed by G-CSF or GM-CSF, and results seem to compare favorably with intermediate-dose Cy with or without G-CSF or GM-CSF. In conclusion, the optimal schedule for PBSC mobilization in MM has not yet been defined, though the most experience is with Cy at 7 g/m2 followed by G-CSF or GM-CSF. A review of the mobilization schedules reported so far in MM patients is presented in Table 6.

**Target of collections and cell monitoring**

CD34+ cell number and CFU-GM dose are both reliable predictors of engraftment time.125-129 The amount of PBSC necessary for engraftment is not clearly defined, but values of 10 to 20×10^6/kg CFU-GM represent a reasonable minimal dose.110,120 Irrespective of disease, rapid neutrophil engraftment has been reported with 20×10^6/kg CFU-GM or 2×10^6/kg CD34+ cells.125,130,131 However, a higher dose may be necessary for rapid and full platelet engraftment.105,126 In a recent study of MM autografts, Triot et al.139 found that platelet engraftment is influenced by previous history and cell dose. In patients with more than 24 months of chemotherapy before the autograft, they found a dose ≥5×10^6/kg to be required for rapid and full platelet recovery post graft. This number of CD34+ cells may be obtained with 1 or 2 apheresic runs, and only a minority of patients, namely those with prolonged pre-mobilization treatment, need a higher number of apheresic procedures. The number of cells needed is obviously greater when a double autograft is planned. When this is the case, since recovery after a second autograft is influenced by the same factors as the first,109 the number of CD34+ cells to be collected simply has to be doubled.
PBSC transplantation in multiple myeloma

Table 5. Key issues in mobilization and collection of PBSCs.

<table>
<thead>
<tr>
<th>Issue</th>
<th>Related aspects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deregulated or suppressed hematopoiesis</td>
<td>Decreased rate of progenitors, * defective monocyte activation, * prior chemotherapy</td>
</tr>
<tr>
<td>Methods for mobilization</td>
<td>Type (and doses) of chemotherapy, use of growth factors</td>
</tr>
<tr>
<td>Toxicity of mobilization therapy</td>
<td>Fever, allergy, infections, thrombosis</td>
</tr>
<tr>
<td>Kinetics of recovery after mobilization</td>
<td>Timed and asynchronous use of WBC, monocytes and platelets</td>
</tr>
<tr>
<td>Prediction of harvest</td>
<td>Prior chemotherapy, G-CSF test</td>
</tr>
<tr>
<td>Progenitor cell assays</td>
<td>CD34+ cells, GFU-C</td>
</tr>
<tr>
<td>Target of collections</td>
<td>Need for &gt;5×10^6/kg CD34+ cells in heavily pre-treated patients*</td>
</tr>
<tr>
<td>Apheresis method</td>
<td>Cell separator, volume processed, schedule of aphereses</td>
</tr>
<tr>
<td>Tumor contamination of harvest</td>
<td>Purging technique</td>
</tr>
</tbody>
</table>

requires skillful personnel and carries a substantial cost. The issue has been reviewed extensively by Rowley.136 Siena et al.133 initially suggested starting the collection program as soon as CD34+ cells were detectable in the peripheral blood. However, in terms of efficiency, the best collections are performed when CD34+ cells are at their peak. In practice, aphereses should be started as soon as the CD34+ cells in the blood exceed a given level. We suggest a value of 20 CD34+ cells/µL combined with a WBC level >1.0×10^9/L and a platelet count >30×10^9/L before starting collections.92,133 Mononuclear cells (MNC) in DNA synthesis also predict a good yield when their level in the blood is >5% (or >250/µL).137

Few studies report detailed data on apheretic PBSC collection in MM. Dimopoulos et al.109 began the aphereses when the MNC count went above 0.3×10^9/L, having as target the collection of >2×10^6/kg CD34+ cells. They were able to collect >3.0×10^6/kg CD34+ cells daily in patients with ≤4 months of prior chemotherapy, but the mean daily yield was uniformly lower (<1×10^6 CD34+ cells/kg) in patients with more than 12 months of chemotherapy. Tricot et al.59 initiated collections upon recovery of a WBC count >0.5×10^9/L, and assumed a target of >6.3,108/kg MNC to support two autografts. In a recent study113 aphereses were started as soon as the WBC count exceeded 5×10^9/L after a CHOP-like regimen followed by G-CSF, and >6×10^9/kg CD34+ cells were collected from all patients in 1 to 3 aphereses.

A predictive test with G-CSF, a single dose of 10 mcg/kg, followed by CD34+ cell monitoring on days 4 and 5 has been proposed.138 The study included patients with MM, but the sample was too small to draw any conclusions. Steady-state CD34+ cell counts seem to predict the yield of PBSCs after mobilization with chemotherapy and G-CSF,139 but not after G-CSF alone.140 Table 7 shows the first apheresis day reported with different mobilization methods.98,108,111,112,117,141 It is clear that the CD34+ cell peak occurs very early (approximately day 5 or 6) during mobilization with growth factors alone. When chemotherapy is included in the mobilization schedule, the CD34+ cell peak day occurs later (approximately day 20), but the subsequent use of growth factors will shorten it by a week or so.

To conclude, we suggest (Table 8) mobilizing PBSC with the combination of chemotherapy and growth factors (G-CSF or GM-CSF), and performing serial determinations of CD34+ cells in the blood. Aphereses should be started as soon as the level of CD34+ cells exceeds 20/µL, and collections should be performed daily with twice the blood volume processed each time. Continuous-flow separators are to be preferred. As target for collections, the figure of 2×10^6/kg CD34+ cells per single autograft should be adopted for patients with <24 months of prior chemotherapy, while a greater number (>5×10^9/kg) should be collected in patients with a longer treatment history.

### Assessment of myeloma cells in the peripheral blood and role of ex vivo purging

PBSC collections are generally believed to have lower tumor cell contamination than BM harvests in cancer patients eligible for autografting. Moreover, the use of circulating progenitor cells has shown more rapid hematopoietic reconstitution than reinfection of BM-derived cells, thus reducing the incidence of serious infections and virtually eliminating mortality.142 Consequently, PBSC is widely used after myeloablative therapy for the treatment of myeloma patients.53,56,58 However, myeloma-related B-cells bearing the same idiotypic determinant as the neoplastic plasma cells have been identified in the blood of MM patients under steady-state conditions,143,149 and they may play a crucial role in the pathogenesis of the disease.144,147 Therefore in this chapter we will review the published data concerning: i) the presence of MM elements in PB and their kinetics in response to mobilization protocols; ii) methods for myeloma cell assessment; iii) methods for ex vivo removal of contaminating tumor cells and the role of purging with respect to disease relapse.
Identification of circulating myeloma cells

Circulating B-cells belonging to the malignant clone were originally thought to be pre-B-cells on the basis of the surface expression of the CD10 (CALLA) Ag, an endopeptidase present on all fetal pre-B and B-cells, on adult pre-B-cells and their neoplastic counterparts. However, the CD10 Ag has also been found on activated adult pre-B-cells and their neoplastic counterparts.148 By physical parameters, CD19+ cells include a small and a large subset that are mainly late B-cells (pre-plasma cells) coexpressing CD20, CD10, CD45RO and CD24 Ag.148 The majority of large B-cells also express the CD56 Ag and high density CD38, whereas small lymphocytes show only minor expression of these 2 antigenic determinants. This phenotypic profile (i.e. CD19+ CD20+ CD38++ CD56–) is not found in normal resting B-cells. Interestingly, malignant cells were detected at diagnosis, irrespective of tumor burden and stage of disease, and treatment had no detectable effect on the large B-cell subset. Conversely, a significant decrease in the number of small B-lymphocytes followed chemotherapy, although these cells returned to baseline value once the therapy was discontinued. In this regard, it was previously shown that circulating CD19+ cells in MM express the functional multidrug transporter p-glycoprotein, thus suggesting that blood B-cells include a highly drug-resistant subset capable of inducing disease recurrence in myeloma patients. However, it should be noted that mature plasma cells do not always express the CD19 Ag, whereas the presence of the CD56 Ag discriminates clonal plasma cells from normal ones. In addition, the recently described monoclonal antibody B-B4 seems to be highly specific for BM and circulating terminal plasma cells.

More recently, the issue of myeloma cell contamination in leukapheresis products and the kinetics of circulating tumor cells in response to mobilization protocols have been addressed. These studies have consistently shown that the majority of PBSC collections, if not all, are contaminated by myeloma cells, which represent up to 10% of PB mononuclear cells by immunophenotyping and molecular analysis using polymerase chain reaction (PCR) with consensus oligonucleotides to the Ig heavy chain complementary determining region III (CDR III) (see below). The same pattern of contamination has been shown following high-dose Cy and either G- or GM-CSF, as well as after G-CSF alone, suggesting that growth factors for stem cell mobilization, regardless of the use of chemotherapy, may influence the expression of adhesion molecules associated with the myeloma cell membrane. Notably, kinetic analysis has demonstrated that following high-dose Cy and G-CSF, the concomitant mobilization of plasma cells and hematopoietic progenitor cells in the PB takes place with the maximum peak of neoplastic elements occurring within the optimal time period for collection of circulating CD34+ cells. Conversely, GM-CSF seems to reduce asynchronous mobilization of neoplastic elements and hematopoietic stem cells into PB, so that the contamination of actively proliferating myeloma cells is minimal in the first two days of apheresis.

Methods for assessment of minimal residual disease

A number of methods have been proposed to detect malignant cells in the blood of myeloma patients, including immunologic assessment by monoclonal antibodies, flow cytometry analysis of DNA and cytoplasmic Ig, studies on gene rearrangement. Each of these techniques has limitations in sensitivity and, in some cases, specificity. For instance, analysis of the hypervariable region of the Ig heavy chain (IgH) gene using a set of family-specific primers (IgH fingerprinting) requires 0.1% mononuclear cells.

Table 6. PBSC mobilization schedules in multiple myeloma.

<table>
<thead>
<tr>
<th>Authors</th>
<th>No. pts</th>
<th>Treatment</th>
<th>Growth factor</th>
<th>Day of progenitor peak</th>
<th>Peaked CD34/µL</th>
<th>Peaked CFU-GM/µL</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reiffers157</td>
<td>15</td>
<td>Cy 7 g/m²</td>
<td>no</td>
<td>nr</td>
<td>nr</td>
<td>nr</td>
<td>5/13 failures</td>
</tr>
<tr>
<td>Jagannath157</td>
<td>36</td>
<td>Cy 6 g/m²</td>
<td>no</td>
<td>nr</td>
<td>nr</td>
<td>nr</td>
<td>better with GM-CSF</td>
</tr>
<tr>
<td>Tanella156</td>
<td>11</td>
<td>Cy 7 g/m²</td>
<td>GM-CSF</td>
<td>15 (13-16)</td>
<td>126</td>
<td>6432</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Cy 2×1.2 g/m²</td>
<td>no</td>
<td>16 (16-18)</td>
<td>31</td>
<td>462</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Cy 2×1.2 g/m²</td>
<td>GM-CSF</td>
<td>14 (14-15)</td>
<td>77</td>
<td>2588</td>
<td></td>
</tr>
<tr>
<td>Ossenkoppele151</td>
<td>6</td>
<td>no</td>
<td>G-CSF×6 g</td>
<td>6</td>
<td>845</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Majidini152</td>
<td>7</td>
<td>VCAD</td>
<td>no</td>
<td>20 (17-30)</td>
<td></td>
<td>622</td>
<td></td>
</tr>
<tr>
<td>Vasta153</td>
<td>7</td>
<td>VCAD</td>
<td>G-CSF</td>
<td>13 (9-17)</td>
<td>22</td>
<td>893</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>VCED</td>
<td>G-CSF</td>
<td>13 (12-15)</td>
<td>70</td>
<td>2391</td>
<td></td>
</tr>
</tbody>
</table>

Legend. Cy: cyclophosphamide; VCAD: vincristine 1 mg, cyclophosphamide 4x500 mg/m², adriamycin 2x50 mg/m², dexamethasone 4x40 mg. VCED was identical to VCAD except that epirubicin 2x60 to 80 mg/m² was substituted for adriamycin. nr: not reported.
PBSC transplantation in multiple myeloma

Table 8. Recommendations for PBSC mobilization and their apheresis harvest in patients with multiple myeloma.

- Mobilization with chemotherapy + growth factors (G-CSF or GM-CSF)
- Serial CD34+ determinations according to institutional protocol
- Start apheresis when CD34+ cells in blood >20×10⁵/L
- Continuous flow separator, volume processed × 2 blood volume per run
- Collect at least 2×10⁶/kg CD34+ cells in patients with <24 months prior chemotherapy, at least 5×10⁶/kg CD34+ cells in patients with >24 months prior chemotherapy

Table 7. Day of cell peak and of first apheresis after PBSC mobilization in patients with MM. The addition of G-CSF or GM-CSF shortens the time to progenitor peak and consequently the time to apheresis. Mean number of apheresic procedures was lower when growth factors were employed.

Ex vivo purging of myeloma cells

Of the purging methods proposed for the elimination of MRD, the cyclophosphamide derivative 4-hydroperoxycyclophosphamide (4-HC) was the first used, on the basis of in vitro models demonstrating that this compound was able to eliminate BM-infiltrating MM cell lines. The main mechanism of action of 4-HC is based on a marked inhibition of myeloma cell growth, whereas it spares normal primitive hematopoietic cells. Moreover, this alkylating agent seems to induce the apoptotic death of tumor cells as well as activate immune mechanisms capable of controlling malignant cell proliferation. Because 4-HC does not affect surface antigen expression of myeloma cells, it is also a potential candidate for combined treatment with monoclonal antibodies (MoAbs), and preliminary in vitro data confirm the additive effect of these two purging techniques. Several MoAbs directed against tumor-associated or cell differentiation antigens not expressed by primitive cells responsible for hematopoietic engraftment have been selected for clinical trials after in vitro studies demonstrated high purging efficacy with the use of complement. GOV et al. developed a series of MoAbs that recognize mature plasma cells as well as B-cell precursors. One of them (8A) was conjugated with the ribosome-inactivat-
ing toxin momordin and clinically tested in 8 advanced stage MM patients to eliminate, \textit{ex vivo}, contaminating myeloma cells prior to ABMT.\textsuperscript{166} Although a marked tumor reduction was observed in all evaluable patients, none of them achieved CR and hematopoietic reconstitution following the myeloablative conditioning therapy was significantly delayed in 3 patients. These preliminary results showed the feasibility of this purging approach despite the poor selection of patients.

The same MoAbs were also employed \textit{in vitro} to remove myeloma cells through the avidin–biotin immunosorption technique, and the result was a greater than 3 log reduction in tumor cells with acceptable recovery of BM progenitors.\textsuperscript{168} More recently, Goldmacher \textit{et al}.\textsuperscript{167} reported the development of an anti-CD38 immunotoxin capable of killing 4–6 logs of human myeloma and lymphoma cell lines. The immunotoxin was composed of an anti-CD38 antibody conjugated to a chemically modified ricin molecule (blocked ricin). However, the CD38 Ag may not be the proper target for purging because it is strongly expressed on myeloma plasma cells (see above) and on committed hematopoietic progenitor cells,\textsuperscript{169} which are thought to be essential for rapid BM reconstitution. More specific antibodies directed either toward B-cells (anti-CD10 and CD20) or mature plasma cells (PCA-1) and complement were used to deplete tumor cells from the graft before ABMT by Anderson \textit{et al}.\textsuperscript{165} Following a TBI-containing conditioning regimen, a neutrophil count greater than $0.5 \times 10^9/L$ and an unsupported platelet count greater than $20 \times 10^9/L$ were reached at a median of 21 days (range 12–46) and 23 days (range 12–53), respectively. Similarly, immunologic reconstitution was not different from that commonly observed in cancer patients receiving unmanipulated autograft. This study documented that high-dose chemo-radiotherapy can produce a high response rate in pretreated patients with sensitive disease, and MoAb-based purging methods do not prevent rapid and sustained engraftment. However, the occurrence of relapses post-ABMT and partial responses will not define the need, if any, for marrow purging until more effective ablative strategies are developed. Taken together, these data demonstrate that the heterogeneity of Ag expression on neoplastic cells and the lack of true tumor-specific determinants may greatly influence the efficacy of antibody-based strategies for the depletion of myeloma cells. Alternatively, long-term Dexter-type marrow cultures have been used to select normal myeloid progenitors from heavily infiltrated myeloma BM, on the basis of the selective growth advantage of benign cells over malignant cells in this system.\textsuperscript{170}

Enrichment of hematopoietic CD34$^+$ cells has lately been shown to be an alternative approach to myeloma cell removal with a limited loss of normal stem cells. The CD34 Ag is a 110–120 kD glycoprotein that is mainly

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**Figure 4.** Schematic representation of the genomic region of rearranged CDRIII of IgH gene and further utilization of the PCR product for detection of MRD. For further details see text.
expressed on the earliest identifiable precursor cells and committed myeloid progenitors. In normal individuals, CD34+ cells represent 1% to 4% of the mononuclear cell fraction in the BM, whereas they are barely detectable in the PB. In addition, the CD34 Ag is not expressed on the surface of mature plasma cells in MM, although the possibility that this glycoprotein may be present on clonally less differentiated B-lymphocytes is still a matter of debate. As reported above, recent data support the hypothesis that MM originates in the later stages of B-cell differentiation when B lymphocytes have lost the CD34 Ag, whereas other studies have found CD34+ cells to be part of the neoplastic clone. It should be underlined, however, that reverse transcription-PCR, which was used to detect MRD in those studies, is an extremely sensitive technique, and the potential contamination of the CD34+ cell fraction by unwanted cells should be carefully avoided.

In this respect, Vescio et al. did not find IgH gene clonal rearrangement in collections of 99.99% pure CD34+ cells obtained after using a combination of two methods of stem cell purification. Schiller et al. and Lemoli et al. reported the first studies on purified, CD34-selected PBSCs conducted in patients with advanced MM. A median of 4.65 and 4 × 10^6 CD34+ cells/kg were infused in the two trials with a median purity of 77% and 88.5%, respectively. The median time to neutrophil and platelet recovery was 12 days and 10 and 11 days, respectively, with no difference with respect to a group of patients receiving unmanipulated PBSCs.

Both reports utilized rigorously quantitative immunofluorescence and/or IgH gene rearrangement analysis, and tumor cell depletion ranging from 2.5 to 4.5 logs was achieved. However, the persistence of myeloma cells in the CD34+ cell fraction was documented by sensitive PCR assay in all cases heavily contaminated before positive selection of CD34+ cells. Thus an additional purging step may be necessary to achieve a virtually tumor-free autograft.

In this regard, studies aimed at optimizing myeloma cell depletion by positive selection of primitive CD34+ Lin-Thy+ cells have already been performed and clinical trials are currently in progress.

In summary, all these studies show the capacity of purging techniques to eliminate a substantial proportion of the myeloma cells from autologous grafts without affecting their engraftment potential. The clinical impact of purging on disease relapse remains to be determined in future randomized trials.

Post-transplant (immuno)therapy

In MM as well as in other hematologic malignancies, the primary objective of high-dose therapy with hematopoietic stem cell support is to prolong survival and possibly to cure an otherwise incurable disease. The aim of post-transplant therapy is to prevent recurrence of the disease while assuring good quality of life. From this latter point of view, there is no room for additional chemotherapy as a preventive means. In addition, high-dose chemotherapy itself involves a risk of secondary myelodysplastic syndrome or acute myeloid leukemia. This risk is apparently related to prolonged alkylating agent therapy prior to transplantation and would undoubtedly increase with additional post-transplant chemotherapy.

In the past few years interferon-α (IFN-α) has been extensively evaluated in the management of MM, either as part of the induction program or as maintenance therapy. Although controversial findings were frequently reported, several clinical trials showed a prolongation of the remission phase, and even of the survival duration, for patients receiving IFN-α after a favorable response to conventional chemotherapy. These results suggested that IFN-α might be particularly useful in patients with low tumor burden or minimal residual disease, and led to clinical investigations of this agent in the autograft setting.

The European Group for Blood and Marrow Transplantation (EBMT) has recently presented a retrospective study of a large series of MM patients treated with autologous stem cell transplantation. Interestingly, post-transplant treatment with IFN-α was independently associated with extended survival of responding patients, i.e. those achieving either CR or partial remission. Moreover, Powels et al. designed a randomized clinical trial aimed at comparing maintenance IFN-α therapy with no maintenance after HD and ABMT. The authors found that IFN-α prolonged remission and improved the survival after autotransplant, and that this effect was particularly marked in the group of patients achieving CR.

Maintenance IFN-α is usually started three months after transplant and is given sc at a dosage of 3 × 10^6 U/m², 3 times weekly. This dose usually induces mild hematological and non-hematological toxicity, thus allowing good quality of life. Available data indicate that about 50% of the MM patients who achieve CR and are then treated with IFN-α remain in remission four years after transplantation.

Alternatively, maintenance treatments aimed at prolonging the duration of disease control after transplantation may also include the administration of interleukin 2 (as nonspecific immunotherapy) or humanized anti-idiotypic monoclonal antibodies, which could allow selective killing of myeloma cells and might be particularly useful for controlling minimal residual disease.

References
4. Evaluating the National Cancer Program: an ongoing


6. Sawyer JR, Waldron JA, Jagannath S, Barlogie B. Cyto


cescence in situ identifies chromosomal abnormalities in plasma cells from patients with monoclonal gam


43. Cavo M, Benni M, Gozzetti A, et al. The role of haema


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Allogeneic hematopoietic stem cells from sources other than bone marrow: biological and technical aspects

Background and Objectives. Identification and characterization of hematopoietic stem cells in peripheral blood (PB) and cord blood (CB) have suggested feasible alternatives to conventional allogeneic bone marrow (BM) transplantation. The growing interest in this use of allogeneic stem cells has prompted the Working Group on CD34-positive Hematopoietic Cells to review this subject by analyzing its biological and technical aspects.

Evidence and Information Sources. The method used for preparing this review was informal consensus development. Members of the Working Group met three times, and the participants at these meetings examined a list of problems previously prepared by the chairman. They discussed the individual points in order to reach an agreement on the various concepts, and eventually approved the final manuscript. Some of the authors of the present review have been working in the field of hematopoietic stem cell biology and processing, and have contributed original papers published in peer-reviewed journals. In addition, the material examined in the present review includes articles and abstracts published in journals covered by the Science Citation Index® and Medline®.

State of Art. Several studies have now shown that hematopoietic stem cells collected from peripheral blood after the administration of G-CSF, or from cord blood upon delivery, are capable of supporting rapid and complete reconstitution of BM function in allogeneic recipients. Perhaps more importantly, reinfusion of large numbers of HLA-matched T-cells from PB collections or T-cells with various degrees of HLA disparity from CB did not result in a higher incidence or greater severity of acute graft-versus-host disease than expected with BM. Based on the data reviewed, operative guidelines for mobilization, collection and graft processing are provided.

Perspectives. It should be remembered that despite the growing interest, these procedures must be still considered as advanced clinical research and should be included in formal clinical trials aimed at demonstrating their definitive role in stem cell transplantation. In this regard, a large European randomized study is currently comparing PB and BM allografts. However, the possibility of collecting large quantities of hematopoietic progenitor-stem cells, perhaps with reduced allo-reactivity, offers an exciting perspective for widening the number of potential stem cell donors and greater leeway for graft manipulation than is possible with BM.

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Key words: hematopoietic stem cells, bone marrow, cord blood, peripheral blood, allogeneic transplantation, graft-versus-host disease

Allogeneic bone marrow transplantation has progressed from a highly experimental procedure to being accepted as the preferred form of treatment for a wide variety of diseases. There have been impressive improvements in this therapeutic procedure in the last two decades, but the most important advances probably took place in the last few years and concern the source of hematopoietic stem cells itself. Whereas this had always been by definition the bone marrow since the very beginning, identification of stem cells in peripheral and cord blood has now provided useful alternatives.

In 1994 the growing interest in the use of peripheral blood stem cells (PBSC) in the setting of allogeneic bone marrow transplantation induced the GITMO (Gruppo Italiano Trapianto di Midollo Osseo) to promote a Study Committee for evaluating the key aspects of allogeneic PBSC collection and transplantation. This Committee produced a list of recommendations that were published as a position paper in this Journal at the beginning of 1995. In summary, the authors strongly recommended the use of allogeneic PBSC in experienced centers, in well-defined clinical settings, and possibly – for the time being – in patients with advanced disease.

As the use of PBSC expanded both in the autologous and the allogeneic setting, expression of the CD34 antigen became increasingly important for their character-
PBSC mobilization and collection in normal donors

Until recently, the collection of hematopoietic cells for allogeneic transplantation has required general or spinal anesthesia and multiple punctures of iliac bones. However, marrow harvesting is not completely devoid of complications, side effects or patient discomfort. In a report on 1270 harvest procedures in Seattle, 6 donors suffered life-threatening complications and 10 showed significant operative site morbidity. As many as 10 percent of donations were associated with fever, and increasing donor age was significantly linked to poor cell harvest. In a different survey, 10 percent of donors recovered completely from marrow donation only more than 30 days after the procedure.7

PBSC transplantation represents an alternative approach. In autologous transplantation peripheral blood is now replacing bone marrow as a source of progenitor cells. The advantage is quicker hematopoietic recovery8,10 with consequently fewer complications and shorter hospital stay.

In the autologous setting, PBSC can be collected after mobilization with chemotherapy,11,12 growth factors, 13 or a combination of the two.14 In a randomized study, leukaphereses created less anxiety and pain than bone marrow harvest.15

Table 1. Relationship between CD34+ cell yield and G-CSF dose in allogeneic PBSC donors. Only clinical experiences are reported.

<table>
<thead>
<tr>
<th>Authors (ref.#)</th>
<th>Donors</th>
<th>G-CSF dose/kg and days of administration</th>
<th>CD34+ collected x 10^6</th>
<th>Apheresis No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weaver (31)</td>
<td>4</td>
<td>16 µg, 4 d</td>
<td>9.6/kg</td>
<td>2</td>
</tr>
<tr>
<td>Korbinger (16)</td>
<td>9</td>
<td>12 µg, 7 d</td>
<td>13.1/kg</td>
<td>3</td>
</tr>
<tr>
<td>Bensinger (18)</td>
<td>8</td>
<td>16 µg, 6 d</td>
<td>13.1/kg</td>
<td>2</td>
</tr>
<tr>
<td>Schmitz (17)</td>
<td>8</td>
<td>5-10 µg, 5-6 d</td>
<td>6.7/kg</td>
<td>1-3</td>
</tr>
<tr>
<td>Russell (34)</td>
<td>9</td>
<td>6-8 µg, 2-4 d</td>
<td>4.7/kg</td>
<td>1-2</td>
</tr>
<tr>
<td>Majolino (27)</td>
<td>5</td>
<td>10 µg, 5 d</td>
<td>754</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>16 µg, 4 d</td>
<td>789</td>
<td>2</td>
</tr>
<tr>
<td>Tabilio (1996)</td>
<td>39</td>
<td>12 µg, 4-7 d</td>
<td>132.6</td>
<td>2-4</td>
</tr>
</tbody>
</table>

In allogeneic transplantation, the use of PBSC has been somewhat delayed by a possible increase in graft-versus-host disease (GVHD) as a consequence of the much higher number of lymphocytes in the graft inoculum, and by the need for a mobilization treatment for healthy individuals in order to obtain a good cell yield. However, the clinical experience of the last two years suggests that the incidence of acute GVHD is not increased with PBSC as compared to marrow, and that in healthy donors a sufficient cell number can be obtained by using growth factors alone, in particular G-CSF.16-20 As a consequence, the number of allogeneic PBSC transplants is increasing rapidly. The European Blood and Marrow Transplant Group (EBMT) registered only 12 PBSC allografts in 1993, but their number increased to 180 in 1994 and to 537 in 1995 (Gratwohl, personal communication).

Collection of PBSC in normal donors

On biological grounds, there are several means of mobilizing progenitor cells into the peripheral blood, but their ultimate modality of action is always detachment of the CD34+ progenitor cell from marrow stroma and endothelium, to which it is normally bound by interactions with different integrin-adhesion molecules.21,22 We may induce detachment either by an inhibition of the link between CD34+ cells and stroma, or by inducing a stress to the hematopoietic system capable of favoring the egress of progenitor cells from marrow to circulation. The former is obtained by means of monoclonal antibodies directed against adhesion molecules,22 while the latter is based on the use of a drug or a combination of drugs. Richman et al.23 demonstrated for the first time in man that chemotherapy-induced cytopenia is followed by a substantial increase of CFU-C in blood.

In normal donors, however, the use of chemotherapy is ethically unacceptable, and only growth factors must be employed. Though a number of other cytokines are able to induce an increase of PBSC, only G-CSF and GM-CSF have been utilized in clinical practice. G-CSF in particular has an excellent mobilizing effect when used alone.13,24-28 The pilot experience with stem cell mobilization in normal donors is the one reported by the Seattle group. They administered G-CSF 300 µg/day or 6 µg/kg/day to increase WBC levels in apheresis collections from granulocyte donors.29 A number of different schedules were later applied to mobilize PBSC for allogeneic transplantation. The results in terms of CD34+ cell collection are reported in Table 1. In most of the studies the G-CSF dose ranged from 10 to 16 µg/kg/day. With 16 µg/kg/day for 5 days, Weaver et al.31 collected 1.6 to 12.6 (median 9.6) x 10^6/kg CD34+ cells with two aphereses. All transplants were syngeneic, and recovery of 0.5x10^9/L granulocytes and 20x10^9/L platelets occurred on day 13 and 10, respectively. With the same dose administered for 4 days, Majolino et al.27 were able to mobilize (median) 147x10^6/L CD34+ cells on day 4, a 65-fold increase over the baseline level. The median collection was 754x10^6.
CD34+ cells and 270×10⁶ CD34+ cells with 2 aphereses. With 12 µg/kg for 6 days, Körbling et al.16 collected a mean of 13.1×10⁶/kg CD34+ cells with 3 aphereses, and their patients recovered >0.5×10⁹/L granulocytes on day 10 and >20×10⁹/L platelets on day 14. However, their short recovery times were also influenced by the absence of methotrexate from GVHD prophylaxis. The relationship between G-CSF dose and CD34+ cell mobilization is supported in part by the study by Dreger et al.,26 who compared 5 µg/kg/day and 10 µg/kg/day G-CSF in normal volunteers. They found 10 µg/kg to be superior in terms of progenitor cell yield. With higher doses the advantage seems to decline, and no statistical difference was found between 10 µg/kg for 5 days and 16 µg/kg for 4 days in a retrospective non-randomized study (Figure 1).22 Dührsen23 has suggested that the maximal effect in terms of progenitor cell increase is that obtained at a dose level of 10 µg/kg/day. This is also the dose recommended by the GITMO in its recently published guidelines.24

The number of apheresic procedures necessary for a good collection is critical for the donor, and may vary with the dose and schedule of G-CSF as well as with the volume processed. Bensinger et al.28 routinely employ a schedule of 16 µg/kg/day for 5-6 days in an effort to minimize the number of apheresic procedures. With this dose, a median of approximately 7×10⁶/kg CD34+ cells are obtained with a single apheresis performed on day 5. At the M.D. Anderson Cancer Center in Houston29 a schedule of 12 µg/kg/day for 4-6 days is used. With a single large volume apheresis the target CD34+ cell dose of >4×10⁹/kg is reached in nearly 80% of the donors. Russell et al.34 mobilized their donors with 6-8 µg/kg/day for 2-4 days. By daily monitoring of CD34+ levels, the target of 2.5×10⁶/kg CD34+ cells was achieved with a single 2-4 hour harvest in 12 out of 14 donors. With 24 µg/kg/day G-CSF for 4 days Waller et al.25 were able to collect 13×10⁶/kg CD34+ with a single apheresis; however, one donor suffered severe side effects and the G-CSF dose had to be halved.

The mobilization kinetics of PBSC under low daily doses of G-CSF has also been investigated. With 2.5 µg/kg/day G-CSF on days 1 to 6 followed by 5.0 µg/kg/day on days 7 to 10, a CFU-GM peak was obtained on day 6, but continuing G-CSF administration at 5 µg/kg/day did not increase the level of circulating CFU-GM.36

With a single G-CSF dose of 15 µg/kg a significant rise in CD34+ cells, CFU-GM and BFU-E was obtained,37 but the reported counts of 250/mL, 3.2×10⁹/mL and 1.75×10⁹/mL, respectively, are not comparable with those obtained with prolonged administration schedules. Bishop et al.38 reported their experience with G-CSF at 5 µg/kg/day. Aphereses began on day 4 of G-CSF administration. However, the target cell CD34+ dose of >1×10⁶/kg required 3 to 4 aphereses. With this method, median time to ANC > 0.5×10⁹/L was 12 days but all patients received G-CSF after the allograft.

With G-CSF doses ranging from 10 to 16 µg/kg/day, the progenitor cell peak occurs on day 4 or 5.2,3,5,6,12,17,18,20,27,32,38 Since the CD34+ cell level rapidly declines after growth factor withdrawal, it is highly recommended that its administration be continued till the end of apheresic harvests.

In both the Seattle and the GITMO experiences, the WBC peak occurred approximately the same day as the CD34+ cell peak. In the GITMO study,29 the level of CD34+ cells reached a peak of (mean) 135.5×10⁶/L CD34+ cells, a 19-fold increase over the mean baseline level. Lymphocytes also increased, doubling their counts on day 5. A number of CD34+ cells >4×10⁹/kg was collected in 51% of donors with a single apheresis, in 85% with two. Optimal collections are obtained on days 4 and 5 of G-CSF administration.28,38 It is likely that starting PBSC collection on day 4 is best when using 16 µg/kg/day, whereas day 5 is better when lower doses are employed (Figure 2).

In normal volunteers GM-CSF has found application less frequently than G-CSF. Lane et al.40 studied G- and GM-CSF alone and a combination of the two. The total number of CD34+ cells collected from the G-CSF group with a single apheresis was 119×10⁶, and was not significantly different from that collected from the group treated with G- and GM-CSF (101×10⁶ CD34+ cells), but both were greater than that from the group treated with GM-CSF (12.6×10⁶). However, a higher fraction of an early CD34+/HLA-DR+CD38+ cell population was found among the CD34+ cells after GM-CSF administration. Whether this early fraction is associated with more rapid engraftment is presently unknown.

Predictive factors for progenitor cell yield have not been studied in normal volunteers. Though there is anecdotal experience of donors failing to respond, only age was reported to influence the quality of collections in a single study.26
The number of PBSC necessary for rapid and stable engraftment is unknown. In the autologous setting a dose of \(>2 \times 10^9/\text{kg} \ CD34^+\) cells has been suggested,\(^{41}\) but the requirement might be higher in allogeneic transplantations as a consequence of the immunological mechanisms involved. In Seattle\(^8\) 4 out of the 53 normal donors yielded only 0.6, 1.49, 1.55 and \(1.74 \times 10^6\) CD34\(^+\) cells/kg. Despite the low cell numbers, successful engraftment was achieved in all cases. We suggest that collection of \(>4 \times 10^6/\text{kg} \ CD34^+\) cells is the target for a safe allogeneic transplantation. Lower doses, however, may be sufficient. A lower limit of \(>2 \times 10^6/\text{kg} \ CD34^+\) cells would be reasonable for those patients whose donors respond poorly to cell mobilization. There are currently no contraindications to cryopreservation of cells for later use after thawing. Though most centers currently infuse freshly collected apheresis products, one may consider the advantage of separating the mobilization/collection phase from transplantation in terms of logistics and patient safety.

**Side effects and toxicity of the procedure**

Early toxic effects of G-CSF in healthy donors are now well known. The GITMO survey\(^{39}\) on 76 healthy subjects aged 6 to 67 years receiving G-CSF for PBSC mobilization reveals that the side effects of G-CSF administration are acceptable, the only problem being moderate to severe bone pain in 13% of donors (Table 2).

For apheresis procedures there is no evidence that any serious complications are related to the apheresis apparatus, though significant local side effects of the aphereses can occur.\(^{34,39}\) Additional problems could include pneumothorax due to jugular vein cannulation and paresthesia.\(^{36}\) Nonetheless, donors who had previously given marrow mostly agreed that they preferred blood cell mobilization and collection to marrow harvest.\(^{34,38}\) A good policy would be to avoid the use of venous catheters. In autologous PBSC harvesting where mobilization treatment often includes chemotherapy, central venous catheter (CVC) occlusion necessitating thrombolytic therapy was the most commonly observed complication, occurring in 15.9% of CVC-aided collections.\(^{42}\)

Variations in blood counts mainly consist of a pronounced WBC increase, a moderate thrombocytopenia and a slight decrease of hematocrit values. In the Italian survey, thrombocytopenia from mild (<70 × 10^9/L) to moderate (<50 × 10^9/L) followed PBSC harvests in 40% and 10% of cases, respectively. WBC counts exceeded 50 × 10^9/L in 40% of cases, and 70 × 10^9/L in 8%. Bensinger et al.\(^{28}\) report their experience with 124 donors treated with G-CSF at various doses and scheduling. Forty-one were granulocyte donors, while 13 were PBSC syngeneic and 63 allogeneic donors. One donor had a myocardial infarction after the first apheresis, but he had a previous history of infarction. Thrombocytopenia was in part related to G-CSF dosage, in part to the volume of blood processed. A count <100 × 10^9/L never occurred in granulocyte donors receiving 4 to 12 µg/kg/day and multiple aphereses.

With higher doses of G-CSF, thrombocytopenia occurred in 5% of donors undergoing 1-2 aphereses and in 100% of those collected for 4 days. When the 4-day collection donors received their platelets back by a second spin of the apheresis product, the incidence of thrombocytopenia fell to 25%.

Biochemical abnormalities follow G-CSF administration and consist primarily of mild elevation of ALT, LDH and alkaline phosphatase.\(^{29}\) This last is directly related to the action of G-CSF on the granulocytic lineage.\(^{43}\) These abnormalities have no clinical effects.

At present, we have little data concerning the late effects of G-CSF administration in healthy donors;\(^{24}\) however, the growing interest in PBSC allogeneic transplantation causes the need for prospective studies on the donor population. This kind of study is difficult for statistical reasons. Recently, Hasenclever and Sextro\(^{45}\) presented a preliminary study on long-term risks of growth factor administration to healthy donors. In order to demonstrate a tenfold increase in leukemia risk, more than 2000 healthy PBSC donors should be followed for over 10 years. A control group of BMT donors of equal size would be necessary. Such a study can only be done on a multi-national basis. However, it is necessary to follow the PBSC donors regularly, and to register carefully any variation in their blood counts.

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**Table 2. Incidence and grading of side effects reported during G-CSF administration in 76 healthy donors from the GITMO.**\(^{39}\)**

<table>
<thead>
<tr>
<th>% donors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Absent</strong></td>
</tr>
<tr>
<td>Bone pain</td>
</tr>
<tr>
<td>Arthralgias</td>
</tr>
<tr>
<td>Headache</td>
</tr>
<tr>
<td>Fatigue</td>
</tr>
<tr>
<td>Fever</td>
</tr>
</tbody>
</table>

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**Figure 2. Variations of blood cell counts in normal donors during G-CSF treatment and apheresis collection of mononuclear cells.** The curves represent mean values. Data are those of 76 normal donors.\(^{49}\)
The studies on leukemia development after G-CSF treatment must be considered with caution. A Japanese group reported data on 170 children with aplastic anemia. Eleven out of the 108 receiving G-CSF had a transformation to MDS or leukemia, while this evolution occurred in none of the 62 patients not receiving G-CSF. Another study reports the evolution to MDS/leukemia in 13 patients with congenital neutropenia treated with G-CSF, with the occurrence of monosomy 7 in 10 of them. However, as suggested by Smith et al. in a study on the leukemic evolution of Kostmann’s disease, the fact that congenital neutropenia may evolve into MDS and AML under G-CSF treatment has no implication for normal donors, since it is the underlying hemopoietic defect that represents a pre-leukemic condition. In fact, in Kostmann’s disease not all the chromosomal aberrations involve chromosome 7, and when other abnormalities are detected leukemia does not develop.

The use of G-CSF for mobilization of PBSC in children should be considered with more attention. Though there may be a specific advantage in collecting PBSC from children in the case of considerable disparity in body weight with the recipient, the GITMO stated that such a practice should be discouraged in standard allogeneic transplants. This is also the opinion of the Italian Association of Pediatric Hemato-Oncology (AIEOP).

PBSC have also been employed for allogeneic engraftment in MUD transplants. A small series was presented by Ringdén et al. in Geneva. Six patients with high risk hematological malignancies received PBSC from unrelated donors, 4 of them as primary treatment and 2 for treatment of graft failure. For PBSC mobilization the donors received G-CSF 5 to 12 µg/kg and leukaphereses were performed using continuous flow devices. One donor complained of rib pain and one of nausea, dizziness and anxiety.

One advantage of using PBSC for MUD transplants could derive from the higher number of progenitor cells, with better engraftment and reduction of failures. For the donor, the chance of obtaining stem cells for unrelated transplants without the need for general anesthesia is certainly appealing, and would probably encourage more volunteers to donate stem cells. It would also be easier to expand especially the number of donors belonging to ethnic minorities. Apheresis-derived mononuclear cells might be stored in liquid nitrogen and shipped when needed. Age limit for donors could be expanded. However, because of the limited experience with G-CSF mobilization in normal donors, National Marrow Donor Registries have not approved the use of PBSC as first choice. We expect this will remain the case in the foreseeable future.

In general, the use of growth factors for any purpose in healthy subjects should be considered experimental. The donor should be informed of the potential short and long-term risks of growth factors and leukapheresis, as well as of anesthesia, and he should be given the possibility of choosing. Donor consent should be asked on the basis of a protocol previously approved by an official ethical committee.

Characterization of CD34* hematopoietic progenitor cells mobilized into peripheral blood of normal donors by rHG-CSF

The preliminary results of clinical trials on allogeneic PBSC transplantation have demonstrated the capacity of G-CSF to mobilize true stem cells capable of long-term reconstitution of marrow function. Moreover, similarly to autografting, the most striking finding of PBSC transplantation has been the faster recovery of hematopoiesis after myeloablative conditioning regimens as compared to transplantation of BM-derived stem cells. Thus, clinical investigators asked the question of whether circulating progenitor cells may differ from their BM counterparts with respect to kinetic status, immunophenotype, frequency of both committed and primitive precursors, and their proliferative response to colony stimulating factors (CSFs).

One early report has shown a high expression of myeloid antigens on PB CD34+ cells (i.e. CD33, CD13) at the expense of B-lineage-associated antigens (i.e. CD10, CD19, CD20), coupled with a high colony-forming capacity of G-CSF-stimulated apheresis products. Moreover, Roberts and Metcalf have clearly shown in an animal model and in humans that only a small minority of mobilized PBSC undergo active DNA synthesis, whereas BM cells contain more than 30% of S-phase clonogenic progenitors. This finding, coupled with the lack of expression of CD71 antigen (transferrin receptor) and the Rhodamine 123 dull status observed in CD34+ cells from cancer patients mobilized with G-CSF, has suggested that PB progenitors may be functionally inactive since they are in deep G0-phase of the cell cycle.

However, these results are somewhat in contrast with clinical data indicating rapid BM recovery after autologous and allogeneic transplantation and the experimental evidence that circulating CD34+ cells represent an optimal target for efficient retroviral infection requiring cell cycling for integration. In addition, it is very important to assess the kinetic profile of the CD34+ cell fractions which are believed to ensure permanent engraftment after PBSC allografting, such as cells phenotypically identified as CD34+/CD38-, CD34+/CD33-/HLA-DR- or very primitive progenitor cells capable of generating clonogenic precursors in secondary semisolid assay after 5 or more weeks of liquid culture, long-term culture-initiating cells (LTC-IC). In this regard, defective long-term repopulating activity of early BM cells induced to S-phase by cytokines has recently been shown.

To further elucidate the phenotypic profile and functional and kinetic characteristics of G-CSF-mobilized hematopoietic progenitor cells, highly purified CD34+ cells from the apheresis products of normal individuals undergoing PBSC collections for allogeneic transplantation were recently analyzed. The results were then com-
pared with those obtained on CD34+ cells enriched from the BM of the same donors under steady-state conditions and after G-CSF administration on the same day as PBSC harvest.\textsuperscript{55} The results confirmed the expression of CD33 and CD13 antigens on a higher percentage of circulating CD34+ cells compared to BM cells (91±31% SD and 85.3±10% SD versus 51.1±21% SD and 64.6±25% SD, respectively; \( p < 0.05 \)) and the significantly lower expression of the B-cell associated antigen CD19 (1.3±0.9% SD in PB and 12.4±12% SD in BM). However, a small but consistent proportion of very immature CD34+/CD38– and CD34+/HLA-DR– cells was readily identified in PB that was no different from BM-derived cells. When we compared primed PB CD34+ cells with those of steady-state BM, we reported the same frequency of colony-forming unit cells (CFU-C). However, both myeloid (CFU-GM) and erythroid (BFU-E) circulating precursors showed increased responsiveness to single growth factors (e.g. IL-3) or combinations of G-CSF/SCF or IL-3/SCF. Analysis of cell-cycle distribution of PB and BM CD34+ cells (Figure 3) demonstrated a negligible proportion of mobilized CD34+ cells in S/G1 phase. However, the vast majority of circulating CD34+ cells were found to be actually cycling, being in G1-phase with a tendency, although not statistically significant, toward the recruitment of primed CD34+ cells out of G0-phase. Moreover, it was observed that G-CSF treatment provided CD34+ cells with a little, yet significant, protection from programmed cell death.

Functional characterization of G-CSF mobilized primitive cells

Using the LTC-IC assay, which allows the detection of very primitive progenitors, it was found that PBSC generate a higher number of CFC after 5 and 8 weeks of long-term culture than their bone marrow or cord blood counterparts. Also, the frequency of 5-week-old cobblestone area-forming cells (CAFC), a surrogate of LTC-IC measurement, within PBSC is similar at week 5 to that of BM and cord blood and higher than the frequency in the latter tissues at week 8.\textsuperscript{56} This suggests that PBSC contain either an adequate (or even a higher) number of primitive progenitors (on a per cell basis) or a higher number of very primitive and therefore very potent cells able to give rise to a high number of daughter cells.

The leukapheresis product in fact is enriched in cells with a very primitive phenotype, i.e. CD34+ Lin– Thy–1+, and contain cells able to repopulate SCID-hu mice, that represent an in vivo model for studying the hematopoietic reconstitutive ability of a given population of cells.\textsuperscript{57} Nevertheless, even though in a cohort of heavily treated cancer patients\textsuperscript{58} the number of LTC-IC was found to be 2–10-fold higher after chemotherapy + GM-CSF than in steady-state collections; a high interpatient variability was observed and the proliferative potential of mobilized LTC-IC (measured as the number of CFC produced by single LTC-IC) was lower than BM or steady-state circulating LTC-IC, suggesting that mobilized LTC-IC are less potent progenitors than their bone marrow and blood counterparts. No correlation was found between the number of LTC-IC in the graft and the number of CFC or CD34+ cells, or with the speed of engraftment. All these findings together show that chemotherapy + cytokine treatment allows the mobilization of progenitors with short- and long-term reconstitutive ability, but it is also evident that previous radiotherapy or stem cell-toxic drugs tend to significantly reduce the number of CFC and LTC-IC that can be harvested by apheresis, even though they do not alter the ability of PBPC to engraft.\textsuperscript{58,59}

Increasing lines of evidence suggest that the faster engraftment after PBSC infusion might be related to both the proliferative status of mobilized progenitors and to the high number of committed progenitors infused. Both in the murine and in the human model, short-term G-CSF treatment increases the proportion of actively proliferating progenitor cells in the bone marrow but not in the blood, where CFC and CD34+ cells appear to be mostly in G0-phase but are easily recruitable into S-phase.\textsuperscript{55}

To directly quantitate the proportion of cycling LTC-IC from the blood of cancer patients undergoing chemotherapy +G-CSF, mobilized CD34+ cells were exposed to tritiated thymidine (\( ^3 \text{H}-\text{Tdr} \)) in a 16–24 hr suicide assay.\textsuperscript{60} At the end of the incubation period aliquots of cells were tested for surviving progenitors in a LTC-IC assay. After 16 hrs of incubation in serum free medium containing growth factors (Steel factor, G-CSF and IL-3) and in the absence of \( ^3 \text{H}-\text{Tdr} \), the number LTC-IC remained at input level (panel A). A lower proportion of mobilized LTC-IC is initially quiescent in comparison to normally circulating LTC-IC (% survival: 30±7, \( n=10 \), and 81±8, \( n=20 \), respectively), showing a cycling status very similar to that of BM LTC-IC (% survival: 21±6, \( n=11 \)) (Figure 4). Similar data were found on PBSC collections from normal donors.\textsuperscript{55} In fact, similarly to more mature progenitor cells, very few circulating LTC-IC were found in S-phase (1±3% SEM as compared to 21±8%...
adhesion molecules critical for mobilization and related to cytokine-induced cell-cycle transit. Moreover, pharmacological doses of steel factor determine a redistribution of stem cells in mice and reduce the avidity of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins on the MO7e cell line, with a consequent inhibition of the specific cell adhesion of MO7e cells to VCAM-164. Other adhesion molecules, like L-selectin and VLA4, might play a role in the mobilization of hematopoietic progenitors in primates.

**Stem cells from umbilical cord blood: biological aspects**

More than 20 years ago it was described in this Journal that hematopoietic progenitors circulate between the fetus and the placenta during gestation. However, placental/umbilical cord blood (CB) from human newborns was not considered as a source of stem cells for clinical use until Broxmeyer et al. enumerated the number of CFU-GM that could be collected from the CB remaining in the placenta after birth and suggested that the total number was sufficient for transplantation in pediatric patients. The Fanconi anemia patient who in 1988 first received a CB transplant from his HLA-matched sibling is still alive at the present time and cured from the hematological point of view, thus demonstrating the long-term engraftment capability of CB-derived stem cells.

In the past five years interest in the biological aspects and clinical applications of CB has grown since large CB banks for unrelated stem cell transplantation have been implemented in the USA and Europe, and more than 300 CB transplants have been performed. However, many aspects of the properties of CB stem cells are still elusive. It is remarkable that a unit of CB used for transplantation contains 1-8 x 10⁶ CD34+ cells and 10-120 x 10⁶ CAFC/LTC-IC (56,69-70), i.e. 1-2 logs fewer than the total number of CD34+ cells and CAFC usually infused into recipients of allogeneic BM or PBSC. On average, recipients of CB transplants are given 0.05-0.5 x 10⁶ CD34+ cells/kg bw., while it has been suggested that recipients of allogeneic PBSC must receive at least 2.5-5 x 10⁶ CD34+ cells/kg b.w. to obtain safe hematopoietic engraftment (71). On the other hand, despite the delay in the reconstitution of the megakaryocytic lineage, the rate of engraftment failure in CB transplant recipients is similar to that observed after BM or PBSC transplants. These observations have prompted a number of investigators to focus on the proliferation potential of CB stem cells. In an elegant study, Lansdorp et al.23 sorted CB-derived, CD34+CD45RA+CD71lo cells, defined as stem cell candidates. In liquid cultures supplemented with SCF, IL-3, -6 and Epo, these purified cells generated a number of CD34+ and mature cells significantly greater than that obtained in cultures of purified CD34+ CD45RAlo CD71hi cells obtained from adult donors. This advantage was clearly ontogeny-related, since the proliferative potential of purified CD34+ CD45RAlo CD71hi cells collected from fetal liver was superior to that of CB-derived cells. In this context, Hows et al.24 demon-

**Figure 4. Recovery and proliferative status of CFC and LTC-IC in steady-state normal blood and bone marrow and in the leukapheresis products of cancer patients obtained after chemotherapy + G-CSF.** The cells were cultured for 16 hrs in a medium containing serum substitutes, SF (100 ng/mL), IL-3 (20 ng/mL) and G-CSF (20 ng/mL) in the presence or absence of [3H]Tdr (panel B and A, respectively).
strated in long-term stroma culture that both progenitor cell cultures and the lifespan of cultures were greater in CB than in adult BM, and Payne et al. showed that the proportion of CD34+ cells that are CD38- (lin-) is significantly higher in CB than in other stem cell sources. In contrast to what has been documented in adult BM, Traycoff et al. demonstrated that LTC-IC and cells presumably capable of in vivo engraftment reside in the CD34+HLA-DR-Rh123dull fraction. The cycling status of CB progenitors is still a matter of debate. In fact, some investigators using the tyrosine suicide technique reported a higher frequency of cells in S phase, whereas others did not find any difference between CB and adult BM in the frequency of actively cycling progenitors. More insight into this area is especially necessary since CB is a very attractive target for the transfer of genes able to correct inherited or non-inherited diseases such as thalassemia, Fanconi anemia, ADA-deficiency, etc., and since entering S phase is required for gene transfer through safety modified retroviruses. Interestingly, a higher efficiency of retrovirus-mediated gene transfer has been reported in CB than in BM progenitors. One possible explanation is the particularly rapid exit from the G0/G1 phases of the cell cycle in response to cytokines described by Traycoff et al. in CB-derived CD34+ progenitors, which might also justify the ontogeny-related advantage in proliferative potential.

The functional meaning of these differences in the in vitro behavior of phenotypically defined CB and BM populations is not yet fully understood, but these findings represent an interesting parameter to consider when assessing the suitability of a CB unit for transplantation in pediatric or adult patients. So far, in fact, most CB transplant recipients have been pediatric patients weighing less than 50 kg. Sporadic reports of CB transplants in adult recipients have indicated that the time to myeloid lineage engraftment is comparable to that of BM recipients, whereas the delay in platelet reconstitution seems to be more pronounced than in pediatric CB transplant recipients. As described in the CB processing section, ex vivo expansion of CB progenitors prior to transplantation might be useful to hasten hematopoietic engraftment; however, since the long-term engraftment capability of ex vivo cultured cells might be lost or impaired, more work seems necessary to reach this important goal.

**CB collection**

Established advantages of CB banks over BM donor registries include the immediate availability of the frozen CB unit, minimal donor attrition, the presence of CB donors from minority groups that are poorly represented in BM donors registries, and a much lower incidence of CMV infected donors. In fact, the time from the request for a CB unit to finding a matched donor is on average less than 2 months, and less than 1% of CB units are contaminated by CMV.

Worldwide, the creation of large CB banks has prompted investigators to improve the methods for CB collection and fractionation. The first method described by Broxmeyer et al. included CB collection in heparinized tubes by gravity. Further studies indicated that this open system is much more prone to bacterial contamination than closed systems based upon CB collection in bags, as first proposed by Gluckman et al. Another approach, proposed by Turner et al., includes catheterization of the umbilical vein. However, in a recent report this procedure was found to cause significant contamination of the CB collection by maternal cells, including potentially harmful T cells. It has been demonstrated, moreover, that CDP/CDP-A have an advantage over ACD and heparin because the former can anticoagulate blood over a wider volume range. Figure 5 describes results obtained using the method of CB collection in closed bags while the placenta is still in utero. Briefly, after the birth the umbilical cord is doubly clamped 1-2 cm from the newborn and transected before the newborn is removed from the operative field. The free end of the cord must be accurately disinfected before CB collection by venepuncture of the umbilical vein. As shown in Figure 5, there is a strict correlation between the time of umbilical cord clamping, the volume and the total number of nucleated cells collected. If the clamping procedure is delayed to the second minute after birth, it seems difficult to collect systematically a number of nucleated cells sufficient for clinical use of the CB unit. In fact, after the birth the newborn is frequently positioned below the level of the uterus, and this determines the so-called transfusion effect from the placenta to the newborn. Interestingly, when newborns are delivered following the Leboyer method there is no transfusion effect since the newborn is kept above the level of the uterus. Under these circumstances, early clamping of the cord is not required for collection of CB for clinical use. At the present time there is no consensus among neonatologists and pediatricians about the more appropriate timing of umbilical cord clamping. However, cord clamping in the first 30-60° after birth seems adequate to most reviewers and recent data on the immediate follow-up of newborns who underwent early clamping of the umbilical cord and CB collection support the safety of this procedure. In this retrospective study, none of the newborns who had CB collected were reported to suffer from weight loss, fatigue while feeding, tachypnea and tachycardia, hypoxia, or cardiac or pulmonary disease with reduced arterial oxygen saturation. The sole significant difference between the group of 59 CB donors and the control group was a slight reduction of Hb values, which corresponded to a loss of about 15 to 20 mg of iron.

**CB processing**

As mentioned above, a CB unit to be used for transplantation contains remarkably fewer CD34+ progenitor cells than BM or PBSC collections. For this reason, during CB manipulation the loss of progenitor cells must be carefully avoided. In pilot projects for large scale bank-
ing, CB was in fact stored as unmanipulated whole blood. The high cost of this procedure, which requires large liquid nitrogen space, has fueled intense research to concentrate CB nucleated cells in a reduced volume. Among the methods recently proposed for CB processing, however, some included density separation by Ficoll109 or red cell sedimentation by means of animal gelatin69,100 i.e. reagents that are currently not (and probably will never be) licensed for use in humans by regulatory agencies like the the FDA. Consequently, procedures involving the use of licensed products like HES101,102 should be recommended and a maximum loss of 10–15% of progenitors accepted. In this context, it must be noted that a number of patients have already been successfully transplanted with red cell-depleted CB.103 Conversely, data on the engraftment potential of purified, CB-derived CD34+ cells are still poorly reproducible in the SCID–hu mouse model104 and totally lacking in humans, so for the time being the storage of purified CD34+ CB progenitors for clinical use is not recommended. However, it has been shown in vitro that the proliferation potential of purified CD34+ cells is markedly superior to that of the low density or Ficoll fraction.70,104 This finding has major implications for the possible ex vivo expansion of an aliquot of the CB unit prior to transplantation. Two different strategies have been evaluated: the goal of some authors was to obtain multiple lineage expansion of progenitors by means of cytokine combinations including SCF, FLT-ligand IL-1, IL-3, IL-6, IL-11, G– and GM–CSF,104–106 while others were interested in selected-lineage expansion of the myeloid107 or megakaryocytic lineage.108

Rubinstein et al.103 have recently proposed a new procedure for washing the CB unit prior to transplantation. Advantages of this approach include removal of free Hb from lysed red cells and a significant reduction in the DMSO infused, a molecule which is particularly toxic for pediatric transplant recipients.109 In vitro data indicate that the washing procedure may improve the engraftment potential of the transplanted cells, but this finding should be further confirmed in an in vivo model.

**Immunological features of cord blood lymphocytes**

The immune system which develops during fetal life is not fully competent at birth and continues the differentiation process after birth in response to various antigenic challenges. At least three important elements control the development of the immune system during fetal life, thus determining the peculiar characteristics of the cord blood lymphocyte (CBL)-mediated immune response: i) limited or even absent antigenic experience, ii) immaturity of the majority of lymphocyte populations, and iii) feto-maternal immunological interactions.110-113 These elements are believed to influence the immunological features most strictly related to cord blood transplantation (CBT) and, in particular, the capacity to develop alloantigen-directed reactivity, antimi- crobial immunity and anti-tumor immune surveillance.

As a consequence of poor antigenic experience during pregnancy, the majority of CBL are naive cells expressing the RA isoform of the CD45 molecule.112 The most prominent immunoregulatory function of CD45RA+ T lymphocytes is suppressor activity.113 These peculiar features of neonatal lymphocytes explain their incapacity to develop, both in vivo114 and in vitro,115 an immune response directed towards recall antigens (i.e. tetanus toxoid, influenza virus).

The immaturity of the CBL population is also a direct consequence of its poor antigenic experience. Compared to adult blood, the distribution of the most relevant CBL subpopulations is characterized by a reduced percentage of CD3+ mature T lymphocytes and by the presence of immature T and NK lymphocyte subsets which are not detectable in adult peripheral blood.116-117

B lymphocytes are present in a normal or even augmented percentage in cord blood as compared with adult blood, even though immunoglobulin production is limited to the IgM class.118 Other CB peculiar features related to their immaturity are low expression of adhesion/costimulation molecules such as CD11a (LFA-1), CD18, CD54 (ICAM-1), CD58 (LFA-3) antigens,113,117,119 poor expression of CD40 ligand on activated T lymphocytes,120 and reduced ability to secrete some cytokines (i.e. γ-interferon, tumor necrosis factor and interleukin-4).117

Spontaneous NK activity is reduced in cord blood as compared to adult blood, possibly because of the low expression of adhesion molecules, known to be useful in promoting the capacity of NK lymphocytes to adhere to target cells.121 On the other hand, antibody-dependent cell cytotoxicity (ADCC) and lymphokine activated killer (LAK) activity of cord blood reach values comparable to or even higher than those observed in adult blood.122 Moreover, recent data demonstrate that the innate

![Figure 5. Effect of the time of umbilical cord clamping on the mean (±1 SD) volume and nucleated cell count of cord blood collections (n=67). As in most European OB/GYN units, soon after birth the newborn is kept below the level of the utero, and delayed clamping of the umbilical cord is associated with a reduction of cord blood volume and cellular content.](image-url)
immunity directed towards Epstein-Barr virus-infected cells is remarkably high in CBL collected from the majority of neonates. The capacity of cord blood NK cells to be promptly activated in vitro suggests that innate immunity plays a key role in immune surveillance during fetal and perinatal life, as long as specific T-cell mediated adaptive immunity can be generated.

From an immunological viewpoint, pregnancy can be considered as a successful HLA-mismatched transplantation. It is well known that the feto-maternal, anatomic-functional barrier allows the reciprocal transfer of lymphocyte subpopulations. It is thus conceivable to hypothesize that a very effective immunological network acts to prevent fetal rejection and graft-versus-host reactions (GVHR). Several lines of clinical and experimental evidence support this hypothesis, in particular: i) CBL preferentially display suppressor rather than helper immunological functions and ii) both B and T lymphocytes maintain a state of hyporesponsivity towards noninherited maternal HLA molecules for a long time after birth. A further confirmation of this peculiar state of tolerance derives from a recently reported observation on the occurrence of acute GVHD in patients given CBT from donors who were disparate for the noninherited paternal allele, and on the absence of significant acute GVHD in recipients whose donors were disparate for the noninherited maternal allele.

The fetal/neonatal period has been postulated to represent a crucial time in ontogeny, during which T and B lymphocytes learn to discriminate between self and nonself through the development of a state of tolerance toward antigens they encounter. The concept of neonatal tolerance was recently re-examined in mice and it was demonstrated that induction of this peculiar state of anergy in neonatal cells. It is well known that the feto-maternal, anatomic-functional barrier allows the reciprocal transfer of lymphocyte subpopulations, feto-maternal immunological interactions, and neonatal tolerance may, altogether, contribute to the generation of a suppressive effect on CBL alloreactivity, thus permitting the use of HLA-partially matched donors for CBT. In agreement with this hypothesis, reduced proliferative and cytotoxic activity towards alloantigens was reported by several authors to be present in cord blood as compared with adult peripheral blood. However, normal CBL alloreactivity has been documented in other studies. The discrepancies observed between the above mentioned reports may depend on the high interindividual variability in the distribution of cord blood T and NK lymphocyte subpopulations. Interestingly, it has been recently reported that, even though proliferative response to alloantigen in a primary mixed lymphocyte culture (MLC) is comparable in adult and cord blood, restimulation in secondary MLC induces increased specificity and activity of adult alloactive lymphocytes and a state of unresponsiveness in CBL. These data strongly suggest that repeated in vitro stimulation with allogeneic cells amplifies the specific immune response of adult lymphocytes, while the same procedure induces a state of anergy in neonatal cells.

Several clinical and experimental data obtained in the setting of allogeneic bone marrow transplantation (BMT) demonstrate that there is a strong correlation between GVHD and graft-versus-leukemia (GVL) effect. Thus, due to their low alloreactive capacity (responsible for the reduced GVHR), CBL could be less efficient in mediating a GVL effect. As far as we know, no data concerning the identification of cord blood T lymphocytes capable of mediating specific anti-leukemic activity have been reported in the literature. This lack of information is not surprising since it is well known that the frequency of these cells is extremely low, even in the peripheral blood of healthy adult donors. However, some studies have recently demonstrated that innate anti-leukemic activity mediated by LAK cells and measured

Table 3. Methods of T-Cell depletion in clinical trials.

<table>
<thead>
<tr>
<th>Method of T-Cell Depletion</th>
<th>Cells Removed</th>
<th>T-Cell Depletion $(x \log_{10})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBA lectin and E-rosette depletion</td>
<td>T and B lymphocytes, monocytes, neutrophils</td>
<td>2.5 - 3.0</td>
</tr>
<tr>
<td>Multiple E-rosette depletions</td>
<td>T lymphocytes</td>
<td>2.0</td>
</tr>
<tr>
<td>Mouse MoAb (anti-CD2, CD8) + rabbit C’</td>
<td>T lymphocytes</td>
<td>2.0</td>
</tr>
<tr>
<td>Mouse MoAb (anti CD6) + human C’</td>
<td>T lymphocytes</td>
<td>1.5-2.0</td>
</tr>
<tr>
<td>Rat MoAb (CAMPATH-1) + human C’</td>
<td>T and B lymphocytes, monocytes</td>
<td>2.5</td>
</tr>
<tr>
<td>Anti-CD5 immunotoxin-Ricin A Immunomagnetic separation (anti-CD3, CD8)</td>
<td>T lymphocytes</td>
<td>2.0</td>
</tr>
<tr>
<td>SBA lectin + immunomagnetic separation</td>
<td>T and B lymphocytes, monocytes</td>
<td>3.1</td>
</tr>
<tr>
<td>AIS CD5/8T-CELLector</td>
<td>T lymphocytes</td>
<td>2.5</td>
</tr>
<tr>
<td>Autologous Immunorosettes</td>
<td>T lymphocytes</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Poor antigenic experience, immaturity of lymphocyte subpopulations, feto-maternal immunological interactions, and neonatal tolerance may, altogether, contribute to the generation of a suppressive effect on CBL alloreactivity, thus permitting the use of HLA-partially matched donors for CBT. In agreement with this hypothesis, reduced proliferative and cytotoxic activity towards alloantigens was reported by several authors to be present in cord blood as compared with adult peripheral blood. However, normal CBL alloreactivity has been documented in other studies. The discrepancies observed between the above mentioned reports may depend on the high interindividual variability in the distribution of cord blood T and NK lymphocyte subpopulations. Interestingly, it has been recently reported that, even though proliferative response to alloantigen in a primary mixed lymphocyte culture (MLC) is comparable in adult and cord blood, restimulation in secondary MLC induces increased specificity and activity of adult alloactive lymphocytes and a state of unresponsiveness in CBL. These data strongly suggest that repeated in vitro stimulation with allogeneic cells amplifies the specific immune response of adult lymphocytes, while the same procedure induces a state of anergy in neonatal cells. Several clinical and experimental data obtained in the setting of allogeneic bone marrow transplantation (BMT) demonstrate that there is a strong correlation between GVHD and graft-versus-leukemia (GVL) effect. Thus, due to their low alloreactive capacity (responsible for the reduced GVHR), CBL could be less efficient in mediating a GVL effect. As far as we know, no data concerning the identification of cord blood T lymphocytes capable of mediating specific anti-leukemic activity have been reported in the literature. This lack of information is not surprising since it is well known that the frequency of these cells is extremely low, even in the peripheral blood of healthy adult donors. However, some studies have recently demonstrated that innate anti-leukemic activity mediated by LAK cells and measured
against long-term tumor cell lines, is comparable in adult and cord blood. They are also crucial. Even though the above mentioned studies are interesting, more experimental data and clinical observations are required to better define the potential GVL effect of CBL.

**PBSC processing**

**T-cell depletion**

The role of T lymphocytes in bone marrow transplantation is very complex. They are in fact responsible for GvHD, a major contributing factor correlated with morbidity and mortality in allogeneic bone marrow transplantation. Indeed when T-cells are removed from the graft before transplantation, the incidence of GvHD decreases sharply. They are also crucial in immune-hematological reconstitution after transplantation because slow or deficient reconstitution may lead to a high incidence of viral infections or other infectious complications. Ex vivo manipulation of the T-lymphocyte content is easier and T-cell depleted allogeneic transplants may in the future be followed by infusion of non-alloreactive T-lymphocytes or of specifically engineered lymphocyte clones exerting an antiviral or anti-neoplastic effect.

**Standard T-cell depletion techniques**

Over the past 15 years, several techniques have been developed for depleting T-cells from human marrow allografts. Table 3 summarizes the principles on which they are based and the degree of T-cell depletion each provides. Unfortunately, these methods may be time-consuming, cumbersome and difficult to standardize in different transplantation centers. Results are therefore often variable and no general consensus has emerged on the use and benefit of bone marrow T-cell depletion.

Very few data are available on the use of standard T-cell depletion methods in heterogeneous nucleated cell populations collected by leukapheresis from the peripheral blood of donors previously stimulated by G-CSF. This approach achieves approximately 3log10 T-lymphocyte depletion, as measured by cytofluorimetric assays. The main drawbacks are its complexity and lengthy laboratory times.

**Stem cell positive selection**

In principle, reducing T lymphocytes in the leukapheresis product by positively selecting CD34+ hematopoietic progenitors appears to be a valid technical alternative. Table 4 shows the basis of the main techniques for positive selection of hematopoietic progenitor cells. All use one monoclonal antibody which identifies an epitope on the human CD34 antigen. Separation is effected by collecting the antibody-sensitized cells onto a solid phase such as magnetic beads, plastic plates or columns of non magnetic particles, while non-target cells remain in suspension. Systems that utilize high speed flow cytometry to sort CD34+ cell populations have also been developed.

The CD34+ stem cell selection systems adopted in clinical use are based on immunoabsorption and indirect immunomagnetic beads.

Most clinical trials to date have been carried out with a Ceprate Stem Cell Concentrator (CellPro Inc., Bothell, WA, USA), which employs biotinylated 12.8 monoclonal antibody. The sensitized cells are applied to a column of avidin-coated polyacrylamide beads. Cells expressing

<table>
<thead>
<tr>
<th>Company</th>
<th>Method</th>
<th>Antibody</th>
<th>Detachment</th>
</tr>
</thead>
<tbody>
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<td>CellPro</td>
<td>Immunoabsorption</td>
<td>12.8</td>
<td>Mechanical</td>
</tr>
<tr>
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<td>Magnetic beads indirect</td>
<td>9C5</td>
<td>Chymopapain</td>
</tr>
<tr>
<td>Baxter</td>
<td>Magnetic beads indirect</td>
<td>9C5</td>
<td>PR34×™ oligopeptide</td>
</tr>
<tr>
<td>Dynal</td>
<td>Magnetic beads direct</td>
<td>B13C5</td>
<td>Anti-antibody</td>
</tr>
<tr>
<td>AIS</td>
<td>Panning</td>
<td>ICH3</td>
<td>(anti Fab of mouse MoAb)</td>
</tr>
<tr>
<td>Immunotech</td>
<td>Magnetic latex beads direct</td>
<td>QBEND10</td>
<td>Not required</td>
</tr>
<tr>
<td>Milteny</td>
<td>Magnetic colloid indirect</td>
<td>QBEND10</td>
<td>Not required</td>
</tr>
<tr>
<td>Terry Fox Laboratory</td>
<td>Magnetic Colloid Indirect</td>
<td>8G12</td>
<td>Not required</td>
</tr>
<tr>
<td>System x</td>
<td>FACS (high speed)</td>
<td>Various</td>
<td>Not required</td>
</tr>
</tbody>
</table>

Table 4. Methods available for stem cell positive selection.
the CD34 antigen are retained and unlabelled cells washed through the column with gentle mechanical agitation. The CD34+ cells are then removed from the beads and collected.

Using this system on the leukapheresis product, Link et al. recovered a mean of 30% CD34+ cells, with a purity of 70%. Peripheral blood CD34+ cells were reduced by 3 logs. Other investigators have reported similar results. This degree of T-cell depletion is known to prevent severe GvHD in severe combined immune deficiency (SCID) patients after matched or mismatched bone marrow transplantation. In leukemia patients undergoing matched transplants it may not be enough to eliminate GvHD completely without the concomitant administration of immunosuppressive drugs. The threshold number of clonable T-lymphocytes in the inoculum should be below 1 × 10⁶/kg b.w., which is difficult to achieve with one-step positive selection of hematopoietic stem cells. For a successful mismatched bone marrow transplant the T-lymphocyte threshold must be < 3-5 × 10⁶/kg b.w. in the inoculum because of the greater likelihood and increased severity of GvHD in these patients. On the other hand, infusing a number well below the threshold value could expose the patient to a high risk of graft failure.

Because T-cell depletion with the Ceprate system applied directly on the leukapheresis product does not reduce T-lymphocyte content from the graft by more than 3 logs, an additional T-cell depletion step is required.

In 10 patients with different types of leukemias, Aversa et al. employed an E-rosetting procedure before positively selecting hematopoietic progenitors with the Ceprate system. This combined method yielded a T-cell depletion of 4.3 logs in the graft and a mean CD34+ recovery of 50-60%. In small-scale experiments, Fernandez et al. applied the E-rosetting procedure after positive selection of CD34+ cells with this same Ceprate system to obtain a mean logₐ T-cell depletion of 4. Slaper-Cortenbach et al. achieved a median recovery of 42.7% CD34+ cells and a T-lymphocyte reduction of 2-3 logs in 13 haploidentical transplants for SCID and in leukemia patients by employing autologous immunosorbettes after positive selection of CD34+ cells.

CD34+ progenitor cell immunomagnetic selection (Baxter, Irvine, CA, USA) achieved a 3 log T-cell depletion in preclinical experiments. However, the main problem with this methodology was bead release from the target CD34+ cells. In fact release mediated by chymopapain may cause intractable cell clumping, particularly when a large number of cells are processed. Recently the PR34+TM stem cell releasing agent, an oligopeptide competing with the anti-CD34 monoclonal antibody for the release of the CD34+ cells from the magnetic beads, has also been proposed. Preliminary results showed a reduction of non-target T cells by a factor of 2-3 logs with yields of CD34+ cells ranging from 31.1 to 85%.

In conclusion, positive selection of CD34+ cells with the Ceprate system reduces the graft T-lymphocyte content under the threshold of risk for GvHD only when combined with standard T-depletion techniques such as E-rosetting with sheep red blood cells or autologous immunorosetting. Indirect immunomagnetic systems have to be evaluated more precisely for the use as a T-cell depletion system.

**Immunogenic activity of CD34+ hematopoietic cells**

Autologous transplantation of selected CD34+ cells induces rapid and complete hematologic reconstitution in myeloablated patients. In addition, isolation of CD34+ cells can be considered as an ex vivo means of purging neoplastic cells from the marrow or peripheral blood of patients with solid tumors or hematologic malignancies.

In the allogeneic setting, selection of CD34+ cells may be aimed at depleting donor T-cells and professional antigen-presenting cells (APC) such as monocytes, activated B-cells and dendritic cells, which are very potent stimulators of T-cell responses. Dendritic cells constitutively express the B7-2 (CD86) costimulatory molecule and upregulate B7-1 (CD80), B7-2 (CD86) and other molecules upon activation. Furthermore, they were recently shown to derive from CD34+ marrow or peripheral blood cells, and can be rapidly generated in vitro in the presence of a specific combination of growth factors. Since it has been demonstrated that T-cell receptor (TCR): antigen interaction, in the absence of

<table>
<thead>
<tr>
<th>Authors</th>
<th>TNC (x 10⁶/kg)</th>
<th>CD34+ (x 10⁶/kg)</th>
<th>CD3+ (x 10⁶/kg)</th>
<th>NK (x 10⁶/kg)</th>
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<tr>
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<td>300</td>
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<tr>
<td>Schmitz et al. (17)</td>
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<td>94.0</td>
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<td>13.1</td>
<td>385</td>
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<td>250</td>
<td>27</td>
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<tr>
<td>Rambaldi et al. (203)</td>
<td>8</td>
<td>6.9</td>
<td>279</td>
<td>N.R.</td>
</tr>
</tbody>
</table>

Table 5. Median value of nucleated cells, CD34+ cells, CD3+ cells and natural killer cells infused in patients undergoing allogeneic peripheral blood stem cell transplantation.

Legend. *Syngeneic transplants. N.R.=Not reported.
appropriate costimulation, may induce T-cell unresponsiveness or even apoptotic deletion.\textsuperscript{171,178–181} The alloantigen presenting function of CD34\(^+\) marrow cells was recently investigated to evaluate whether transplantation of purified CD34\(^+\) cells could minimize the immune sensitization of an allogeneic recipient.\textsuperscript{182} CD34\(^+\) marrow cells have been purified to >98\% by a two-step procedure consisting of a first enrichment on an immunoaffinity chromatography column, followed by fluorescence activated cell sorting. Cytfluorimetric analysis of purified CD34\(^+\) marrow cells revealed the expression of HLA-DR and CD86 on >95\% and 6\% of the cells, respectively. Primary mixed leukocyte cultures demonstrated that irradiated CD34\(^+\) marrow cells induce brisk proliferation of allogeneic T-cells isolated from HLA-DR incompatible donors. On the basis of previous reports,\textsuperscript{183,184} expression of CD18, the common chain of a family of leukointegrins, was investigated on CD34\(^+\) marrow cells and CD34\(^+\)/CD18\(^-\) cells were sorted to investigate whether this cell population was enriched in early hematopoietic precursors incapable of immunostimulating activity.

On average, 25\% of CD34\(^+\) marrow cells were CD18\(^-\) by direct immunofluorescent analysis. Purified CD34\(^+\), CD34\(^+\)/CD18\(^-\) and CD34\(^+\)/CD18\(^+\) marrow subsets were tested in bulk MLC with allogeneic T-cells, and it was observed that CD34\(^+\), CD34\(^+\)/CD18\(^-\) and unseparated marrow mononuclear cells have a similar capacity to stimulate a T-cell response. Conversely, CD34\(^+\)/CD18\(^-\) cells do not elicit any T-lymphocyte proliferation. Moreover, limiting dilution assay (LDA) experiments showed, on a per cell basis, that CD34\(^+\)/CD18\(^-\) and CD34\(^+\)/CD86\(^-\) marrow cells have a very poor ability to induce a T-cell response, as opposed to CD34\(^+\)/CD18\(^+\) and CD34\(^+\)/CD86\(^-\) marrow cells. Since most marrow LTC-IC were included in the CD34\(^+\)/CD18\(^-\) cell fraction, it was concluded that CD34\(^+\)/CD18\(^-\) or CD34\(^+\)/CD86\(^-\) marrow cells, may represent a useful source of progenitor cells for allogeneic transplantation because of their high stem cell activity combined with reduced immunogenicity. Data on normal human G-CSF mobilized CD34\(^+\) peripheral blood (PB) cells show that on average 30\% of the cells express CD18 and only 3\% express CD86, while functional in vitro results are consistent with what was previously observed in marrow. Thus, CD34\(^+\) PB cells can potently stimulate T cells, likely through the B7:CD28 pathway, and CD34\(^+\)/CD18\(^-\) PB cells still have very weak immunostimulating activity.

In a preliminary study sibling baboons were fully engrafted with allogeneic CD34\(^+\) marrow cells without GVHD, after receiving total body irradiation as conditioning regimen and standard GVHD prophylaxis.\textsuperscript{184} Development of mobilization regimens capable of increasing the number of peripheral blood hematopoietic stem cells in normal healthy donors allowed sufficient amounts of CD34\(^+\) PB cells to be harvested for allogeneic transplantation in humans. In fact, transplantation of enriched populations of G-CSF mobilized CD34\(^+\) cells resulted in rapid engraftment, similar to that observed in allogeneic PBSC transplants.\textsuperscript{185–190} Purification of CD34\(^+\) cells on the Ceprate column obtains on average a 3 log depletion of CD3\(^+\) T cells in the graft; however, several studies reported contrasting rates of acute GVHD. In particular, > 80\% of the patients transplanted with CD34\(^+\) PB cells in Seattle experienced aGVHD grade II–III after receiving a median number of 0.7×10\(^6\) T-cells/kg in the graft and GVHD prophylaxis with cyclosporin-A (CsA)\(_\text{A}\) methotrexate (MTX).\textsuperscript{187} Another study reported 2 cases out of 5 who died from aGVHD.\textsuperscript{188} By contrast, other groups reported a very low incidence of GVHD.\textsuperscript{189,190} One of the reasons for these disparities may be that small numbers of patients, often with different malignancies and clinical characteristics, are included in these studies. Nevertheless, two hypotheses could be addressed: the first one suggests that infusion of as little as 0.5–1×10\(^6\) CD3\(^+\) T cells/kg could be potentially capable of initiating GVHD, which would be prevented by further steps in T-cell depletion.\textsuperscript{189} The second hypothesis, still to be tested, is whether APC in marrow or peripheral blood could play a role in the development of GVHD by presenting allogeneic peptides to donor T cells.

In this regard, a subset of CD34\(^+\) cells in the graft may induce the activation and proliferation of a limited number of T cells, such as those still present after CD34 purification.

**Peripheral blood stem cells: immunological aspects**

Very few data are available on the effects of hematopoietic growth factors used to mobilize PBSC on peripheral blood lymphocytes. Weaver et al.\textsuperscript{193} analyzed the influence of G-CSF on peripheral blood lymphocytes from 13 individuals (11 autografts and 2 normal donors). In all cases they observed a slight increase in CD3, CD4, CD8, CD19 and CD20-positive lymphocytes, with a return to pretreatment values by days 4 and 5 of G-CSF administration. The change in the CD4/CD8 ratio was not statistically significant.

The expression of CD2, CD3, CD4, CD7, CD8, CD20, CD25, CD57 and HLA-DR antigens was evaluated during administration of G-CSF (12 \(\mu\)g/kg/day for 5–7 days) to healthy donors. No significant variations were observed in the different lymphocyte subsets, in the CD4/CD8 ratio or in the expression of CD25 and HLA-DR antigens (unpublished data). G-CSF administration does not cause direct activation of T lymphocytes in vivo. This might be expected because lymphocytes do not possess the G-CSF receptor.\textsuperscript{192} However, it is possible that activation could be caused by cytokine release from cells stimulated by G-CSF.

Other important aspects of the PBSC allograft include the lymphocyte content, particularly T lymphocytes and natural killer cells, in the apheresis product. Table 5 reports data on the total number of CD3\(^+\) lymphocytes derived from peripheral blood stem cells that were infused for allogeneic transplants. The number of
infused T lymphocytes was always 1.5–2 logs greater than that derived from bone marrow.193

The exact relationship between the T-lymphocyte content in the graft and the development and severity of GVHD remains unclear. A linear relationship between the number of T lymphocytes infused and the development of GVHD has long been hypothesized,139,194,195 but this correlation has not always been confirmed.196,197 Findings in allogeneic peripheral blood stem cell transplantation seem to suggest that the number of T lymphocytes is less important than donor–cell specificity in triggering GVHD.16,17

Another aspect of peripheral blood stem cell transplantation concerns the number of natural killer cells (NK) infused (Table 5) since they are important effector cells in graft-versus-leukemia activity.198 The number of infused NK cells is about 20 times greater in an allogeneic peripheral blood stem cell transplant than in a bone marrow graft.16,17 The question of whether this will translate into more potent GvL activity in patients allografted with peripheral blood stem cells compared to unmanipulated bone marrow cannot be answered at this time, but needs further study. However, preliminary data from a murine model demonstrated strong GvL activity for allogeneic NK cells without the induction of GVHD.199

An important technical point is the effect of freezing and thawing of the graft or of keeping the apheresis product at 4 °C overnight on T-lymphocytes inactivation. Van Bekkum200 described selective elimination of immunologically competent cells from bone marrow after storage at 4 °C. Eckardt et al.201 also noted that cryopreservation of allogeneic marrow may reduce the risk of acute GVHD. Selective depletion or induction of energy in GVHD-inducing cells was hypothesized.201 Storage of the apheresis product at 4 °C overnight does not modify the surface expression of the CD3, CD4, CD8, or the CD57 antigens in a significant manner (Tabilio, 1996, unpublished results); however, how cryopreservation affects the alloreactivity of peripheral blood stem cells needs to be investigated further.

Conclusions

Several studies have now shown that hematopoietic stem cells collected from PB after the administration of G-CSF, or from CB upon delivery, are capable of supporting rapid and complete reconstitution of BM function in allogeneic recipients.16-20,204,205 The faster recovery of hematopoiesis as compared to BM-derived allografts, together with a lower incidence of aGVHD than expected with BM transplantation, raises the question of whether PBSC collections may differ from conventional BM harvests with respect to the number of stem cells and their functional characteristics, lymphoid cell composition and T-cell reactivity. Moreover, recent clinical studies on transplantation of CB-derived cells from unrelated HLA-mismatch donors support the notion that sources of hematopoietic stem cells other than BM represent a feasible alternative to conventional transplantation. In this paper, the phenotypic, functional and kinetic features of circulating and CB hematopoietic cells were reviewed. We also emphasized the technical aspects of CB collection and processing, as well as the protocols for PBSC mobilization and collection from normal donors. Notably, novel data on the immunogenic and kinetic profile of BM and PB CD34+ cells may shed new light on stem cell biology and may help clinical investigators to design future trials on transplantation of purified hematopoietic progenitors.

It should be remembered that despite growing interest these procedures must still be considered as advanced clinical research and should be included in formal clinical trials aimed at demonstrating their definitive role in stem cell transplantation. In this regard, a large European randomized study is currently comparing PBSC and BM allografts. However, the possibility of collecting a large quantity of hematopoietic progenitor stem cells from PB, perhaps with reduced allo-reactivity, offers an exciting perspective for widening the number of potential stem cell donors and greater leeway for graft manipulation than is possible with BM.

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References

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6. Buckner CD, Clift RA, Sanders JE, et al. Marrow har-
8. Gratwohl A, Schmitz N. Introduction to First Interna-
tional Symposium on allogeneic peripheral blood pre-
cursor cell transplants. Bone Marrow Transplant 1996; 17(suppl 2):1-3.
9. To LB, Roberts MM, Haylock DN, et al. Comparison of haematological recovery times and supportive care requirements of autologous recovery phase peripheral blood stem cell transplants, autologous bone mar-
10. Indovina A, Majolino I, Buscemi F, et al. Engraftment kinetics and long term stability of hematopoiesis follow-
13. Sheridan WP, Begley CG, Juttner CA, et al. Effect of peripheral blood progenitor cells mobilized by fil-
macrophage colony-stimulating factor can mobilize sufficient amounts of peripheral blood progenitor cells for allo-
tion of peripheral blood stem cells and their apheresic collection for allogeneic transplantation. Haematolo-
16. Bensinger WI, Buckner CD, Rowley S, Storb R, Appel-
baum FR. Treatment of normal donors with recombi-
inant growth factors for transplantation of allo-
18. Bensinger WI, Prince TH, Dale DC, et al. The effects of daily recombinant human granulocyte colony stimu-
19. Weaver CH, Buckner CD, Longin K, et al. Syngeneic transplantation with peripheral blood mononuclear cells collected after the administration of recombi-
23. Waller CF, Bertz H, Engelhardt M, et al. Mobilization of peripheral blood progenitor cells (PBPC) for allo-
25. Schwinger W, Mache C, Urban C, Beaufort F, Toogl-
hofer W. Single dose of filgrastim (rhG-CSF) increas-
es the number of hematopoietic progenitors in the peripheral blood of adult volunteers. Bone Marrow Transplant 1993; 11:489-92.


42. Goldberg SL, Mangan KF, Klumpp TR, et al. Compli-

43. Fossa SD, Poulsen JP, Anders A. Alkaline phosphatase

44. Sakamaki S, Matsunaga T, Hirayama Y, Kuga T, Niit-


47. Kalra R, Dale D, Freedman M, et al. Monosomy 7 and

48. Smith OP, Reeves BR, Kempski HM, Evans JP. Kost-

49. Ringdén O, Potter MN, Oakhill A, et al. Transplanta-

50. Tjonnfjord GE, Steen R, Evensen SA, et al. Character-

51. Bender JG, To LB, Williams S, Schwartzberg LS. Defin-


64. Kovach NL, Lin N, Yednock T, Harlan JM, Broudy VC. Stem cell factor modulates avidity of a4b1 and a5b1 integrins expressed on hematopoietic cell lines. Blood 1995; 85:159-67.


71. Aversa F, Tabibio A, Terenzi A, et al. Successful engraftment of T-cell-depleted haploidentical “three loci” incompatible transplants in leukemia patients by...


Clinical use of allogeneic hematopoietic stem cells from sources other than bone marrow

Background and Objectives. Peripheral blood stem cells (PBSC) are being increasingly used as an alternative to conventional allogeneic bone marrow (BM) transplantation. This has prompted the Working Group on CD34-Positive Hematopoietic Cells to evaluate current utilization of allogeneic PBSC in clinical hematopoietic transplantation.

Evidence and Information Sources. The method employed for preparing this review was that of informal consensus development. Members of the Working Group met three times, and the participants at these meetings examined a list of problems previously prepared by the chairman. They discussed the single points in order to reach an agreement on different opinions and eventually approved the final manuscript. Some of the authors of the present review have been working in the field of stem cell transplantation and have contributed original papers in peer-reviewed journals. In addition, the material examined in the present review includes articles and abstracts published in journals covered by the Science Citation Index® and Medline®.

State of the Art. Review of the current literature shows that unmanipulated allogeneic PBSC give prompt and stable engraftment in HLA-identical sibling recipients. Despite the much higher number of T-cells infused, the incidence and severity of acute GVHD after PBSC transplant seems comparable to that observed with bone marrow (BM) cells. In comparison to the latter, PBSC probably ensure faster immunologic reconstitution in the early post-transplant period. Controversial results on the incidence and severity of acute GVHD have been reported when CD34+ selection methods are used. Prospective randomized trials are underway to compare the results of PBSC and BM allogeneic transplantation. In mismatched family donor transplants, T-cell depleted PBSC successfully engrafted immune-mevaluated recipients through a mega-cell-dose effect able to overcome the HLA barrier. Experience with PBSC in the context of unrelated donor transplants is currently anecdotal and prospective trials should be completed before that practice becomes routine. Finally, there is also limited evidence that, following induction chemotherapy, the addition of PBSC to donor lymphocyte infusion (DLI) for treatment of leukemia relapse after allogeneic transplant may improve the safety and effectiveness of DLI itself. Concerning cord blood (CB) transplants, the most interesting aspects are the ease of CB collection and storage, the low risk of viral contamination and the low immune reactivity of CB cells. This last property has its clinical counterpart in an apparently reduced incidence and severity of acute GVHD both in sibling and unrelated CB transplants, probably making the level of donor/recipient HLA disparity acceptable to what is required for transplants from other sources.

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Key words: hematopoietic stem cells, bone marrow, cord blood, peripheral blood, allogeneic transplantation, graft-versus-host disease

In the field of allogeneic transplantation the use of alternative sources of stem cells, namely peripheral blood stem cells (PBSC) and placental cord blood (CB) stem cells, is rapidly expanding. The European Group for Blood and Marrow Transplantation (EBMT) registered only 12 allogeneic PBSC transplants in 1993, but this number increased to 180 in 1994 and to 571 in 1995. Concerning cord blood (CB) transplants, following initial attempts considerable experience has now been achieved in the USA and Europe so that this modality is entering a phase of extensive clinical application, with hundreds of procedures registered both from sibling and unrelated donors. The ease of collection and storage of CB stem cells and the apparent tolerance-inducing property of CB CD8 suppressor cells are the most interesting aspects of this latter source.

Stem cells in peripheral blood and CB both possess a migratory status and differ in part from those found in bone marrow with respect to their biological and functional properties. However, while PBSC are envisaged as a means of improving results by increasing the number of cells available, placental CB stem cells open the realistic perspective of increasing the number of transplants thanks to the availability of thousands of cord samples for patients who lack a compatible donor among family
members.

This search for new stem cell sources also arises from the fact that allogeneic bone marrow transplantation still carries a high procedure-related mortality and disease recurrence rate. Cell dose has an influence on engraftment and chance of survival. In a retrospective study Bacigalupo et al.\(^9\) showed that patients with hematologic malignancies who receive allogeneic bone marrow grafts with higher CFU-GM numbers have significantly higher platelet counts on day +80 and a lower mortality rate than those who receive fewer CFU-GM. The effect of CMV infection on platelet counts also appears to be less pronounced when the number of progenitor cells is higher. The use of more hematopoietic progenitors would then result in improved transplant outcome.

The growing interest in allogeneic PBSC induced the Italian Bone Marrow Transplant Group (GITMO) to draw up a list of recommendations that were originally published in 1995\(^10\) and recently revised in light of the increasing experience gained worldwide during the last two years.\(^11\) Moreover, in a previous issue of this Journal\(^12\) a review article analyzed the biological and technical aspects of PB and CB stem cells. The key aspects dealt with were the mobilization and collection methods, the capacity for stable hemopoietic reconstitution, kinetic characteristics and immunological features.

### Historical background

Interest in allogeneic transplantation of PBSC began thirty years ago. In the late sixties, based on an earlier demonstration that autologous PBSC were capable of restoring irradiation-myeloablative hematopoiesis,\(^13\)-\(^15\) the Seattle group reported the first successful attempts at allogeneic PBSC transplantation in dogs\(^16\),\(^17\) and non-human primates.\(^18\) However, due to the high GVHD incidence, those experiments were unable to demonstrate long-term stability of the graft. Only a decade later did purification of PBSC and application of cytogenetic methods allow a group of German investigators\(^19\),\(^20\) to document in dogs the stability of donor-derived hemopoietic function for more than ten years after PBSC allogeneic transplantation.

A key issue at that time was the low number of progenitors in steady-phase peripheral blood, and clinical application of PBSC was limited to autologous transplantation in CML,\(^21\) where hematopoietic progenitors, mostly of the leukemic counterpart, circulate in high numbers and can easily be collected by apheresis without any prior stimulation. A further step was the demonstration that the PBSC level increases dramatically during the post-chemotherapy recovery phase;\(^22\) however, it was the advent of G-CSF and GM-CSF that provided the rapid expansion of PBSC technology and led to their use in allogeneic transplantation as well. The pioneer work of Socinski et al.\(^23\) and Gianni et al.\(^24\) established the ability of hematopoietic growth factors to expand the circulating progenitor cell pool either when used alone or in conjunction with chemotherapy. However, transferring growth-factor PBSC mobilization strategy from auto-graft patients to normal donors took some years. In fact, the safety of growth factors and the clinical applicability of allogeneic PBSC in terms of GVHD incidence and long-term engraftment represented serious reasons for caution. Clinical PBSC allogeneic transplantation began in 1989, when Kessinger et al.\(^25\) reported the first attempt in an HLA-matched recipient with ALL. The patient was an 18-year-old man in third remission after CNS and testicular relapse. His sibling female donor preferred to donate PBSC rather than bone marrow, and she underwent 10 apheresic procedures without any mobilization treatment. The apheresis product was T-depleted by sheep erythrocyte rosetting and infected after conditioning with high-dose Ara-C and TBI. The patient achieved full donor engraftment as demonstrated by cytogenetic studies but died on day +32, and sustained engraftment could not be demonstrated.

Four years later, in 1993, Russell et al.\(^26\) reported another transplant in a patient whose sibling donor presented an increased risk of complications from anesthesia. In this case, 10 mg/kg/day of G-CSF were given to mobilize PBSC. The cells were collected at two leukaphereses containing 36.8×10\(^6\)/kg CFU-GM and infused without any prior manipulation. Engraftment occurred rapidly and GVHD did not develop despite the high T-cell content of the graft sample. The same year, a group of investigators from Kiel University\(^27\) also successfully employed allogeneic PBSC. A 47-year old AML patient who failed to engraft after bone marrow transplantation from an HLA-identical sibling donor was infused with the unmanipulated product of 3 leukaphereses performed after the donor with 6 mg/kg/day G-CSF. Engraftment occurred on day +14, with moderate acute GVHD that responded to immunosuppression starting on day +18. Restriction fragment length polymorphism (RFLP) typing demonstrated full donor engraftment up to 60 days following transplantation.

Another important step was the five PBSC transplants from syngeneic donors performed in Seattle and reported in 1993:\(^28\) with a median of 9.6×10\(^6\)/kg CD34\(^+\) cells infused, the patients engrafted 0.5×10\(^6\)/L granulocytes on day 13 and 20×10\(^3\)/L platelets on day 10. In 1995 three separate reports appeared in the same issue of Blood, one from Seattle,\(^29\) a second from Houston\(^30\) and the other from Kiel;\(^31\) a total of 25 patients were allografted with PBSC from their HLA-identical sibling donors. Acute GVHD was apparently not increased in those series. Molecular analysis of engraftment\(^30\),\(^31\) furnished definitive proof of the experimental data suggesting that allogeneic PBSC contain true long-term repopulating stem cells. The high engraftment potential of PBSC was exploited by the Perugia team\(^32\) to successfully transplant leukemia patients from their haploidentical, three-loci-incompatible family donors through T-cell depletion. Finally, Ringdén et al.\(^33\) recently reported the use of allogeneic PBSC in selected unrelated donor transplants.

The kinetics of PBSC under cytokine mobilization was extensively analyzed in a previous review published in
Fludarabine has been associated with severe side effects in healthy donors employed for allogeneic PBSC mobilization. Variations in blood counts included a sharp elevation of WBC and CD34+ cells, while CD3+ cells and a moderate transitory thrombocytopenia. One problem, however, is the lack of data on the late effects of G-CSF. At the Geneva conference on allogeneic PBSC, Hasenclever and Sextro presented a feasibility study of long-term risk analysis. In order to demonstrate a tenfold increase in leukemia risk, more than 2000 healthy PBSC donors would have to be followed for over 10 years. A control group of BMT donors of equal size would also be necessary. Such a study could only be carried out on a multi-national basis.

Transplantation of allogeneic PBSC from HLA-identical siblings

**Conditioning regimens and GVHD prophylaxis**

Conditioning regimens employed in PBSC transplantation are the same as those used for bone marrow transplantation (BMT). As listed in Table 1, the majority of patients received CY-TBI or BU-CY with CY at 120 or 200 mg/kg. Indeed, 33.8% and 24.5% of reported patients were conditioned with CY-TBI and BU-CY, respectively. Analogously to BM transplantation, patients with SAA and aplastic anemia are treated with G-CSF. The aspect of donor selection is the same as those used for bone marrow transplantation, however, using Ceprate-cell separation, a 2-3 log depletion of T-lymphocytes is not sufficient to avoid the risk of GVHD and further immunosuppression is generally required.

Recently, some innovative regimens have been developed in order to: i) increase antitumor activity; ii) reduce treatment-related toxicity. For instance, high-dose Ara-C or VP16 has been employed for patients with more advanced disease. Others considered thiopeta, a potent myeloablative drug first introduced in conditioning by the Perugia group. This latter compound was used along with classical BU-CY and with 4 µg/kg/day of G-CSF for 5 days enabled the collection of >4×10^6/kg CD34+ cells with two aphereses in 85% of donors. Variations in blood counts included a sharp elevation of WBC and CD34+ cells and a moderate transitory thrombocytopenia. One problem, however, is the lack of data on the late effects of G-CSF. At the Geneva conference on allogeneic PBSC, Hasenclever and Sextro presented a feasibility study of long-term risk analysis. In order to demonstrate a tenfold increase in leukemia risk, more than 2000 healthy PBSC donors would have to be followed for over 10 years. A control group of BMT donors of equal size would also be necessary. Such a study could only be carried out on a multi-national basis.

<table>
<thead>
<tr>
<th>Regimens</th>
<th>No.</th>
<th>%</th>
<th>GVHD prophylaxis</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>CY-TBI</td>
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<td>33.8%</td>
<td>CsA-MTX</td>
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</tr>
<tr>
<td>BU-CY</td>
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<td>CsA-MP</td>
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<td>21.2%</td>
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<tr>
<td>TiBUCY</td>
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<td>FK506-MTX</td>
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<td>2.3%</td>
</tr>
<tr>
<td>VP16TBI</td>
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<td>3.3%</td>
<td>FK506-MP</td>
<td>44</td>
<td>14.6%</td>
</tr>
<tr>
<td>TiCY</td>
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<td>2.6%</td>
<td>CsA</td>
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</tr>
<tr>
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<td>MTX</td>
<td>1</td>
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</tr>
<tr>
<td>Others</td>
<td>43</td>
<td>16%</td>
<td>Others</td>
<td>2</td>
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</tr>
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</table>

**Table 1. Conditioning regimens and GVHD prophylaxis: 301 patients.**

mg and methylprednisolone 2 g over two days.

A non-myeloablative regimen with busulfan and fludarabine combined with immunosuppression with the CD3 monoclonal antibody was used by Tan et al., while Slavin et al. employed fludarabine and ATG as intensive immunosuppression associated with busulfan at 4 mg/kg/day over 2 days. Although these regimens are not specifically designed for PBSC transplantation, the high number of inoculated PB stem cells overcomes graft rejection, favoring rapid and stable chimerism.

The large amount of stem cells in the inoculum might explain the full engraftment observed in a patient who could not complete the whole regimen and received busulfan alone as preparation.

The large number of CD3+ T cells present in the PB-derived inoculum has raised some concerns about the severity of GVHD following PBSC allograft. However, GVHD prophylaxis has not been substantially modified from the standard regimens used for bone marrow transplants. Cyclosporin A (CsA) has been used alone (2.9%) or in association with either methotrexate (MTX) (49.8%) or methylprednisolone (MP) (21.2%) in 71% of patients (Table 1).

New immunosuppressive regimens including tacrolimus (FK506) in association with methylprednisolone, methotrexate or monoclonal antibodies have been explored in 17% of cases.

Finally, some centers have developed techniques for in vitro stem cell enrichment. However, using Ceprate-cell separation, a 2-3 log depletion of T-lymphocytes is not enough to avoid the risk of GVHD and further immunosuppression is generally required.

In some institutions cryopreservation of collected PBSC is preferred to freshly collected material for several reasons. First, cryopreservation allows precise evaluation of the hematopoietic progenitor content in the harvested material. Second, the allograft may be scheduled at the proper time once adequate quantities of PBSC are collected. Finally, although still unproven, a reduced risk of acute GVHD in patients transplanted with cryopreserved BM cells has been suggested.
**Engraftment**

Engraftment kinetics following PBSC allograft has been extensively investigated. None of the studies include patients dying before day 21. The median time to reach an absolute neutrophil count (ANC) above $0.5 \times 10^9/L$ ranges between 10–16 days. The reported incidence of graft failure is definitely low: $1/59$ in the EBMT survey,$^{50} 1/41$ in the MD Anderson series,$^{51} 1/26$ in the Canadian experience.$^{52}$ The few rejections occurred in transplants with 1–2 antigen disparity. Platelet engraftment is also prompt, with median time to achieve an absolute platelet count (APC) of $20 \times 10^9/L$ ranging between 10 and 18 days in the reported series.

Platelet more than neutrophil engraftment may be affected by acute GVHD or CsA toxicity as well as by early relapse or progressive disease.$^{40}$ In a recent report by the Genoa group a second infusion of PBSC without conditioning was required to achieve full engraftment of platelets in three out of thirty-one patients.$^{40}$

The prompt engraftment offered by PBSC implies a reduction in transfusion need. The reported transfusion requirements range from 2 to 10 packed red cell units and from 3 to 12 platelet units.

Furthermore, GVHD prophylaxis may adversely influence the time to engraftment. In particular, methotrexate given for GVHD prophylaxis delays neutrophil and platelet engraftment.$^{53,54}$

Growth factors, mainly G-CSF, have been employed in several studies to speed-up engraftment. Urbano-Ispizua reported that G-CSF given to patients not receiving methotrexate accelerates neutrophil recovery ($p=0.001$); median time to $>20 \times 10^9/L$ platelets was significantly delayed ($p=0.01$), although the time to reach $50 \times 10^9/L$ platelets was not affected. No difference in engraftment kinetics was seen between cryopreserved and fresh PBSC when G-CSF was administered following transplantation.$^{55}$

The studies carried out so far are not sufficient to draw definitive conclusions about engraftment with PBSC as compared to engraftment with BM. Randomized studies are still in progress and results are not yet available. Most information comes from comparisons of PBSC results with historical data from BM transplants.

A highly informative study was reported by the Seattle group, which compared 37 PBSC transplanted patients with a historical group of 37 bone marrow recipients.$^{56}$ Patients were well matched for diagnosis, disease stage, age and graft versus host prophylaxis. Faster neutrophil engraftment, 14 versus 16 days to reach more than $0.5 \times 10^9/L$ ($p=0.0063$), and earlier achievement of platelet engraftment independence, 11 versus 15 days ($p=0.0014$), were observed in PBSC recipients compared to the BM control group. Consequently, the median number of platelet units transfused was 24 versus 118 ($p=0.0001$) and the median number of red blood cell units transfused was 8 versus 17 ($p=0.0005$) in the PBSC group and in the BM group, respectively.

Similar results have been reported by Russel et al.$^{52}$

platelets ($p=0.0003$) was significantly reduced in patients receiving PBSC compared to BM recipients. Interestingly, the advantage of PBSC was also maintained if methotrexate was used as GVHD prophylaxis.

A recent report by Rosenfeld et al.$^{57}$ evaluated 19 patients transplanted with PBSC. No growth-factor was employed in the post-transplant phase. Significantly faster neutrophil recovery was observed in PBSC transplanted patients compared to historical control group transplanted with BM ($p=0.01$). However, the difference was not significant when the PBSC group was compared to BM recipients given G-CSF in the post-transplant phase.

More recently, a prospective non-randomized study was carried out by the M.D. Anderson group.$^{39}$ The study included 74 adults transplanted with HLA-matched related donors. Thiopeta, busulfan and cyclophosphamide were employed as preparative regimen. The patients were divided into 3 cohorts: Group 1 received BMT using CsA and MTX as GVHD prophylaxis, Group 2 received marrow using CsA and MP, and Group 3 received PBSC with CsA and MP. All patients were given G-CSF post-transplant. Median time to neutrophils $>0.5 \times 10^9/L$ was 17, 9 and 10 days, and to platelets $>20 \times 10^9/L$ was 32, 25 and 18 days in Groups 1, 2 and 3, respectively. The use of CsA and MP for GVHD prophylaxis, rather than the source of engrafted cells was shown to be the most important factor for rapid neutrophil and platelet recovery. Provided that CsA/MP was used for GVHD prophylaxis, platelet transfusion requirement was found to be significantly lower in PBSC than in BMT recipients ($p=0.04$). Significant differences concerning regimen-related toxicity were seen for grade 2–4 stomatitis only between the BMT group using MTX in GVHD prophylaxis and the PBSC group using MP.

Correlation between engraftment kinetics and quantity of PB cells infused is still an open question. The absolute number of nucleated PB cells or CD34$^+$ cells did not correlate with time to neutrophils $>0.5 \times 10^9/L$ or with time to platelets $>20$, $>50$ or $>100 \times 10^9/L$ in a study by Rosenfeld et al.$^{57}$ Similarly, Urbano-Ispizua et al. did not find any correlation using several cut-off values of CD34$^+$ cells at 2.5, 3, 4, 5.5 and $7 \times 10^9/kg$. In contrast, Roy et al.$^{58}$ reported a correlation between CD34$^+$ cells infused and engraftment using a mobilization regimen with G-CSF at a dose of 5 µg/kg. In a large series published by the M.D. Anderson group,$^{37}$ in univariate analysis of patients not given MTX prophylaxis the number of total nucleated cells infused positively affected ANC recovery. Moreover, platelet recovery was positively influenced by the number of CD34$^+$ cells, as well as by young age and sex mis-matching.

**Immune reconstitution after transplantation of peripheral blood stem cells**

Patients undergoing allogeneic BMT experience a prolonged period of profound cellular and humoral immunodeficiency, mainly due to complete pre-transplant destruction of the host lymphohemopoietic system, the
use of immunosuppressive drugs for GVHD prophylaxis and the development of GVHD.\textsuperscript{59-61} This immunodeficiency lasts until stem cells and mature lymphocytes contained in the transplanted marrow repopulate and reconstruct the hematopoietic and lymphopoietic systems which had been destroyed by the pre-transplant conditioning regimen. In particular, immunological reconstitution after BMT is considered to be dependent on two distinct phenomena.\textsuperscript{59,60} In the early post-transplant period, there is an expansion of mature donor-derived lymphocytes transferred with the graft, a process influenced by both the recipient’s environment and the cytokine storm\textsuperscript{62} related to the transplant procedure. Thereafter, naive lymphocytes derived from the differentiation of donor hematopoietic stem cells colonize the lymphoid organs of the recipient and sustain the late immune response.

The crucial role of the first step in immunological recovery is demonstrated by the observations that patients receiving a T-cell depleted transplant are at particular risk for infections and that patients transplanted using donors either recently vaccinated against or immune to a certain pathogen usually have a more rapid recovery of specific T-cell response than those who received bone marrow from unprimed donors.\textsuperscript{63-66} Formal proof of the contribution of transferred donor-derived lymphocytes to recipient immune reconstitution has been recently reported.\textsuperscript{62} In fact, using the combination of a cell culture method and a PCR amplified technique to study tetanus toxoid (TT)-specific T-cells clones, it was possible to demonstrate that patients after BMT display a small response that can be accounted for by a few donor-derived clones and that the T-cell clones transferred with the transplant were still detectable within the donor polyclonal T-cell lines for up to at least 5 years after BMT. Moreover, the vaccination of donors with TT before BMT resulted in a more relevant transfer of antigen-experienced T-cells.\textsuperscript{66}

The expansion of mature donor-derived lymphocytes transferred with the graft in recipients of peripheral blood stem cell (PBSC) transplantation could be expected to be more efficient than patients given BMT, in view of the higher number of donor lymphocytes transferred. However, at present, few reports specifically addressing the question of immune recovery after transplantation of PBSC are available.

Ottinger et al.\textsuperscript{67} demonstrated that, compared to BMT recipients, patients who were given a PBSC transplant had a more rapid recovery of both naïve and memory CD4\textsuperscript{+} cells (expressing the RA and RO isoforms of the CD45 molecule, respectively) whose counts significantly exceeded those observed following marrow transplantation. This determined that in patients receiving PBSC transplantation the characteristic inversion of the CD4\textsuperscript{+}/CD8\textsuperscript{+} ratio observed after BMT was not encountered. Furthermore, the B-cell levels and, at least for the first 2 months after transplantation, the monocye counts were augmented. Since monocytes of granulocyte colony-stimulating factor (G-CSF)-mobilized donors have been demonstrated to reduce the responsiveness of alloantigen specific T-cells, the increase in their count could contribute to the low incidence and reduced severity of acute GVHD reported after transplantation of PBSC.\textsuperscript{68} Moreover, it must be noted that there is a prompt recovery of the lymphocyte counts after transplant of PBSC coupled with an enhanced in vitro response of lymphocytes to aspecific polyclonal activators (phytohemagglutinin and pokeweed mitogen) and to recall antigens (TT, Candida). The most likely hypothesis for explaining this accelerated recovery of helper T cells, B lymphocytes and monocytes is that the number of lymphocytes infused for each subset is more that one magnitude higher in recipients of PBSC transplant than in patients given BMT. However, alternative mechanisms cannot be excluded.

Similar results in terms of more rapid recovery of CD4\textsuperscript{+} cells have also been reported by Bacigalupo et al.\textsuperscript{69} in adults with advanced leukemia who received high-dose chemotherapy followed by G-CSF mobilized PBSC. More recently, two additional reports have further confirmed that recipients of PBSC transplants have a faster recovery of both naïve and memory helper T cells.\textsuperscript{69,70} Moreover, one of these studies documented that patients experiencing a more rapid recovery of the lymphocyte count had a significantly better probability of survival after transplantation.\textsuperscript{69}

Whether the improved immune reconstitution observed after transplantation of PBSC is associated with a lower incidence of infectious complications still remains to be documented. In one of the previously mentioned studies,\textsuperscript{69} the actuarial risk of reactivation of human cytomegalovirus (HCMV) infection in patients given a PBSC transplant was comparable to that observed in a historical control group of BMT recipients. This could be attributed to a greater viral load infused with the graft and correlated with the very large number of nucleated cells that can harbor HCMV transfused. Nonetheless, since the use of donor-derived adoptive immune therapy has been shown to be able to cure or prevent HCMV-related interstitial pneumonia and EBV-induced lymphoproliferative disorders,\textsuperscript{71-73} it can be hypothesized that patients given PBSC transplants, with a more efficient transfer of antigen–experience lymphocytes, may have a reduced incidence and/or reduced severity of infectious complications. Support for this theory is provided by the study reported by Bensinger et al.\textsuperscript{74} in which a lower number of deaths from infectious complications was observed in patients given PBSC as compared to a historical group of BMT recipients.

**Acute and chronic GVHD**

PBSC collections contain a large number of T-cells – approximately 10 times more than unmanipulated marrow grafts.\textsuperscript{75} Therefore concern for increased incidence and severity of GVHD after their infusion into an allogeneic host has been and still is a major issue after PBSC transplantation. Here we analyze the results reported so far in the most recent peer-reviewed studies pub-
lished. Because of the relatively short follow-up of these studies, the assessment of chronic GVHD (cGVHD) is less complete and less accurate than that of the acute form. Some of the studies in fact do not address the problem of cGVHD. Acute GVHD, on the other hand, can now be evaluated in a rather significant number of patients. We shall look first at the characteristics of the studies, then analyze acute and cGVHD separately and finally make comparisons between marrow and PB blood transplants. A set of tentative comments will be made at the end of the chapter.

Type of studies. Selected studies of PBSC transplantation for hematological malignancies are reported in Tables 2 and 3. Several of them have been analyzed in the section on hematological recovery. Table 2 gives details of the transplant procedure and results of acute and chronic GVHD where applicable. Six studies are from a single institution, while three are from several centers. One study from the EBMT Group multicentric in nature, also reports several patients included in four of the other studies – a typical example of double reporting – so that its results reinforce what has already been observed. The figures from this last study were not calculated in any further statistical analysis in order to avoid the error of counting some of the patients twice. However, they are useful for comparisons and have been left in the tables. The total number of patients is 212. None of the studies is prospective or randomized, but four compare the results of PBSC with those of marrow, although using different methods. We shall have to wait some time before seeing the results of the two prospective randomized studies comparing marrow and PBSC transplantation which are now in progress in Europe and the US; for the moment, the reports analyzed here represent the best we have. The 8 studies took place recently, between late 1993 and 1995, and mostly dealt with adults (median age 38 yrs, with a range from 1–57), but some included pediatric patients. Transplants were from fully HLA-identical siblings in 96% of the cases, but a minority received cells from family donors mismatched for one HLA antigen; a minority of patients (5 to 10%) also received a second allo transplant, usually from the original sibling who had donated the marrow. Patients showed a typical spectrum of hematological malignancies for which transplant is indicated. The majority (median 83%) were in advanced phases of their diseases, although definitions are quite variable with the term high-risk being used as a synonym for advanced phase, but 17% had early phase or low-risk disease at the time of transplant. These proportions differed widely within studies, some including 100% advanced diseases and others only 60%, with many more early phase patients. Pre-transplant regimens were obviously different, but despite their apparent disparities they can be grouped into those based on busulphan (54%) or TBI (31%). Only two studies differ considerably from the rest of the series: the Genoa group purposely employed a low intensity regimen based on thiotepa and cyclophosphamide to reduce toxicity in a rather old patient population. The MD Anderson Hospital, on the other hand, used a very intensive regimen combining busulphan, thiotepa and cyclophosphamide in a pop-

<table>
<thead>
<tr>
<th>Ref</th>
<th>Type Of study</th>
<th>Period of study</th>
<th>Median Follow-up days (range)</th>
<th>N° pts</th>
<th>Median age yrs (range)</th>
<th>2nd transplants</th>
<th>Hla family mismatches</th>
<th>Phase of the disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>74</td>
<td>SC</td>
<td>12/93-11/95</td>
<td>nr</td>
<td>37</td>
<td>38 (20-52)</td>
<td>NO</td>
<td>NO</td>
<td>Advanced 100%</td>
</tr>
<tr>
<td>52</td>
<td>MC</td>
<td>5/93-6/95</td>
<td>nr</td>
<td>26</td>
<td>40 (1-54)</td>
<td>3 (11%)</td>
<td>6 (23%)</td>
<td>High risk 23 (88%); Standard risk 3 (12%)</td>
</tr>
<tr>
<td>40</td>
<td>SC</td>
<td>nr</td>
<td>136 (6-228)</td>
<td>31</td>
<td>44 (19-55)</td>
<td>NO</td>
<td>3 (10%)</td>
<td>Advanced 28 (90%); Early 3 (10%)</td>
</tr>
<tr>
<td>55</td>
<td>SC</td>
<td>nr</td>
<td>270 (180-600)</td>
<td>25</td>
<td>43 (17-57)</td>
<td>1 (4%)</td>
<td>NO</td>
<td>Relapse 21 (84%); Remission 4 (16%)</td>
</tr>
<tr>
<td>76</td>
<td>MC</td>
<td>3/94-7/96</td>
<td>nr</td>
<td>24*</td>
<td>37 (16-57)</td>
<td>1 (4%)</td>
<td>1 (4%)</td>
<td>Early 10 (42%); Advanced 14 (58%)</td>
</tr>
<tr>
<td>54</td>
<td>SC</td>
<td>1/94-4/95</td>
<td>nr</td>
<td>33</td>
<td>36 (12-53)</td>
<td>8 (24%)</td>
<td>NO</td>
<td>Early 12 (36%); Advanced 21 (64%)</td>
</tr>
<tr>
<td>39</td>
<td>SC</td>
<td>32 months</td>
<td>nr</td>
<td>19</td>
<td>Not detailed</td>
<td>1 (5%)</td>
<td>NO</td>
<td>Early 18 (82%)</td>
</tr>
<tr>
<td>77</td>
<td>MC</td>
<td>3/94-4/95</td>
<td>111 (15-402)</td>
<td>17</td>
<td>33 (16-52)</td>
<td>NO</td>
<td>NO</td>
<td>Early 6 (35%); Advanced 11 (65%)</td>
</tr>
<tr>
<td>50</td>
<td>SC</td>
<td>1994</td>
<td>nr</td>
<td>51*</td>
<td>39 (2-54)</td>
<td>NO</td>
<td>NO</td>
<td>Early 15 (25%); Advanced 44 (75%)</td>
</tr>
</tbody>
</table>

LEGEND: SC = single center; MC = multi center; n.r. = not reported; * includes 1 pt with SAA; ** includes patients from studies #40, 76 and 54.
ulation of similar age. Another difference is represented by the processing of the collected PBSC: in 130 cases (61%) they were infused fresh and in 82 cases (39%) they were cryopreserved instead until infusion. Finally, GVHD prophylaxis was not uniform: it was based on a combination of CsA and short-course methotrexate in 130 (62%) of the cases, and on CsA plus prednisone in 42 cases (20%); only one study reports 19 patients (9%) who received a combination of tacrolimus and prednisone. CsA alone was used in three patients (1.4%).

Acute GVHD. The incidence of aGVHD, grade II to IV, was about 40% on average. The Genoa study reported a 55% incidence, but it also included the oldest patients in the series; the lowest incidence, 22%, was reported in the series from the MD Anderson Hospital where tacrolimus was used in a combination of tacrolimus and prednisone. CsA alone was used in three patients (1.4%).

Chronic GVHD. The number of patients analyzable for cGVHD is smaller than for aGVHD; survival > 90, 100 or 150 days is the requisite for evaluation. In addition, some studies give many details on cGVHD while others do not address the issue or mention it very briefly.

### Table 3. Transplant modalities, aGVHD and cGVHD.

<table>
<thead>
<tr>
<th>Ref</th>
<th>Fresh or cryopreserved cells</th>
<th>Conditioning regimen*</th>
<th>G-CSF post TX</th>
<th>GVHD prophylaxis N° pts</th>
<th>n. evaluable</th>
<th>Acute GVHD</th>
<th>Chronic GVHD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n. grade II-IV</td>
<td>grade III-IV</td>
<td>related/total deaths</td>
</tr>
<tr>
<td>74</td>
<td>F</td>
<td>TBI 32</td>
<td>no</td>
<td>CsA/MTX 19&lt;br&gt;CsA/PDN* 18</td>
<td>35</td>
<td>13 (37%)&lt;br&gt;5 (14%)&lt;br&gt;1/15</td>
<td>17</td>
</tr>
<tr>
<td>52</td>
<td>C</td>
<td>TBI 18</td>
<td>no</td>
<td>CsA/MTX 26</td>
<td>nn</td>
<td>37%</td>
<td>nn</td>
</tr>
<tr>
<td>40</td>
<td>F</td>
<td>Thio-CTX 31*</td>
<td>no</td>
<td>CsA/MTX 31</td>
<td>31</td>
<td>17 (55%)&lt;br&gt;4 (13%)&lt;br&gt;4/12</td>
<td>28</td>
</tr>
<tr>
<td>55</td>
<td>C</td>
<td>Bus 25</td>
<td>yes</td>
<td>CsA/PDN 25</td>
<td>25</td>
<td>11 (42%)&lt;br&gt;6 (22%)&lt;br&gt;3/7</td>
<td>nn</td>
</tr>
<tr>
<td>76</td>
<td>F</td>
<td>Bus 22 Other 3</td>
<td>no</td>
<td>CsA/MTX 23&lt;br&gt;CsA 1</td>
<td>22</td>
<td>10 (45%)&lt;br&gt;2 (9%)&lt;br&gt;0/7</td>
<td>16</td>
</tr>
<tr>
<td>54</td>
<td>F</td>
<td>TBI 17</td>
<td>yes (11 pts)</td>
<td>CsA 2&lt;br&gt;CsA/MTX 22&lt;br&gt;CsA/PDN 9</td>
<td>32</td>
<td>11 (34%)&lt;br&gt;7 (22%)&lt;br&gt;nr</td>
<td>11</td>
</tr>
<tr>
<td>39</td>
<td>C</td>
<td>Bus 19</td>
<td>yes</td>
<td>FK-506/PDN 19</td>
<td>nn</td>
<td>22%</td>
<td>nn</td>
</tr>
<tr>
<td>77</td>
<td>F</td>
<td>Bus 17</td>
<td>no</td>
<td>CsA/MTX 17</td>
<td>10</td>
<td>3 (33%)&lt;br&gt;4 (24%)&lt;br&gt;4/4</td>
<td>10</td>
</tr>
<tr>
<td>50</td>
<td>F</td>
<td>TBI 22 Bus 22 Thiotepa 11</td>
<td>yes (14 pts)</td>
<td>CsA/PDN 7&lt;br&gt;CsA 6&lt;br&gt;Other 9&lt;br&gt;Other 4</td>
<td>57</td>
<td>30 (50%)&lt;br&gt;14 (23%)&lt;br&gt;7/29</td>
<td>49</td>
</tr>
</tbody>
</table>

*Conditioning regimen mainly based on; #Regimen not including TBI or busulphan; CTX = cyclophosphamide; ^cells infused over 2 days: apheresis of day 1 stored at 4°C until infusion; °PDN=prednisone; n.r.=not reported

Within the single studies as discussed by their authors or by combining data as in this review. Of interest, on the specific issue of PBSC, no correlation was found with the number of T-lymphocytes infused or with the use of fresh or cryopreserved cells. However, it should be noted that the highest incidences of severe aGVHD (22–24%) were reported when prophylaxis was based on CsA/prednisone, which is perhaps less effective than CsA/MTX or in the Spanish study which reported data from multiple institutions with a good proportion of patients receiving CsA/prednisone for GVHD prophylaxis. Nevertheless, a high incidence was also reported in a study from Brazil where CsA/MTX was used in all cases. Mortality from aGVHD is not reported in all studies, as shown in Table 3; those giving the causes of death often do not mention, when infection was the main cause, whether GVHD was associated. However, considering the causes of death of 47 events analyzable in detail, GVHD was the main cause in 12 (25%). Incidentally, this figure is higher than the overall incidence of grade III–IV GVHD, but data on death are reported for fewer patients than data on GVHD.
We calculate that slightly more than 110 patients are evaluable. The overall incidence ranged from 36% to 78%; considering the four studies which give detailed information, the extensive form of the disease occurred with nearly the same frequency or less than the limited form in three studies, while one series reported a striking incidence of the extensive form, with the limited one being only minimally represented. In that study cGVHD developed de novo in 5 out of 10 patients, at variance with the low incidence of aGVHD observed earlier. Another interesting observation is contained in a study from Seattle: of 10 patients at risk, who had received CsA/prednisone for prophylaxis, 6 developed cGVHD, while only 1 of 7 given CsA/MTX did so.

Comparisons between marrow and PBSC transplantation. Four studies compared the incidence of GVHD after PBSC or marrow transplantation. These studies were carried out using matched-pair analysis with a historical control group of marrow recipients who were matched for diagnosis, disease and disease phase at transplant, age, GVHD prophylaxis or age and disease status. One study does not give the characteristics of the marrow recipients. The Seattle study found a lower incidence of grade II-IV aGVHD for PBSC recipients – 37% vs 56% – severe GVHD (grade III-IV) was even more impressively lower in PBSC recipients, 14% vs 33% of marrow transplants. However, due to the small number of patients these data are not statistically significant. The overall incidence of cGVHD was similar in the two groups, with a tendency toward more severity in the PBSC than in the marrow group (42% vs 26% for any grade of clinical cGVHD), but again this was not statistically significant. The striking effect of MTX in the GVHD prophylaxis regimen observed in this study has already been mentioned. The multicenter Canadian study reported a higher incidence of both aGVHD and cGVHD for the PBSC group than for the marrow recipients: 37% vs 21% grades II-IV aGVHD and 53% vs 48% for cGVHD, respectively. A different kind of comparison can be made in a study from the MD Anderson Hospital, where patients with advanced hematological malignancies received, over a 3-year period, the same conditioning protocol but different forms of GVHD prophylaxis and different sources of allogeneic stem cells. The PBSC patients could be compared to the marrow group who received CsA/prednisone as GVHD prophylaxis: severe aGVHD was slightly less in the PBSC group, 22% vs 33%, but this difference was not statistically significant. No data on cGVHD were provided. The Brazilian study reported more aGVHD in the PBSC than in the marrow group, grade III-IV 4/17 vs 3/21, but again this was not statistically significant. No comparative data on cGVHD were given.

Comments. The fear of an unacceptably high rate of severe and perhaps uncontrollable acute GVHD after allo PBSC can now be allayed with a certain degree of confidence on the basis of the data analyzed here. In a population of adults with mainly advanced hematological malignancies who sometimes received second transplants or not fully HLA-identical grafts and, most importantly, were often not given the best GVHD prophylaxis available, acute GVHD was no more than what is expected with marrow transplants. Similar conclusions had been reached in an earlier review on the subject, although on a much smaller number of patients. It is more difficult to say whether GVHD is slightly more severe than after conventional marrow grafting, considering the wide variation in its occurrence, due to the multitude of factors which influence it. A broad comparison of the published data indicate that GVHD observed after PBSC is higher than in the best marrow series but not worse than what is described in the large reports from registries. Four studies have attempted a comparison with retrospective marrow transplants and none found significantly increased aGVHD incidence or severity. It is interesting to speculate why after infusion of 1 log more T-lymphocytes as compared to marrow aGVHD is not increased. One explanation is that any number of T-cells, once the 10^6/kg threshold has been surpassed, is already high enough to cause GVHD and even a 10-fold increase, with respect to the marrow, does not make any difference. Perhaps an extraordinarily large number of T-cells infused, that is usually not reached after G-CSF mobilization, for example 3 or 4 logs, could be the next threshold above which more severe GVHD would regularly occur. Data from a study in Seattle, where 1500×10^6/kg donor buffy-coat mononuclear cells were intentionally infused soon after BMT in patients with advanced disease to enhance a graft-versus-leukemia effect did show that acute, severe GVHD was indeed increased; in that study, unfortunately, GVHD prevention was based on MTX only so comparisons with today’s practices are not possible. Other explanations for why aGVHD does not increase relate to the possible biological modifications of lymphokine production induced by G-CSF. For example, in mice G-CSF has been demonstrated to induce a polarization of T-lymphocytes towards the production of type-2 cytokines (namely IL-4 and IL-10), which display an anti-inflammatory effect. Such polarization was shown to be long-lasting and was associated with a significant reduction in the severity of GVHD after transplantation of these cells into allogeic mice recipients. These results do not seem to be attributable to a direct effect of G-CSF on T-cells, since this subset of lymphocytes rarely expresses G-CSF receptors. Instead, these findings could be explained by the anti-inflammatory effects of G-CSF; in fact, administration of G-CSF decreases tumor necrosis factor (TNF) secretion. Moreover, in normal subjects G-CSF is able to increase the production of two important cytokine antagonists such as soluble TNF-receptor and IL-1 receptor antagonists.

Finally, leukapheresis products from G-CSF-mobilized donors contain a large number of monocytes. These cells have been demonstrated to significantly reduce the alloantigen specific proliferative response of T-lymphocytes. Also, monocytes from subjects treated with G-CSF or GM-CSF can induce the apoptosis of T-lymphocytes via the interaction of the FAS molecule with its ligand.
use of G-CSF may inhibit the function of monocytes as antigen presenting cells and this, in turn, may explain the ability of this cytokine to polarize T-cells towards an anti-inflammatory cytokine profile. These findings could also contribute to explaining the unexpectedly low incidence and reduced severity of GVHD after transplantation of PBSC. However, more studies on the characterization of G-CSF mobilized lymphocytes are needed.

With regard to cGVHD, it appears from this analysis that there is a trend toward a slightly increased incidence after PBSC, although not all individual studies had the same results. The clinical presentation of cGVHD was reported as peculiar in two studies, with many de novo cases\textsuperscript{51,75} but also with a high response to treatment.\textsuperscript{49} It should be noted that early data from the M.D. Anderson on PBSC transplants also reported an increase in cGVHD, with more liver and gastrointestinal manifestations compared to the marrow.\textsuperscript{87} At the time of this writing, several patients were still on immunosuppressive treatment so the full magnitude of cGVHD will be appreciated only in the future after withdrawal of CsA, which is a critical time for the development of the syndrome. Furthermore, in a study on aplastic anemia\textsuperscript{88} the infusion of donor buffy coat cells was associated with a significant increase of cGVHD. However, this increase of cGVHD was not observed in malignancies in the study.\textsuperscript{89} Clearly a longer follow-up of a much larger number of patients is needed to answer the question of cGVHD.

**Transplantation of enriched allogeneic CD34\textsuperscript{+} cells**

As reported above, allogeneic PBSC transplantation results in the infusion of approximately 1 log more T-cells than conventional BM transplantation. Thus, in order to reduce the potential risk of severe aGVHD, several investigators have attempted to remove T-lymphocytes from allogeneic grafts. The only technique that has been utilized so far for T-cell depletion has been the positive selection of hematopoietic CD34\textsuperscript{+} cells.

The CD34 antigen is present on the earliest identifiable progenitor cells and committed myeloid precursors, whereas it is not expressed on mature myeloid and B- and T-lymphoid cells.\textsuperscript{90} However, CD34\textsuperscript{+} cells co-expressing both T-lymphoid (CD2, CD3, CD7) and B-lymphoid markers (CD19) are likely to be the early precursors of the T- and B-lymphoid lineages.\textsuperscript{90} Preclinical studies have also shown the capacity of positive selection of CD34\textsuperscript{+} cells to eliminate 3 to 4 logs of T-cells, coupled with a substantial recovery of hematopoietic progenitors.\textsuperscript{91,92} More recently, transplantation of autologous CD34\textsuperscript{+} cells has been proven to reconstitute normal hematopoiesis in cancer patients treated with myeloablative regimens.\textsuperscript{93-96}

Based on these premises, Link et al.\textsuperscript{97} transplanted 5 patients with unmodified marrow and CD34\textsuperscript{+} selected PBSC and 5 patients with enriched marrow and PB CD34\textsuperscript{+} cells. They concluded that hematopoietic recovery was accelerated with respect to marrow allografts without an apparent increase in aGVHD following conventional CsA and MTX prophylaxis. In a subsequent study,\textsuperscript{98} the same authors transplanted 10 individuals with positively selected circulating CD34\textsuperscript{+} cells alone. The patients were grouped according two different regimens of aGVHD prophylaxis: CsA alone or CsA and MTX. The median grades of aGVHD were 3 in group I (CsA) and 1 in group II (CsA plus MTX). Two patients in group I died from aGVHD and 2 leukemic relapses occurred in group II. Complete and stable donor hematopoiesis was shown in all patients with a median follow-up of 370 days (range 45-481). It was concluded that despite a 3-log reduction of T-cells by CD34\textsuperscript{+} cell enrichment, CsA alone was not sufficient to avoid severe aGVHD.

More recently, Bensinger et al.\textsuperscript{99} transplanted 16 patients with advanced hematologic malignancies with HLA-identical highly enriched PB CD34\textsuperscript{+} cells. Prophylaxis against aGVHD was CsA alone for 5 patients and CsA plus MTX for 11. A median of 8.96 x 10\textsuperscript{6} CD34\textsuperscript{+} cells/kg of patient body weight were infused with a median purity of 62\%. Positive selection of stem cells resulted in a median 2.8-log reduction of T-cells. Despite the prompt and sustained engraftment, 8 out of 16 patients died between 3 and 97 days post-transplant of transplant-related causes and 1 of progressive disease. Grade 2-4 aGVHD occurred in 86\% of patients and 6 out of 8 evaluable patients developed chronic GVHD.

More promising results have been reported by Urbano-Ispizu et al. (1997), who recently transplanted 20 acute and chronic leukemia patients with allogeneic CD34\textsuperscript{+} cells. The median number of CD34\textsuperscript{+} cells and CD3\textsuperscript{+} cells infused was 2.9 x 10\textsuperscript{9}/kg and 0.42 x 10\textsuperscript{9}/kg, respectively. The patients were conditioned with fractionated TBI (total dose 13 Gy in 4 fractions) and cyclophosphamide 120 mg/kg. Additional GVHD prophylaxis included CsA and methylprednisolone. No patients developed grade II-IV aGVHD. The overall procedure was associated with low morbidity and no transplant-related deaths occurred within the first 100 days. Although the median follow-up (7.5 months) is rather short for a full evaluation of cGVHD incidence and disease relapse, the absence of extensive cGVHD and the low rate of disease recurrence (only 3 out of 20 patients relapsed) encourage further studies in this direction. In comparison with previous studies,\textsuperscript{99} it should be noted that the median age of the patient population was 40 years and only 35\% of the individuals were older than 45 years. Moreover, the majority of the leukemic patients were transplanted in the early phase of their disease. Both these parameters are generally associated with lower transplant-related mortality and a lower incidence of severe GVHD.

Although further studies involving larger numbers of patients are currently in progress, these results, taken together, demonstrate that infusion of CD34\textsuperscript{+} selected PBSC results in rapid and stable engraftment. However, transplantation of purified stem cells may induce a higher rate of acute and chronic GVHD than expected, thus requiring full GVHD prophylaxis. Therefore this approach for T-cell depletion should be carefully evaluated in the setting of HLA-identical PBSC transplantation and weighed against the potential increased risk of disease...
relapse, and perhaps delayed immunological reconstitution, and the increased cost of the procedure.

**Allogeneic PBSC from haploidentical familial donors: the mega-stem-cell dose concept**

Allogeneic BMT has been largely confined to patients who are HLA-identical to their donors. At present, only about 30–35% of patients who might benefit from allogeneic BMT have an HLA-identical sibling. The establishment of large registries of HLA-typed individuals during recent years has led to a substantial increase in transplants from unrelated donors.\(^{100-102}\) Although 40 to 50% of patients are successful in locating HLA-A, B, DR-matched unrelated donors, many patients still fail to find an appropriate donor.\(^ {103,104}\) In contrast, nearly all patients have an HLA-haploidentical relative (parent, child, sibling,) who could serve as a donor.

The feasibility and safety of transplants from partially matched family members have been investigated and the results of these studies have demonstrated that HLA matching is a critical and limiting factor in marrow transplantation.\(^ {105-107}\) In published works on mismatched transplants, there has been no large study involving patients mismatched with their donors by one full haplotype. These experiences have been limited because the problems of transplant increase with the number of antigenic disparities between donor and host.\(^ {106}\) In 2- or 3-antigen mismatched transplants, studies by the Seattle program\(^ {108}\) and the **International Bone Marrow Transplant Registry**\(^ {109}\) reported graft failure in 20 to 30% of cases. The reported incidence of acute GVHD (grade II or greater) varied from 34% to 100% overall, but in 2- and 3-antigen mismatched patients the incidence was at least 80%.\(^ {105,106}\) Severe GVHD was a greater problem than graft rejection, preventing more widespread use of mismatched related transplants during the latter 1970s and 1980s.

By contrast, extensive experience in severe combined immunodeficiency (SCID) patients has shown that GVHD is largely preventable, even in 3-antigen mismatched transplants, when a 3-log T-cell depletion of the donor bone marrow is achieved.\(^ {109,110}\) In 1981 Reisner et al. reported the first case of leukemia treated with a T-cell depleted marrow transplant from a haploidentical, 3-loci incompatible, parental donor.\(^ {111}\) There was full engraftment and no GVHD. Subsequently, clinical trials in mismatched-sibling BMT for patients with leukemia were begun using the lectin or other T-cell depleting methods which included monoclonal antibodies with complement or conjugated to toxins, and counterflow centrifugal elutriation (reviewed in ref. #112). It was determined that the threshold dose below which GVHD was not seen in matched patients was 2.0×10\(^ {10}\) T cells/kg.\(^ {113}\) However, early enthusiasm for all methods was soon tempered by an increased incidence (>50%) of graft rejection.\(^ {114}\)

In exploring the problem of failure in mismatched grafts, the inadequacy of immunosuppression was documented by the observation of residual host lymphocytes in patients who failed to engraft after being conditioned with conventional preparative regimens and given T-cell depleted mismatched transplants. Work in experimental models has shown that inhomogenous T-cell depleted transplants can be successfully performed by manipulating the conditioning regimen and/or the graft composition.\(^ {115}\) The immunologic response of the remaining host immune system against the graft can be overcome by increasing the total dose of TBI\(^ {116}\) or by adding selective anti-T measures with minimal toxicity, such as splenic irradiation\(^ {117}\) or in vivo treatment with anti-T monoclonal antibodies.\(^ {118}\) Engraftment is also improved by increasing the myeloablative effect of the conditioning regimen through the use of dimethylmyleran, busulfan or thiotepa, given with TBI.\(^ {119,120}\)

Different cytodestructive agents or radiation regimens were therefore added to the basic conditioning protocols used for conventional BMT. Although a marked beneficial effect was found in recipients of T-cell depleted HLA-identical bone marrow upon adding ATG and thiotepa to TBI and cyclophosphamide,\(^ {121}\) none of these agents were found to be useful in recipients of T-cell depleted haploidentical 3-antigen incompatible transplants. Others, using the monoclonal antibody Campath-1G instead of ATG, have observed similarly disappointing rejection rates.\(^ {122}\)

Concerning the composition of the graft, Lapidot et al. showed that megadoses of T-cell depleted incompatible bone marrow inoculum could obtain full donor-type engraftment in mice treated with sublethal irradiation, or presensitized with donor lymphocytes or partially reconstituted before the transplant by adding back a controlled number of host-type mature thymocytes.\(^ {123}\) The means of overcoming graft failure elucidated in the experimental model can be applied in the clinical setting by combining approaches that increase both the conditioning of the host and the size of the stem cell inoculum. The major advance that finally made full haplotype-mismatched transplantation possible in leukemia patients was the availability of rhG-CSF\(^ {124}\) and the experience in autologous transplants in which G-CSF was used to mobilize high numbers of stem cells into the blood of patients without significant side effects.\(^ {125}\) Their employment made it feasible to increase the number of donor stem cells to a level which, in animal models, made transplantation across the histocompatibility barrier possible.\(^ {117}\) On the basis of these concepts, the BMT team at the University of Perugia first introduced the megadose cell transplant in full haplotype-mismatched leukemia patients.\(^ {126}\) After a conditioning regimen which included 8 Gy TBI in a single fraction at a fast dose rate (16 cGy/m), thiotepa (10 mg/kg), rabbit ATG (20 mg/kg in 4 days) and cyclophosphamide (100 mg/kg in 2 days), advanced leukemia patients, mostly adults, were given the combination of marrow and G-CSF-mobilized blood stem cells. Donors compatible with the patients for only one haplotype and 3-antigen disparate on the other haplotype
underwent bone marrow harvest followed within a few days by treatment with G-CSF (12 µg/kg/d × 7 days). Four leukaphereses of progenitor cells were performed starting on the fourth day. The marrow as well as the leukapheresis product were each depleted of T-cells using soybean lectin agglutination and E-rosetting. Both the CD34+ cells and CFU-GM were increased 7- to 10-fold over bone marrow alone, and the average number of CD3+ cells infused was 2.2×10^9/kg recipient body weight. Following conditioning and stem cell infusion, patients received no additional GvHD prophylaxis. The results of the first 17 patients were reported in 1994 and subsequently 27 additional leukemia patients, most of them in chemoresistant relapse at the time of transplant, were treated. For the first time a very rapid hematopoietic engraftment was observed in more than 80% of patients and, without any post-transplant prophylaxis, acute GvHD occurred in only 27%, and there was no significant chronic GvHD. As for survival, 7 patients are currently alive and disease free at a median follow-up of more than 3 years. The major complications observed in this pilot study were interstitial pneumonitis, which occurred in 43%, and infections in the setting of GvHD. Both were responsible for the 60% transplant-related mortality.

This pilot experience showed that the megadose cell strategy, together with a highly immunosuppressive and myeloablative conditioning, resulted in a high incidence of durable engraftment with significantly reduced GvHD comparable to historical experience with unmanipulated transplants. It also confirmed that in humans, as in mice, the stem cell dose plays a crucial role in overcoming HLA-histocompatibility barriers. This concept is also supported by the recent work by Rachamin et al. demonstrating that purified CD34+ cells have a very powerful veto activity. They are able to specifically reduce, in a mixed lymphocyte culture, the frequency of CTL precursors against the stimulatory cells of the same subject and thereby help to overcome allogeneic rejection and enhance their own engraftment.

The approach to haplotype-mismatched transplants has evolved since Aversa et al. originally proposed the megadose cell concept. With their initial protocol GvHD was decreased but not eliminated, and it contributed to transplant-related mortality, which was significantly greater than in matched patients receiving similar conditioning (Aversa et al., unpublished data). These remaining problems were addressed in a subsequent trial, where it was possible to completely abrogate GvHD by improving the T-cell depletion method. By processing the peripheral blood progenitor cells with an initial debulking of both mononuclear and T-cells with one-round E-rosetting followed by positive selection of CD34+ cells with the Ceprate stem cell concentrator (CellPro Inc. Bothell, WA, USA), it was possible to infuse a median of 3×10^6 CD3+ cells/kg and 13×10^9 CD34+ cells/kg in 24 high-risk leukemia patients. Conditioning-related toxicity was also reduced by modifying pre-transplant chemotherapy. As a substitute for cyclophosphamide, which was considered a possible factor in the early mortality in the first pilot study, fludarabine was tested. In fact, it had been shown to have powerful immunosuppressive effect in patients treated for lymphoproliferative disorders, even at doses which were not associated with significant extra-hematologic toxicity. Furthermore, in a mouse model TBI+fludarabine (40 mg/m^2/d × 5) was shown to provide an immunosuppressive effect comparable to TBI+cyclophosphamide.

At present, a regimen including TBI in a single fraction, thiopeta, fludarabine and ATG followed by the infusion of T-depleted bone marrow plus T-depleted CD34+-selected blood cells is being evaluated for toxicity and efficacy. The preliminary results of this study were recently presented at the American Society of Hematology meeting in Orlando. As hoped, with the decrease in the number of T-cells infused and the modifications in conditioning, the problem of GvHD was largely prevented (only 2 patients developed grade II acute GvHD and one progressed to chronic GvHD); the engraftment rate was 95% and there was a decrease in transplant-related mortality to 29% compared to the previous 60%.

A more recent update on 48 patients was presented in Mannheim. The abstract reports that 22/28 patients were in chemoresistant relapse at the time of transplant; age ranged from 4 to 53 years (median 27). Forty of 48 patients engrafted, grade II–IV acute GvHD occurred in only two patients and no one developed chronic GvHD. Twenty patients were alive and disease free at a median follow-up of 5 months (range 1–16). There were 11 relapses and 17 nonhematologic deaths. Transplant-related mortality was 35%.

An unsolved problem remains the slow immunologic recovery of engrafted patients that is responsible for infections. Counting of peripheral blood lymphocytes which exhibited a phenotype of NK cells (CD56+), helper T cells (CD3+/CD4+), cytotoxic T-cells (CD3+/CD8+), cytotoxic T-cells (CD3+/CD8+) and B-cells (lgM+) revealed early recovery (within 2–4 weeks) of NK cells and extremely delayed recovery of T cells. In particular, CD4+ cells reached near normal values after 10 to 12 months. In addition, the frequencies of T-cells responding to polyclonal activators in a sensitive limiting dilution assay were approximately 1 in 100 within the first post-grafting month and 1 in 10 at 10 months post-transplant (control responder cell frequencies are in the range of 1 in 2). The low number of T-cells, combined with their functional peculiarities (i.e. failure to respond to TcR stimulation) are certainly implicated in the high frequency of infectious complications and are strongly indicative of a markedly distorted T-cell maturation process.

Interestingly, looking at post-transplant immune reconstitution, Albi et al. observed a large donor-type TcR-αβ+ CD8+ cell population that co-express NK-like receptors for specific MHC class I alleles. NK cells expressed multiple, clonotypically distributed membrane receptors with different specificities for families of MHC
class I alleles (termed **killer cell inhibitory receptors**, KIR). The interaction between these receptors and the appropriate alleles produces a signal which inhibits killing of the target cells. Analysis of more than 900 clones revealed that 40% to 80% of these KIR-T-cells exhibit NK-like functions, i.e. they were able to lyse class I-negative targets and were functionally blocked by the expression of specific class I alleles on target cells. Furthermore, these cells do not lyse autologous hematopoietic cells, but are able to lyse fresh leukemia cells. This might suggest that they could provide a graft-versus-leukemia effect without causing GVHD.

In a period of twenty years transplants across the histocompatibility barrier have advanced from being experimentally to clinically possible. The principles outlined at the beginning – adequate cell dose, adequate immunosuppression and myeloablation, avoidance of GvHD – have been successfully combined. Two other groups have recently reported on successful engraftment in haplotype mismatched transplants by combining bone marrow and G-CSF-mobilized blood stem cells after CD34-positive selection for patients with advanced leukemia. Refinements of this protocol should make haplotype mismatched transplants an attractive therapeutic option for patients with high-risk leukemia without a matched related or unrelated donor.

Furthermore, there are enormous potential applications of the concept of the stem cell dose for the future treatment of non-neoplastic diseases like aplastic anemia, Fanconi’s anemia, SCID, thalassemia, and for induction of tolerance in organ transplantation. This approach should be applicable not only in mismatched transplants but also for overcoming problems which remain in the matched transplant setting, such as rejection in aplastic anemia, regimen-related toxicity in Fanconi’s anemia and thalassemia.

**Transplantation of allogeneic PBSC from unrelated donors**

Two retrospective studies have recently suggested that the number of hematopoietic cells present in BM harvest correlates with the clinical outcome in the setting of stem cell transplantation from both HLA-identical siblings and from HLA-matched unrelated donors. In the latter case, the number of cells infused has proven to be the most potent prognostic factor for survival. Therefore, given the much higher number of progenitor cells collected in primed PB as compared to conventional BM harvest, the use of PBSC appears to be a promising alternative for improving the results of transplantation from unrelated donors. In this regard, Ringden et al. and Stocksclader et al. recently reported their preliminary experience with transplantation of allogeneic PBSC from full-matched or 1-antigen mismatched unrelated donors. In particular, Ringden et al. transplanted 6 patients with high-risk hematologic disease. Four of them received allogeneic PBSC as primary treatment while 2 others were treated after a BM graft failure. Five PBSC collections were infused without any manipulation; in 1 case Campath-1 monoclonal antibody was used for T-cell depletion. In the German study, 1 AML patient in 2nd CR received purified CD34+ cells from an HLA-matched unrelated donor. The total number of patients transplanted is too small and the follow-up too short to draw any conclusion; however, these preliminary data showing a rapid rate of engraftment are encouraging, whereas the role of T-cell depletion remains to be clarified.

**Transplantation of umbilical cord blood progenitor cells**

The existence of hematopoietic progenitors circulating between the fetus and the placenta during gestation was first described in this Journal more than 20 years ago, but their clinical application began only when it became evident that the progenitor cell content of CB was sufficient for bone marrow repopulation in pediatric patients given myeloablative chemo-radiotherapy. In 1988, a patient affected with Fanconi’s anemia was first transplanted with CB progenitor cells from his HLA-matched healthy sibling. Subsequently, successful CB transplants (CBT) were sporadically reported in patients affected by both malignant and nonmalignant disorders. The establishment of large CB banks in Europe and USA, and improvement of the methods of cell collection, manipulation and freezing have permitted a rapidly increasing use of CB progenitor cells, which are now extensively employed for allogeneic transplantation.

The biological and functional characteristics of CB hematopoietic stem cells have been already reviewed by the Working Group.

**Clinical results after cord blood transplantation**

As mentioned above, the use of human umbilical CB hematopoietic progenitors represents an alternative modality of transplantation. Advantages of CBT include ease of hematopoietic stem cell collection, absence of donor risks, low risk of viral contamination (cytomegalovirus, Epstein-Barr virus, etc.) and, for transplantation among unrelated individuals, prompt availability of hematopoietic stem cells. Over the past decade, placental blood has been used to transplant hundreds of patients (mainly children) and information on the rate and kinetics of engraftment and on the risk of severe acute or chronic GVHD is now available for CBT recipients from both related and unrelated donors.

In the two largest cohorts of patients transplanted from an HLA-identical sibling reported to date, the probability of engraftment of donor hematopoiesis was 79% and 85%, respectively, even though it must be underlined that in the cohort analyzed by Wagner and colleagues rejections were mainly observed in patients affected by bone marrow failure syndromes or globinopathies, which are diseases with a high risk of graft failure. In the cohort reported by Wagner et al., the
median time to achieve granulocyte (PMN >0.5×10^9/L) and platelet (PLT >50×10^9/L) recovery was 22 and 49 days, respectively; these values were greater than those observed with BMT. Comparable time for PMN and PLT recovery were observed in the European experience. In particular, in this latter report, patients receiving a higher number of nucleated cells (i.e. more than 37×10^6/kg) experienced faster engraftment than those given a lower number of cord blood progenitors, suggesting that the number of cells infused is the main factor influencing the rate of hematologic recovery. More prolonged periods of profound leukopenia and thrombocytopenia have also been described in children receiving CBT from unrelated donors. In fact, in the first two series of patients transplanted from an unrelated donor, PMN recovery occurred in a median of 22 and 24 days, respectively, whereas the median time for PLT recovery was 82 and 67 days, respectively. The importance of the number of cells infused on the kinetics of PMN and PLT engraftment in the Eurocord Transplant Group experience was also observed in the group of patients given an unrelated CBT. Moreover, unlike BMT, where the use of hematopoietic growth factors has been demonstrated to hasten myeloid recovery significantly,151,152 administration of these cytokines has produced conflicting results in CBT recipients. In fact, in the cohort of patients receiving CBT from HLA-identical or disparate family donors studied by Wagner et al., the use of G-CSF or GM-CSF did not influence the kinetics of PMN reconstitution.142 In contrast, in a group of children transplanted using unrelated CB units reported by the same authors, patients receiving hematopoietic growth factors experienced faster myeloid recovery than those who were not given the cytokines.150

The delayed rate of neutrophil engraftment and the conflicting data mentioned above could be explained by the infusion of fewer progenitor cells with CBT with respect to BMT, as suggested by the European experience, or, alternatively, by the particular characteristics of the proliferative, self-renewing and differentiating capacity of CB cells. A practical consequence of the above observation is that specific attention should be paid to the risk of infectious complications in children receiving CBT.

During the first few months after transplant CBT recipients show a steady, impressive increase in HbF whose values are significantly higher than those observed in patients receiving BMT. Moreover, the subsequent decline is usually less pronounced than that observed in normal children in the first year of life (Figure 1).153,154 This preferential production of γ chains in erythroid progenitors seems to reproduce the normal ontogeny of erythropoiesis, even though the persistence of HbF levels higher than those observed in the first year of age suggests a more delayed switch from fetal to adult hemoglobin synthesis.

The dose of CB progenitor cells necessary to ensure early and sustained hematopoietic engraftment and favorable clinical outcome has still not been precisely defined. Wagner et al.148 claimed that the lowest dose of CB nucleated cells reported to be capable of yielding complete and sustained engraftment is 1×10^7/kg of recipient body weight. However, as previously mentioned, the Eurocord Transplant Group documented that a dose of nucleated cells available before thawing of fewer than 3.7×10^7/kg recipient body weight was highly predictive of both graft failure and poor survival after CBT.7 The importance of this value also emerges from Kurtzberg et al’s experience.149 Ten out of 13 patients undergoing CBT from an unrelated donor and having

![Figure 1. HbF levels observed in the 5 CBT recipients in the first year after transplant. Normal percentiles of HbF values (dotted line, mean±2SD) observed in the first year of age (Galanello et al., 1981) are also reported (F. Locatelli, personal data).](image-url)
received fewer than $3.7 \times 10^7$/kg nucleated cells failed to benefit from the procedure. Although the importance of this cellular dose appears evident from these two reports, it should be noted that rarely is such a number of cells available in the case of adult patients. In fact, since the average leukocyte count in placental blood is about $10 \times 10^6$/mL and the average volume of donated blood is about 80 mL, the average number of nucleated cells before thawing in one cord blood unit may reach $800 \times 10^6$. About 30% of nucleated cells are lost during the thawing and washing procedure, and even though the loss mostly involves mature cells which have no role in transplantation, it is reasonable to expect fewer than $3.7 \times 10^7$/kg viable cells for patients with body weight greater than 30–40 kg.

The reduced immune reactivity of cord blood cells found a clinical counterpart in 38 children reported by Wagner et al.\textsuperscript{144} who received CBT from an HLA-identical or 1-antigen mismatched sibling. In these patients, the incidence of grade II–IV acute GVHD and limited chronic GVHD was 3% and 6%, respectively, with no patient dying of GVHD. Confirmatory results were obtained by the Eurocord Transplant Group, which reported a 9% incidence of grade II–IV acute GVHD in CBT recipients from an HLA-identical relative. However, it is noteworthy that the same group documented a 50% incidence of grade II–IV acute GVHD in patients transplanted from an HLA nonidentical family donor.

In CBT performed between unrelated subjects with, in some cases, a disparity of 2–3 HLA antigens, the incidence of acute grade III–IV GVHD is reduced (approximately 10–20%)\textsuperscript{2,149,150} with respect to that observed after unmanipulated BMT between unrelated subjects for whom, notwithstanding complete HLA identity between recipient and donor, the observed risk of acute grade III–IV GVHD reaches at least 30–40%. In particular, in the cohort of patients given CBT from an unrelated donor reported by Kurtzberg et al.,\textsuperscript{149} no patient developed grade IV acute GVHD or experienced hepatic involvement or died of acute GVHD, and only 4 out of 65 patients given an unrelated CBT reported by the Eurocord Transplant Group showed grade IV acute GVHD.

From the data collected up to now, therefore, it clearly appears that CBT, from both familial and unrelated donors, is associated with a reduced risk of acute and chronic GVHD.\textsuperscript{2,148-150} In view of this observation, different centers tend to adopt less intensive schemes of GVHD prophylaxis. Typically, children transplanted with a CB unit collected from an HLA-identical sibling receive GVHD prophylaxis consisting of CsA alone, whereas for patients undergoing unrelated CBT the most widely used regimens are those based on a combination of CsA with either low- or high-dose steroids.\textsuperscript{149,150} The association of CsA with short-term methotrexate as proposed by the Seattle group in BMT recipients\textsuperscript{155} is not generally employed due to concerns about the prolongation of time required for engraftment and possible damage to hematopoietic progenitors with a reduction in the potential for marrow repopulation. Procedures involving T-cell depletion of CB cells are also discouraged.

The reported low incidence of GVHD\textsuperscript{148-155} might, on the other hand, be a major drawback to the use of CB as a source of stem cells for allogeneic transplantation in leukemic patients. In fact, since the role of allogeneic lymphocytes in the control and/or eradication of malignancy is well established, the potential absence of GVL activity could represent a theoretical concern in leukemic subjects given CBT. Currently available data do not conclusively establish whether CBT really predisposes patients to an increased risk of leukemia relapse. However, considering the concern mentioned above, the choice of less intensive GVHD prophylaxis schemes could represent a possible means for partially sparing the immune-mediated GVL effect, which may significantly contribute to preventing regrowth of leukemia cells.

**Immunological reconstitution following cord blood transplantation**

Although immunological reconstitution after BMT has been extensively studied,\textsuperscript{58,59} few data are available on the kinetics of immune recovery in CBT recipients.\textsuperscript{144,153,156} After CBT, recovery of T-cell immunity, as well as that of natural killer subpopulations, mimicks what is described in BMT recipients.\textsuperscript{153} In particular, in the early post-transplant period recovery of CD8\textsuperscript{+} lymphocytes seems to be faster than that of CD4\textsuperscript{+} cells, determining a characteristic inversion of the ratio between the two subpopulations during the first 6 months after CBT, similarly to what is described in BMT recipients. Considering the much lower number of lymphoid cells transferred with CBT as compared to BMT, the recovery of T-lymphocyte number and function towards normal must be considered rapid. The prompt recovery of T-cell immunity following CBT could be positively influenced by the reduced incidence and severity of both acute and chronic GVHD, which per se adversely affects the acquisition of lymphocyte function. However, it must be noted that the prompt recovery of lymphocyte function in vitro does not necessarily correlate with effective in vivo immunity. In fact, at present there are insufficient data to prove that this rapid T-cell recovery translates into a low incidence of viral and fungal infections after CBT.

In contrast to what is observed in BMT recipients,\textsuperscript{58,59} an impressive increase in the percentage and absolute number of B-lymphocytes, apparently not related to viral infections, has been documented in children receiving CBT.\textsuperscript{153,157} Possible hypotheses to account for this observation could involve the physiological characteristics of B-cell ontogeny in the first year of life and/or different distribution of mature memory lymphocytes in bone marrow and CB.\textsuperscript{158,159}

**Ethical problems of cord blood transplantation**

Like any other innovative treatment, CBT also poses some ethical questions that have still not been completely resolved. In particular, these ethical considera-
tions can be subdivided into those concerning transplants between HLA-compatible siblings and those regarding CBT from unrelated donors.

The two main ethical problems regarding transplantation from a family donor are those of conceiving a sibling with the hope of producing a compatible donor for a previous child who requires transplantation of hematopoietic stem cells, and of his/her HLA typing in utero. Of course, any decision to conceive a child for the sole purpose of making it become a cord blood donor entails belittling the value of the individual to be born. However, it cannot be ignored that it is extremely difficult to separate the reasons that lead to conceiving a child solely for the joy of procreating from those linked to the possibility of saving a living, sick child. On the other hand, even this last reason does not lessen the importance of the future child who will bring happiness to the family in addition to being the person who, in the case of successful transplantation, allowed the family to save the life of a child who would have otherwise been lost.160

In the meantime, it is important to stress the inappropriateness of performing HLA typing in utero; because of the increased abortion rate due to the procedure (about 1–2%), it entails the risk of causing the death of a healthy human being and would in any case be deeply despicable if it were used to dispose of a conceived child found to be HLA-incompatible with the sick patient. From the point of view of the unborn child, HLA typing in utero quite obviously poses critical problems and offers no advantages, but only tangible risks for that unborn child’s survival. HLA typing in utero should be carried out only when other, far more important reasons (for example, advanced age of the mother with consequent higher risk of chromosome-21 trisomy for the fetus) suggest performing prenatal diagnostic procedures.

Since the donor is a newborn infant, the use of cord blood for an unrelated sick patient has raised many questions of ethical interest. These ethical aspects go beyond the scope of this review, but we would like to comment briefly on some of them. Particular attention has been devoted to the problems raised by tests required to determine whether cord blood is suitable and usable without the risk of transmitting to the recipient any disease carried by the donor cells (namely infectious diseases and genetically transmissible disorders). In fact, for this specific aspect the ethical question is: what kind of behavior should be adopted by the medical operator who works with a woman (or with the parents of a child) if a disease for which there is no therapy is detected in the infant?161 Such possibly dramatic news must cause as little damage as possible. We must by all means prevent our increasingly profound biological awareness of our selves from leading to a culture of anguish. One might recall in this regard that, for example, it has been stated that minors should not be tested for abnormal genes unless there is an effective curative or preventive treatment that must be instituted early in life.162

Another heavily debated problem regarding unrelated transplants is the case of a cord blood unit assigned to allotransplantation, making these cells unavailable in the case the donor needs them for an autologous transplant. However, to ensure that every CB donor has the right to use the donated blood for himself if necessary, there would be no way to provide cord blood units for allotransplants. Therefore the very nature of the technique originally conceived for allotransplants would be profoundly transformed, and this would punish all donation ethics at their very core.

Strictly linked to these considerations is the problem of private banking of cord blood cells.163 In any case, we firmly believe that involving money-making aspects in CB transplantation technology is unacceptable. In particular, as stated by other authors as well,164 no part of the human body should be commercialized and CB should not be used for the benefit of financial speculators.

Rational use of PBSC in the treatment of leukemic relapse after allogeneic transplant

The cure rate of patients receiving an allogeneic transplant for hematological malignancies is negatively affected by relapse. The incidence and time of disease recurrence depend on several factors such as diagnosis, disease phase at the time of transplant, conditioning regimen, GVHD prophylaxis and T-cell content of the infused graft.165

The response rate and clinical outcome of relapsing patients with acute leukemia treated with chemotherapy alone are extremely poor.166,167 Interferon therapy significantly prolongs the survival of CML patients relapsed after transplant, but its benefit is not durable over long-term follow-up.168–170 Finally, a second transplant offers some possibilities of cure for relapsed patients but it carries high morbidity and regimen-related mortality.171–173

During the past few years the therapeutic approach to post–transplant relapse has been substantially modified. Following its first report by Kolb et al.,174 donor lymphocyte infusion (DLI) is currently being used as a form of adoptive immunotherapy for patients with hematological malignancies who relapse after transplant175–188 or develop EBV lymphoproliferative disorders.72,189

A number of observations in allogeneic transplants support the evidence that a GVL effect, whether associated or not with GVHD and mediated by donor immunocompetent cells, contributes to the eradication of the neoplastic clone.82,190–195

The rationale for the use of DLI in post–transplant relapse is based on two main factors: 1) the persistence of an immunotolerant status versus donor cells in the relapsing host; 2) the cytotoxic activity exerted by HLA-unrestricted NK and LAK cells or by HLA-restricted T-cells of donor origin against host malignant cells.188,196

However, the effectiveness of DLI therapy is variable since it greatly depends on the type of disease and its stage at the time of relapse. Following DLI, a high pro-
portion of CML patients with molecular, cytogenetic or chronic phase hematologic relapse will likely experience a long-term disease free survival, but the success rate is substantially lower in recurrent AML and virtually absent in patients relapsing with ALL or blast crisis CML. Furthermore, the therapeutic success of DLI is counteracted by related severe complications such as GVHD and myelosuppression, which occur in up to 90% and 50% of cases, respectively. The mortality due to DLI may approach 20%.

Therefore in order to optimize adoptive immunotherapy with DLI and to improve the general management of post-transplant relapse, several biological and clinical conditions should be considered.

One of the most important factors is the time required for GVL to destroy the host neoplastic cells. In early stage CML this time seems to be enough to allow a GVL reaction to build up and eliminate residual CML cells. By contrast, neoplastic growth is so fast in acute leukemia that it may not be challenged by the GVL effect.

A further variable influencing the response to DLI is the potential of the neoplastic clone to mature and differentiate into dendritic cells, which contribute to the GVL reaction by enhancing the antigen presentation capacity of tumor cells. This property is spontaneously attained by CML cells in chronic phase and, to some extent, by AML cells, particularly when cell differentiation follows the tumor reduction induced by chemotherapy. Dendritic cells derived from bone marrow or produced in vitro by CD34+ cell cultures in the presence of cytokines can exert their action through an HLA restricted mechanism. Therefore, in treating leukemia relapse weakly expressing HLA or tumor-specific antigens, donor hematopoietic progenitor cells may improve the immunologic effect mediated by DLI.

Finally, bone marrow chimerism detected by PCR prior to DLI may predict either response to treatment or the occurrence of myelosuppression. Although long-term persistence of donor T-cells in the peripheral blood during relapse has been reported, this observation does not provide any prognostic information on the post-DLI clinical outcome. Southern blot RFLP analysis, erythrocyte phenotype and cytogenetics have been employed to detect residual donor cells, but no correlation was found among pre-DLI BM chimerism, response to treatment and the risk of myelosuppression. However, pre-DLI BM chimerism assessed by quantitative PCR of VNTR sequences in relapsed CML patients is associated with cytogenetic and molecular remission and strongly predicts the development of aplasia, thereby providing an early indication for the reinfusion of PBSC from the donor.

Donor PBSC reinfusion has frequently been adopted as from rescue of DLI-associated myelosuppression. As to the combined use of donor PBSC and DLI, the reported experiences are limited to a small number of patients who relapsed with acute leukemia. In these studies, donors were stimulated with G-CSF at doses ranging from 2.5 mg/kg for 10 days to 16 mg/kg for 5 days. The apheresis products obtained over 1 to 3 consecutive days contained a median of 4×10^9/kg CD34+ cells and a median of 3.5×10^9/kg CD3+ cells. All patients received chemotherapy prior to PBSC infusion and most of them achieved CR with prompt hematopoietic reconstitution which in the cases analyzed originated from donor cells. The majority of patients developed acute or chronic GVHD and related complications. In one of the reported series, the median duration of CR after this combined treatment was longer than the median time from transplant to relapse. These results compare favorably with those recently reported in patients receiving DLI alone for relapse of acute leukemia or myelodysplasia after BMT. Of the eight patients receiving this treatment, only one achieved CR and 7 died of progressive disease.

In conclusion, these preliminary experiences suggest that patients relapsing with acute leukemia or advanced phase CML after BMT should be treated with intensive chemotherapy regimens, not necessarily including immunosuppressive drugs, followed by donor mobilized PBSC.

This approach might result in certain therapeutic advantages such as: 1) reduction of the tumor burden; 2) slowing down of the neoplastic growth; 3) acceleration of donor hematopoiesis recovery and promotion of dendritic cell differentiation; 4) the possibility for the immunocompetent donor cells to express their GVL activity to a greater extent. Whether the additional administration of cytokines (IFN, IL-2, G-CSF, GM-CSF) would improve the efficacy of chemotherapy and PBSC is unknown at present and awaits further investigation.

Finally, the GVL reaction exerted by donor lymphocytes against CML cells which retain biological features of early stage disease is potent enough that patients might be spared a repetition of previous chemotherapy. However, donor PBSC infusion should be considered for CML patients with either cytogenetic or chronic phase relapse who show minimal (< 10%) or no BM chimerism. In such cases the use of donor PBSC is mainly indicated to counteract the risk of severe BM aplasia following the infusion of DLI alone.

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All authors equally contributed to the conception and writing of this review article.

**Disclosures**

Conflict of interest: this review article was prepared by a group of experts designated by Haematologica and by representatives of two pharmaceutical companies, Amgen Italia SpA and Dompé Biotec SpA, both from Milan, Italy. This co-operation between a medical journal and pharmaceutical companies is based on the common aim of achieving an optimal use of new therapeutic procedures in medical practice. In agreement with the Journal’s Conflict of Interest Policy, the reader is given the following information. The preparation of this manuscript was supported by educational grants from the two companies. Dompé Biotec SpA sells G-CSF and rhEpo in Italy, and Amgen Italia SpA has a stake in Dompé.
Clinical use of allogeneic hematopoietic stem cells

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Biotec SpA.
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References

45. Tan P, Yeow Tee G. Stem cell allografting using a non-
43. Giralt S, Estey E, Terenzi A, et al. Induction of graft-
40. Bacigalupo A, Van Lint MT, Valbonesi M, et al. Thio-
39. Aversa F, Tabilio A, Terenzi A, et al. Successful engraft-
37. Rosenfeld C, Collins R, Piñeiro L, Agura E, Nemunaitis
36. Hasenclever D, Sextro M. Safety of alloPBPCT donors:
35. Majolino I, Cavallaro AM, Bacigalupo A, et al. Mobi-
34. Ringdén O, Potter MN, Oakhill A, et al. Trans-
33. Aversa F, Tabilio A, Terenzi A, et al. Successful engraft-
32. Ringdén O, Potter MN, Oakhill A, et al. Trans-
31. Majolino I, Cavallaro AM, Bacigalupo A, et al. Mobil-
30. Majolino I, Cavallaro AM, Bacigalupo A, et al. Trans-
96. Lemoli RM, Fortuna A, Motza MR, et al. Concomitant mobilization of plasma cells and hematopoietic prog...
enitors into peripheral blood of multiple myeloma patients: positive selection and transplantation of enriched CD34+ cells to remove circulating tumor cells. Blood 1996; 1625-34.
128. Bachar-Lustig E, Rachamim N, Hong-Wei Li, Lan F,


Ex vivo expansion of hematopoietic cells and their clinical use

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Background and Objectives. Hematopoietic stem cells are being increasingly used for treatment of malignant and nonmalignant disorders. Various attempts have been made in recent years to expand and manipulate these cells in order to increase their therapeutic potential. A Working Group on Hematopoietic Cells has analyzed the most recent advances in this field.

Evidence and Information Sources. The method used for preparing this review was an informal consensus development. Members of the Working Group met three times, and the participants at these meetings examined a list of problems previously prepared by the chairman. They discussed the single points in order to achieve an agreement on different judgments, and eventually approved the final manuscript. Some authors of the present review have been working in the field of stem cell biology, processing and transplantation, and have contributed original papers in peer-reviewed journals. In addition, the material examined in the present review includes articles and abstracts published in journals covered by the Science Citation Index® and Medline®.

State of Art. Over the last decade, recombinant DNA technology has allowed the large scale production of cytokines controlling the proliferation and differentiation of hema-lymphopoietic cells. Thus, in principle, ex vivo manipulation of hemopoiesis has become feasible. The present review covers three major area of interest in experimental and clinical hematology: manipulation of hematopoietic stem/progenitor cells, cytotoxic effector cells and antigen presenting dendritic cells. Preliminary data demonstrate the possibility of using, in a clinical setting, ex vivo expanded hematopoietic cells with the aim of reducing, and perhaps abrogating, the myelo-suppression after high-dose chemotherapy. Conversely, other important potential applications for ex vivo manipulation of hematopoietic cells have recently been investigated such as the generation and expansion of cytotoxic cells for cancer immunotherapy, and the large scale production of professional antigen presenting cells capable of initiating the process of immune response.

Conclusions and Perspectives. Present and future challenges in this field are represented by the expansion of true human stem cells without maturation, to extend this strategy to allogeneic stem cell transplantation as well as the manipulation of cycling of primitive progenitors for gene therapy programs. The selective outgrowth of normal progenitor cells over neoplastic cells to achieve tumor-free autografts may ameliorate the results of autologous transplantation. The selective production of cellular subsets to manipulate the graft versus-host and graft versus-tumor effects and anti-tumor vaccination strategies may be important to improve cellular adoptive immunotherapy.

Key words: hematopoietic stem cells, bone marrow, cord blood, dendritic cells, peripheral blood, allogeneic transplantation

Hematopoietic stem cells
Following the discovery that bone marrow transplantation could be used to rescue irradiated mice, the identification and characterization of the hematopoietic stem cell has become essential in order to achieve new developments in stem cell expansion and transplantation (SCT). The potential for using stem cells as vehicles for gene therapy has further increased the efforts of a number of research groups working on stem cell identification, characterization, cloning and manipulation.

Self-renewal and differentiation
Marrow and blood hematopoietic cells are heterogeneous and belong to different lineages at different stages of maturity. The structural and functional integrity of the hematopoietic system is maintained by stem cells that, by definition, comprise a relatively small cell population, located mainly in the bone marrow, which can (i) undergo self-renewal to produce stem cells or (ii) differentiate to produce progeny which is progressively unable to self-renew, irreversibly committed to one or other of the various hematopoietic lin-
Clinical use of hematopoietic stem cells

...eages, and able to generate clones of up to 10^5 lineage-restricted cells that mature into specialized cells. Although in recent years, (i) the development of in vitro and in vivo assays for hematopoiesis, (ii) the identification and characterization of hematopoietic growth factors, and (iii) the development of strategies for enriching hematopoietic cells have expanded our knowledge and understanding of hematopoiesis, the definition of the stem cell, originally proposed by Lajtha and McCulloch, has not substantially changed.

In addition to self-renewal and differentiation, a number of properties are ascribed to hematopoietic stem cells, including a high migratory potential, the ability to undergo asymmetric cell divisions, the capacity to exist in a mitotically quiescent form and extensively regenerate the different cell types that constitute the tissue in which they exist.

The issues of asymmetrical and symmetrical cell divisions and the regulation of self-renewal/differentiation process are crucial when analyzing stem cell behavior and the potential for stem cell manipulation. Asymmetric cell divisions produce one differentiated daughter (progenitor cell) and another daughter that is still a stem cell (Figure 1A). When all cell divisions are necessarily asymmetric and controlled by cell-intrinsic mechanisms, no amplification of the stem cell size is possible. Asymmetric divisions are referred to unequally distributed transcription factors in daughter cells, and have been shown to be possible in hematopoietic progenitors by clone-splitting experiments. Symmetric cell divisions produce either two progenitor cells or two stem cells according to a 0.5 probability of self-renewing versus differentiative divisions (Figure 1B). In this case, it can be assumed that the size of the stem cell pool can be modified by factors affecting the 0.5 probability value, i.e., factors that control the probability of self-renewing versus differentiative divisions. A third model postulates that individual cell divisions can be, but not necessarily are, asymmetric with respect to daughter cell fate (Figure 1C). This model also implies that daughters behave differently due to different local environments. Although it is not known whether a single cell can switch from an asymmetric to a symmetric mode of cell division, available evidence in the hematopoietic stem cell system favors a predominance of symmetric cell divisions.

The decision of a stem cell to either self-renew or differentiate as well as the selection of a specific differentiation lineage by a multipotent progenitor during commitment have been proposed to be regulated according to either stochastic or deterministic (inductive) models. Based on computer simulation and the distributions of colony-forming units in spleen (CFU-S) in individual spleen colonies, stochastic models postulate that the decision of a stem cell to self-renew (birth) or to differentiate (death) is randomly regulated by a probability parameter "p" which is equal to 0.5 in steady-state conditions. Deterministic models postulate the existence of lineage-specific anatomic niches that direct the differentiation of uncommitted progenitors. There is experimental evidence suggesting that the hematopoietic system may employ both stochastic and deterministic strategies, probably depending upon the stage of lineage differentiation.

Based on a number of studies performed in the last three decades, the regulation of self-renewal, commitment, proliferation, maturation, and survival can be assumed to reflect highly integrated processes under control of extracellular mechanisms, including regulatory molecules and microenvironment, as well as intracellular mechanisms, including protooncogenes, cell cycle regulators, tumor suppressor genes, transcription factors. Regulatory molecules include positive (growth factors) and negative (interferons, TGF-β, MIP-1α) factors which interact in complex ways (synergism, recruitment, antagonism). Molecules that maintain the stem cell state are beginning to be identified. These include ligands of the Notch family receptors that act from outside the cell as regulators of proliferation or maintenance of the undifferentiated state, and factors like PIE-1 that act from within the cell. However, despite the efforts which have been devoted to elucidating the issue of self-renewal control, no factors have yet been identified that are

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**Figure 1.** Possible patterns of stem cell division. "S" indicates a stem cell; "C" indicates a committed progenitor cell.
capable of maintaining self-renewing divisions and the molecular basis of self-renewal capacity remains to be elucidated. Growth factors so far identified more probably act as regulators of proliferation and survival. Theoretically, growth factors and cell-cell interactions can influence the outcome of fate decisions by stem and progenitor cells in a selective or instructive manner. According to selective mechanisms, the stem cell commits to a particular lineage independently of the growth factors and the factors act to control survival and proliferation of committed progenitors. In instructive mechanisms, growth factors cause the stem cell to choose one lineage at the expense of others. The relative contribution of these two mechanisms to hematopoietic regulation remains controversial, but experimental evidence suggests that at least some subsets of stem/progenitor cells can be instructed by growth factors to choose one differentiation pathway at the expense of others. In the absence of still unidentified instructive signals, it can be hypothesized that environmental signals may act by increasing or decreasing the probability of choosing a particular fate, rather than promoting or repressing it in an all-or-none manner.

Although growth factors play a key role in stem/progenitor cell proliferation and differentiation it seems improbable that hematopoiesis is regulated by a random mix of growth factors and responsive cells. Indeed, it is likely that regulatory molecules and localization phenomena within marrow stroma are required to sustain and regulate hematopoietic function. Stromal cells of the hematopoietic microenvironment provide the physical framework within which hematopoiesis occurs. They play a role in directing the processes by synthesizing, sequestering or presenting growth-stimulatory and growth-inhibitory factors, and also express a broad repertoire of adhesion molecules which mediate specific interactions with hematopoietic stem/progenitor cells. Differential expression of adhesion molecules could cause different stem cell subsets to home to different marrow microenvironments capable of differently affecting self-renewal.

While much progress has been made in identifying cytokines and stromal factors, little is known about intracellular mechanisms regulating hematopoietic stem/progenitor cells self-renewal and differentiation. Structure-function analysis of growth factor receptors as well as identification of novel signal transduction molecules have provided new insights into the processes involved in signal transmission pathways. Post-translational modifications of pre-existing proteins, in particular tyrosine phosphorylation, play a key role in transmitting signals and thereby linking extracellular signals to the activation of nuclear effector molecules which govern gene expression. Accumulating evidence points to transcription factors such as AML-1, Ikaros, SCL/Tal-1, Rb1n-2, Tan-1, GATA-2, and HOX homeobox genes as important regulators of these processes. Overexpression of HOX84 in murine bone marrow cells markedly increases the regenerative potential of long-term repopulating cells and causes an expansion in clonogenic progenitor cell numbers, without altering their ability to differentiate normally into mature myeloid, erythroid and lymphoid cells.

In contrast, overexpression of HOX83 causes defective lymphoid differentiation and progressive myeloproliferation. The glucocorticoid receptor, in combination with an activated receptor tyrosine kinase, seems to be a key regulator of erythroid self-renewal. Shc overexpression increases GM-CSF sensitivity and prevents apoptosis of the GM-CSF-dependent acute myeloid leukemia cell line GF-D8, thus suggesting that Shc is an important regulator of cell survival and proliferation. Different levels of protein kinase C modulate progenitor cell phenotype by favoring myelomonocytic or eosinophil differentiation. Recently, it has been shown that telomerase expression correlates with hematopoietic self-renewal potential. Hematopoietic stem cells show decreasing telomere length with increasing age. Thus, telomerase may regulate self-renewal capacity by reducing the rate of DNA shortening. Overall, intracellular mechanisms of hematopoietic control result in the repression or de-repression of lineage-specific genes regulating growth factor responsiveness and/or proliferation potential. The exact knowledge of these mechanisms will greatly modify our approach to stem/progenitor cell manipulation.

In summary, stem and progenitor cell behavior is the result of highly integrated phenomena based on extracellular signals triggering intracellular transduction phenomena. The properties of self-renewal and differentiation give stem cells their remarkable ability to repopulate the hematopoietic tissue of lethally irradiated or genetically defective recipients. Understanding the interplay between extracellular and intracellular regulatory factors in controlling lineage determination remains an important challenge for the future clinical use of hematopoietic cells.

**Stem cell antigen(s)**

CD34 is a surface glycoposphoprotein expressed on early lympho–hematopoietic stem and progenitor cells, small-vessel endothelial cells, as well as embryonic fibroblasts. CD34+ hematopoietic cells are morphologically and immunologically heterogeneous and functionally characterized by the in vitro capability to generate clonal aggregates derived from early and late progenitors and the in vivo capacity to reconstitute the myelo–lymphopoietic system in a myeloablated host. The CD34+ cell population contains virtually all the myeloid and lymphoid progenitors as well as a small subset of cells that can initiate and maintain stromal cell-supported long-term cultures. Expression of the CD34 marker has dominated attempts to isolate, purify and characterize human hematopoietic stem cells by a variety of immunologic means.

Several monoclonal antibodies (MoAbs) assigned to the CD34 cluster identify a transmembrane glycoprotein antigen of 105–120 kd expressed on 0.5–2% normal BM cells, 0.01–0.1% peripheral blood cells and 0.1–0.4% cord blood cells. The function of the CD34 antigen is...
not yet known, although it seems that CD34 is involved in stem/progenitor cell localization/adhesion in the marrow. CD34 antigen expression is associated with the concomitant expression of several markers, including the lineage non-specific markers Thy1, CD38, HLA-DR, CD45RA, CD71 as well as T-lymphoid, B-lymphoid, myeloid and megakaryocytic differentiation markers. Analysis of the expression of CD38, Thy-1, CD71, the isoforms of CD45, and uptake of rhodamine-123 have resulted in a consensus stem cell phenotype which is CD34 bright, Thy-1 +, CD38 −, CD45RA −, rh-123 dull, Hoechst 33342 dull, Lin −. CD34 + cells also express receptors for a number of growth factors classified as tyrosine kinase receptors, such as the stem cell factor receptor (SCF-R) or the stem cell tyrosine kinase receptors (STK), and hematopoietic receptors, not containing a tyrosine kinase domain. Tyrosine kinase receptors are of particular relevance since their ligands might represent new factors able to selectively control stem cell self-renewal, proliferation and differentiation. Recently, CD34 − cells have been shown to have functional characteristics associated with stem cells and differentiate, in vivo, to CD34 + cells.

**Stem/progenitor assays to evaluate engraftment potential**

Different types of progenitors can be measured directly by multiparameter phenotyping of CD34 + cells or subpopulations. Although this approach has the significant advantage that the results may be quickly available and can be used to guide clinical decisions, correlations between progenitor cell phenotype and functional activity are not yet refined enough to be clinically applicable. In addition, although CD34 antigen is expressed by virtually all progenitor cells, the percentage of CD34 + cells with clonogenic activity in vitro ranges from 10 to 50%. Non-clonogenic CD34 + cells include lymphoid progenitors as well as subsets of cells which are unresponsive to conventional growth factors and might require the presence of still unknown factors able to activate stem cell specific genes. Figure 2 shows a schema of the cellular organization of hematopoiesis based on in vitro and in vivo functional properties of progenitor cells.

Recently, a new human hematopoietic cell, termed the SCID repopulating cell (SRC), that is capable of extensive proliferation and multilineage repopulation of the bone marrow of non-obese diabetic (NOD)/SCID mice has been identified. The SRCs which are detected exclusively in the CD34 + CD38 − cell fraction have been shown to be biologically distinct from CFC and most LTC-IC. With the exception of transplantation of human cells into immunodeficient mice, the identification of putative human stem cells has essentially relied on in vitro assays. Short-term in vitro assay systems require appropriate nutrients and growth factors and are particularly suitable for measuring quantitative changes of the different progenitor cell types as well as for evaluating growth factor responsiveness or investigating differential effects of regulatory molecules on progenitors at different stages of differentiation or on different hematopoietic pathways. Short-term assays are not suitable for analyzing self-renewal or interactions of hematopoietic progenitors with stromal cells.

By using the long-term culture (LTC) technique, a sustained production of myeloid cells can be readily achieved in vitro, provided that a stromal layer is present, when marrow (or blood) is placed in liquid culture at relatively high cell concentration, with appropriate supplements, temperature and feeding conditions.

The LTC system, based on the re-establishment in vitro of the essential cell types and mechanism responsible for the localized and sustained production of hematopoietic cells in the marrow in vivo, offers an approach able to investigate not only the proliferative and differentiative events but also self-renewal of any clonogenic cell types. A 5- to 8-week time period between initiating cultures and assessing clonogenic progenitor numbers allows a very primitive, self-renewing human cell, the...
so-called long-term culture-initiating cell (LTC-IC) to be quantified. Limiting dilution assays allow the frequency of LTC-IC and their proliferative potential (number of CFU-GM generated by each LTC-IC) to be calculated. Another assay system, the cobblestone area-forming cell (CAFC), uses a pre-formed stroma as a support for hematopoiesis. In this system, the primitive cells are measured directly by their ability to form characteristic colonies of cells resembling cobblestones.

With the possibility of studying not only the differentiation but also the self-renewal of primitive progenitors, the LTC system will play an increasingly important role in the design and assessment of new strategies involving the genetic engineering of hematopoietic cells and marrow stromal cells. Hematopoietic cells that can generate active hematopoiesis for weeks in vitro or months in vivo after transplantation are considered stem cells. This seems a clinically useful criteria, because it characterizes those cells which are important for sustained hematopoietic recovery following SCT. However, it reflects an oversimplification of the rather complex process of hematopoietic function. In fact, the ability of a cell to provide long-term hematopoietic activity can either be due to a long period of quiescence after the initiation of the culture or be a function of the probability of stem cell self-renewal which influences the long-term survival of stem cell clones. Thus, the number of primitive cells measured in an LTC assay will be the product of the number of stem cells present at the onset of the culture and the probability of stem cell self-renewal. Although LTC assays will likely predict the in vivo repopulating activity of the graft, the clinical definition of a stem cell does not consider those stem cells that differentiate and die soon after transplantation or initiation of a culture.

### Preparation of hematopoietic cells for ex vivo expansion

In the vast majority of cell culture systems, the presence of inhibitory mature and accessory cells limits the degree of ex vivo expansion of the progenitor cell compartment. Thus, a higher production of total cells, clonogenic cells and more immature hematopoietic progenitors has been observed when purified progenitor cells (namely CD34+ cells) rather than the whole BM, cord blood, or peripheral blood stem cell (PBSC) collections are cultured ex vivo. Haylock et al. selected and cultured 1,000 CD34+ cells in presence of a 10-fold excess of contaminating CD34-, CD3+, CD14+ cells and found no differences in total cell production after 14 days of culture, as compared to the production of 1,000 CD34+ cells grown alone. However, when CD34+ cells were mixed with increasing concentrations of CD3-, CD3+ CD14+ cells, a marked decrease in the total cell output was observed after two weeks of culture suggesting an inhibitory activity of monocytes and T-cells. Despite the lack of information on CFU-C production, these results point out that the purity of the starting population is an important variable and it was recommended that at least 50% of cells should be CD34+ in the initial cellular input.

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<th>Table 1. Hematopoietic cell separation systems.</th>
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<td>Negative selection</td>
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<td>Avidin-biotin immunoabsorption</td>
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<td>FACS (high-speed cell sorting)</td>
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<td>Recovery (% of initial cells) (% CD34+ cells)</td>
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Also practical advantages in using a purified stem cell population as starting material for ex vivo manipulation, such as the ease of cell handling and the amount of cytokines consumed in the culture. Other variables that play an important role in stem cell expansion, which will be discussed in detail in the following paragraphs are: initial cell density, different cytokines used and their concentration, the presence of stromal cells, the composition of the culture medium and the refeeding schedule. Conversely, it has been recently demonstrated that CD34+ cells can be safely and efficiently processed after cryopreservation suggesting that the availability of fresh hematopoietic cells may not be an essential prerequisite for ex vivo expansion. Moreover, whereas the majority of preclinical and clinical studies have attempted to optimize the expansion of highly enriched CD34+ cells, under certain circumstances it may be advisable to select earlier subfractions of progenitor cells such as CD34+ DR- cells in chronic myelogenous leukemia (CML) or CD34+ Thy-1+lin- cells in multiple myeloma (MM). In these diseases the CD34+ cell population is still contaminated, to various degrees, by malignant cells; therefore, isolation of primitive progenitors prior ex vivo expansion may provide a starting cell population with a high proliferative potential free of tumor cells.

### Methods for hematopoietic progenitor cell (HPC) enrichment

Several methods have been proposed for purification of HPC (Table 1). Their final target is a cell population with optimal purity, viability and high proliferative potential obtained by means of a low cost, rapid and simple separation technique. Early attempts toward the purification of HPC were based on the cell physical properties. Density-gradient centrifugation, velocity sedimentation and elutriation are methods that separate cells based on cell size and buoyant density. More recently, immunologic selection techniques which take advantage of the expression of specific antigens on HPC membrane, have allowed a much better degree of enrichment. Specifically, the

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demonstration of the presence of the CD34 antigen on HPC has led to a number of positive selection systems which use MoAbs to purify hematopoietic precursors. Alternatively, CD34+ cells can be enriched by depletion of CD34- accessory and mature cells.

**Fluorescence activated cell sorter (FACS)**

Flow cytometry can be used to separate HPC from a heterogeneous population after incubation of cells with fluorochrome-conjugated MoAbs directed to cell-surface markers. Moreover, multiparameter enrichment can be obtained by combining physical properties such as cell size and cytoplasmic granularity and intracellular characteristics indicating cellular function (e.g. propidium iodide to determine cell viability; rhodamine-123 to assess metabolic quiescence and nucleic acid dyes to evaluate cycling status). This cell sorting technique can yield a highly purified (> 99%) cell population combining positive and negative markers for HPC using clinically-graded MoAbs. The main criticisms to the use of flow cytometry for selecting large numbers of cells are the low recovery of target cells and the length of time required to process the whole BM harvest or the leuka-pheresis products. The development of multiparameter high-speed cell sorting has been described and recently upgraded for clinical use. Viable cells have been sorted at rates as high as 40,000 cells/sec as compared to 2,000-5,000 cells/sec of commercially available cell sorters. Thus, the sample processing time can now be reduced to 8-12 hours. The sorted cell fraction also maintains its hematopoietic potential based on the presence of CFU-C, more immature CAFC and long-term repopulating cells in mice. Presently, selection of HPC (i.e. CD34-Thy-1- lin-) from clinical samples is directed toward the purification of cell populations highly enriched for HPC free of contaminating malignant cells in myeloma patients.47

**Panning**

Anti-CD34 MoAbs bound to the bottom of cell culture flasks have been used to select CD34+ cells. The target cell population present in a heterogeneous cell suspension is blocked on the plastic surface while CD34- cells remain in the supernatant and can be easily eliminated. Despite the good results reported, the availability of more efficient methods of cell separation have made this technique largely redundant.

**Immunomagnetic systems**

A variety of magnetic cell-separation methods have been described. Some of these systems are commercially available and have been used in clinical trials. The main differences between the currently used magnetic cell-separation methods are the composition and size of the magnetic particles used for labeling the cells and the separation process. Superparamagnetic beads can be equally used for negative and positive cell separation depending on the specificity of MoAbs. The rosetted target cells can be easily isolated from unlabeled cells by a magnet applied on the outer wall of the test tube or a magnet applied on the outer wall of the test tube or

| Table 2. High efficiency of Mini-MACS separation system for the enrichment of BM or circulating CD34+ cells. |
|---------------------------------|--------|--------|--------|--------|--------|
| Source                          | Pre (%) | Post (%) | Recovery (%) | CE* (x10^6 BM/CD34+) |
| BM                              | 2.3±1  | 97.3±7 | 88±9  | 3.6±0.3 | 62.5±54 |
| PB                              | 0.7±0.4 | 98.9±1 | 90±8  | 3.9±0.4 | 48.2±35 |

*Abbreviations: CE, clonogenic efficiency; BM, bone marrow; PB, peripheral blood. The results are expressed as mean±SD.

blood bag. Large magnetic beads (diameter >0.5 µm) have been used clinically for the purging of neuroblastoma and lymphoma cells from stem cell harvests prior to autologous transplantation.53,54 Immunomagnetic beads coupled with anti-CD34 MoAb can be used for positive selection of HPC.55 However, before the clinical use of the enriched cell fraction, the cell-bound particles must be removed to avoid damage to the cells and/or toxic events to the patient. Beads can be released using chymopapain or a peptide competing with the CD34 Ab. In alternative, HPC can be enriched by negative depletion of mature and accessory cells targeting lineage-specific antigens.

More recently, the magnetic cell-sorting (MACS) system has been proposed as an efficient and more manageable alternative to flow cytometry for cell separation.56 It uses colloidal-sized superparamagnetic particles made of dextran and iron oxide with 60 nm of diameter. The use of very small beads minimizes unspecific binding and allows the efficient isolation of rare cells. In addition, the magnetic particles are readily internalized by the labeled cells without affecting their physical, phenotypic and functional capacity.57 Table 2 reports the results of a large number of experiments (=14) comparing the efficiency of the Mini-MACS system for selecting CD34+ cells from two different cellular sources (R.M.L., unpublished observations).

**High-affinity chromatography based on avidin-biotin immunoabsorption**

This technique relies on the high affinity between the protein avidin and the vitamin biotin whose interaction has an extremely high dissociation constant (≈ 10^{-15} M). In this system, a heterogeneous cell population is incubated with a biotinylated antibody to the CD34 antigen. The cell mixture is then passed through a disposable column containing avidin-coated polyacrylamide beads. CD34+ cells are retained on the column due to the high affinity binding of biotin to avidin while negative cells are washed away. Target cells are then recovered by mechanical agitation of the column which disrupts the antibody-antigen link. Thus, bound cells are eluted from the column mainly free of antibody. An automated version of the device controlled by a computer which guar-
Ex vivo expansion of myeloid progenitor cells

Ex vivo expansion of hematopoietic progenitors might result in:

- amplification of the population of committed progenitors due to an extensive, although controlled, differentiation process;
- amplification of the stem cell pool through extensive self-renewal of the early progenitor cell population.

Obviously, both processes can take place simultaneously mimicking, in vitro, the complex interplay of regulatory mechanisms that allows hemopoiesis in vivo. The latter situation, so far, has never been obtained in vitro, whereas different approaches have permitted the first goal to be reached (at least, to a certain extent) and some recent data suggest that relevant self-maintaining processes can be triggered.

The clinical relevance of extended differentiation versus self-renewal is obviously different. The induction of an increased in vitro production of committed progenitors might hasten the early phases of hemopoietic reconstitution which occur after myeloablative treatment and stem cell transplantation. Moreover an increased number of infused cells might modulate graft versus-host disease (GVHD) intensity in the allogeneic setting.

Although relevant, the potential clinical benefit of techniques allowing only committed progenitor cell expansion is outweighed by the possibility of triggering the self maintenance, and perhaps amplification, of early hemopoietic progenitors. In this situation, starting from a limited number of progenitors, long-term reconstitution of hemopoiesis might become feasible. Moreover, ex vivo manipulation of primitive hemopoietic cells could be performed under experimental conditions suboptimal for the growth of neoplastic cell contaminating autologous grafts. Thus, a purging effect could be obtained.

Several attempts of in vitro expansion of hemopoietic progenitors have been published in the last years. Recent reviews summarize early experiences.

The first studies on ex vivo generation of hematopoietic progenitors involved liquid culture in the presence of cytokines such as SCF, IL-1, IL-3, IL-6, G-CSF, GM-CSF and Epo. These experiences showed that a relevant increase (from 10 to 1,000 fold) of CD34+ cells and of committed progenitors can be obtained. The expansion of committed progenitors does not mean, however, that the long-term reconstitution of hemopoiesis is achievable. Indeed, several data support the concept that an uncontrolled commitment decreases the stem cell pool. Yonemura et al. have reported that IL-3 or IL-1 abrogates the reconstituting ability of hematopoietic stem cells. Furthermore, Peters et al. have demonstrated that ex vivo expansion of murine marrow cells with IL-3, IL-6, IL-11 and SCF leads to impaired engraftment in irradiated hosts.

As indicated by Traycoff et al., ex vivo expansion of hematopoietic cells using SCF, IL-1α, IL-3 and IL-6 generates classes of cells possessing different levels of BM repopulating potential based on their cycling status. Along this line, Young et al. correlated a higher proliferative capacity with a quiescent status after ex vivo expansion in the presence of SCF, IL-3, IL-6 and LIF. Taken together, these data suggest that cultures in the presence of cytokine combinations based upon SCF, IL-1 and IL-3 involve differentiation of BM or mobilized CD34+ cells entering the S phase. Different results were obtained by Di Giusto et al., who found that ex vivo...
expanded cord blood CD34+ cells repopulated the marrow of immunodeficient mice as well as non-expanded cells. However, it must be remembered that cord blood is rich in hematopoietic progenitors that have an increased proliferation potential.

New strategies to induce the expansion of CD34+ cells with little (or no) differentiation might involve different approaches. The use of stirred suspension or hollow fiber bioreactors has been proposed in order to grow cells in a more physiologic environment, and inhibitors such as TGF-β and MIP-1α have been the object of intense studies. Recently, MIP-1α has been found to exert a weak inhibitory effect on CD34+CD38+ cells and to enhance the proliferation of CD34+CD38+ cells, whereas TGF-β strongly inhibits both cell populations. The most promising results, however, have been obtained with the recent introduction of FL and Tpo. FL, a recently discovered member of the class III tyrosine kinase receptor family, is able to induce proliferation of very early hematopoietic progenitors that are non-responsive to other early acting cytokines, and to improve the maintenance of progenitors in vitro. This is also supported by the finding that FL significantly reduced the number of cultured cells undergoing apoptosis. Analysis of the effects of 16 cytokines on CD34+CD38+ cells showed that FL, SCF and IL-3 produced a 30-fold amplification of the input of LTC-IC. Yonemura et al. compared FL- and SCF-driven ex vivo expansion. They reported that both cytokines, in combination with IL-11, enhanced the production of progenitors, but with different kinetics. In fact, the maximal expansion by FL required a longer incubation than with SCF. Interestingly, in these studies the combination of SCF/IL-11, together with IL-3, reduced the ability of cultured cells to reconstitute hematopoiesis in irradiated hosts. Other recent data have compared the effect of FL and SCF. FL acts as a self-renewal or proliferation/expansion signal for CD34+CD38+ cells while the effect of SCF is more likely to transduce a differentiation signal, resulting in more rapid repopulation at the expense of cell expansion. Gene transfer studies in mice have also demonstrated that FL maintains the ability of human CD34+ cells to sustain long-term hematopoiesis. In fact, incubation of CD34+ cells with FL before transduction was associated with long-term provirus expression, whereas provirus expression declined in recipients of CD34+ cells transduced in the absence of FL. The expansion ex vivo of early progenitors seems to be affected at the single cell level by changes in cytokine concentrations. In a recent paper by the Vancouver group, maximal LTC-IC expansion was obtained in the presence of 30 times more FL, SCF, IL-3, IL-6 and G-CSF than could concomitantly stimulate the near-maximal amplification of CFC.

Tpo, the ligand of the mpl receptor expressed on both early and committed hematopoietic progenitors, is known to support megakaryocytogenesis. Moreover, it has been shown to be capable of enhancing ex vivo expansion of early/committed progenitor cells. As single factors, FL and Tpo stimulated a net increase of LTC-IC generated from CD34+CD38+ cells within 10 days. Furthermore, as demonstrated in mice recipients of BM cells transduced with the mpl receptor, Tpo does not induce lineage-restricted commitment of mpl-receptor positive pluripotent progenitors but permits their complete erythroid and megakaryocytic differentiation. Tpo has also been found to increase the multilineage growth of CD34+CD38+ cells from 3%, in absence of the cytokine, up to 40% when Tpo is added to SCF and FL. The presence of additional cytokines such as IL-3, IL-6 and Epo does not significantly enhance clonal growth above that observed in response to Tpo, SCF and FL. Interestingly, the soluble form of Tpo receptor and G-CSF receptor directly stimulate the proliferation of primitive hematopoietic progenitors of mice in synergy with SCF and FL.

A step toward extensive ex vivo amplification of early human progenitor cells has been reported by Piacibello et al. They first demonstrated that IL-3 induces an early production of committed progenitors but is not able to sustain true self maintenance of hematopoietic stem cells even in the presence of other early acting cytokines (FL, Tpo, SCF). Afterwards, several combinations of early acting cytokines were tested for their ability to sustain long-term hematopoiesis in stroma free cultures. Among the various combinations tested on purified cord blood CD34+ cells, the mixture of Tpo + FL was found to be able to maintain early progenitors up to six months. These data indicate the enormous potential of cord blood progenitors and the key role of Tpo and FL in the regulation of early hematopoiesis. However, several issues remain to be clarified:

- is it true self-renewal or a slow differentiation of cord blood cells, which are rich in immature progenitors?
- what is the in vivo repopulating capacity of ex vivo expanded cells?
- is such expansion possible using CD34+ cells obtained from the marrow or peripheral blood of adult subjects?

In this view, while a number of papers have already reported that committed progenitors can be generated and safely administered to transplant recipients, there are no reports on expansion of cells with long-term repopulating capacity in humans. Brugger et al. reported the successful reconstitution of hematopoiesis in ten cancer patients transplanted with autologous cells generated from CD34+ cells cultured in the presence of SCF, IL-1β, IL-3, IL-6 and Epo. However, the conditioning regimen given to these patients was not fully myeloablative, and this study offered no insight into the long-term engraftment potential of cells generated in this fashion. A similar approach was followed by Alcorn et al. In ten patients with malignancy, an aliquot of the PBSC harvest was recovered from liquid nitrogen and CD34 were selected. Cells were cultured for 8 days in the presence of the same cytokine combination. A mean of 379×10^6 expanded cells were reinfused in addition to unmanipulated
cells. The authors reported that the total LTC-IC number was not increased and most of the CD34+ cells were differentiated in front of an average 15-fold CFU-GM expansion (range 4-39). Similarly, averages of 71 (range 27-151)-fold megakaryocytic cell expansion and 1,040 (19-16,000)-fold erythroid cell expansion were reported. Although adverse reactions were not reported, no difference in the kinetics of engraftment was observed in comparison with historical controls.

Different results come from preclinical studies where the infusion of large numbers of ex vivo expanded committed hematopoietic progenitors, together with unmanipulated cells might speed engraftment after chemotherapy and/or total body irradiation (TBI). Data from Uchida et al.90 have suggested in the past that most of the short-term as well as the long-term engraftment potential resides in uncommitted progenitors. More recently, Szi1vassy et al.91 have demonstrated that partially differentiated ex vivo expanded cells accelerate hematologic recovery in myeloblated mice transplanted with highly enriched long-term repopulating stem cells. In humans, Williams et al.92 reinforced 9 breast cancer patients with unmanipulated apheresis products together with a mean of 44×10^6/kg mature CD15+ cells generated ex vivo by CD34+ cells cultured in the presence of PIXY-321. No toxicity was observed after reinfusion, and time of white cell recovery was similar to that observed in the retrospective control group. In a more recent study,78 megakaryocytic progenitors (MP) were obtained from CD34+ cells cultured in serum-free medium in the presence of Tpo, FL, SCF, IL-3, -6, -11 and MIP-1α. Proliferation peaked on day 7 in culture, and a 8×5-fold expansion of CD34+/CD61+ cells, a 17×5-fold expansion of CFU-MK and a 58×14-fold expansion of the total number of CD61+ cells was obtained. Ten cancer patients undergoing autologous PBPC transplant received MP generated ex vivo (range 1-21 CD61+ cells ×10^6/kg) together with unmanipulated PBSC. Platelet transfusion support was not needed in 2 out of the 4 patients receiving the highest dose of cultured MP and this result compared favorably with a retrospective control group of 14 patients, all requiring platelet transfusion support. A major concern is the potential expansion of contaminating tumor cells along with hematopoietic progenitors. In fact, it has been demonstrated that CD34+ cell selection decreases (but does not abrogate) neoplastic cell contamination from apheresis of myeloma patients.93 For instance, in the majority of B cell lymphoma patients CD34+ cell selection does not eliminate contaminating t(14;18)1+ cells. However, during ex vivo expansion residual lymphoma cells do not proliferate and become undetectable by molecular analysis in the majority of cases.94 Similarly, Vogel et al. recently indicated that exogenously mixed epithelial tumor cell lines might have a relative disadvantage over CD34+ cells during ex vivo expansion.95

**Future directions**

Future challenges in this field are represented by the expansion of true human stem cells without maturational education. This strategy to allogeneic stem cell transplantation, and especially cord blood allografts, as well as the manipulation of cycling of primitive progenitors for gene therapy programs.

Selective amplification of specific myeloid lineages (e.g. platelets or granulocytes) may improve the results of autologous transplantation. Moreover, although early results need confirmation, the amplification of early/committed hematopoietic cells coupled with the removal of neoplastic cells contaminating autologous grafts appears to be feasible.

**Expansion of cytotoxic effectors**

Human cytotoxic effector (CE) cells can be divided in two major groups:

1. Cells requiring prior antigen sensitization, which recognize their target in the context of the major histocompatibility complex (MHC) molecules;
2. Cells not requiring prior antigen sensitization being spontaneously cytotoxic against tumor target cells (e.g. K-562 cell line) in a non-MHC restricted setting.

While the first group includes only some subsets of T-lymphocytes (CD8+ or CD4+ cells), the second one is more heterogeneous and includes both T-cells and natural killer (NK) cells, expressing the CD56 antigen.95

The so-called antibody-dependent cellular cytotoxicity (ADCC) can be mediated by cells expressing the Fcγ receptor II and the Fcγ receptor III (e.g. NK cells and CD3+CD16+ cells). Although this activity is not exhibited by non MHC-restricted cells, it cannot be considered aspecific and it is also exerted by monocytes.

The lymphokine-activated killer cells (LAK) are capable of killing NK-resistant cellular targets (e.g. Daudi cell line). Although some tissue-resident lymphocytes may have spontaneous LAK activity, normal blood mononuclear cells do not show any LAK activity, which can be acquired after incubation with Interleukin-2 (IL-2).96

Therefore the LAK assay is a measure of the capacity of T and NK cells to become activated and to express cytolytic function.

**Killing mechanisms of cytolytic effectors**

Cytotoxic T-Lymphocytes (CTL) and NK cells possess at least two distinct, fast-acting, lytic mechanisms.97,98

1. The granule exocytosis pathway involves the secretion of perforin and granzymes which penetrate throughout the target cell pores, inducing cell death;
2. A non-secretory mechanism which is mediated by the interaction between the Fas-ligand, expressed by the killer-cell and Fas (CD95) which triggers a cascade of proteolytic enzymes leading to apoptosis of both the killer and the target cell.

A third cytolytic pathway, involving TNF, has recently been described.99

A series of apoptosis-resistant clones of human lym-
phoma cells has been described. These cells express fas/APO-1 receptors lacking the intracytoplasmic signaling domain.\(^{100}\)

NK cells, as well as CTL, can recognize MHC class I molecules. However, recognition of MHC on target cells downregulates the NK cell function, suggesting the presence of inhibitory receptors.\(^{101}\)

LAK cells are not MHC-restricted and are also capable of killing freshly isolated tumor cells. Similarly to CTL and NK cells, their activity is mediated by both lytic pathways (perforin/granzyme) and Fas-mediated apoptosis.

**Cytokines involved in CE function**

Several cytokines affect CTL and NK cell response. In particular, IL-2 expands the pool of alloreactive CTL precursors and IL-15 (produced by monocytes) mimics IL-2 action by inducing γ-IFN production, the activation of memory T cells and CTL proliferation.\(^{102}\)

In addition, GM-CSF can affect certain T-lymphocyte functions by enhancing their cytotoxicity and γ-IFN production. This multifunctional cytokine can also augment NK cell function and the expression of adhesion molecules on the surface of leukemic cells.\(^{103,104}\) Moreover, it has been hypothesized that the association of GM-CSF/IL-2 can also be useful for the activation of cytotoxic effectors by circulating progenitors, preserving the clonogenic potential of normal hemopoietic precursors.\(^{105}\)

IL-12 elicits the production of γ-IFN by CTL thus enhancing their antineoplastic efficacy and promotes the differentiation of T-helper-1.\(^{106,107}\) Finally, IL-7 seems to be critical for the development of CTL and for a fast immune reconstitution after bone marrow transplantation (BMT).\(^{108}\)

In conclusion, these cytokines play a pivotal role in the immune response against tumor cells by expanding, activating and recruiting CE and secondary effector cells (macrophages) or by directly inhibiting tumor cell growth.

**Role of cytotoxic effectors in immuno-surveillance**

There are several in vitro and in vivo data supporting the role of immunosurveillance in tumor growth control.\(^{109}\) The graft-versus-leukemia (GVL) effect has been demonstrated to play a critical role in the eradication of minimal residual disease (MRD) after allogeneic transplantation and there is evidence supporting the role of both T cells and NK cells in preventing disease relapse.\(^{110}\)

Today, there is no doubt that CTL have a major role in killing allogeneic tumor cells in a MHC-restricted manner. For example, in CML, the higher relapse incidence after T-cell depleted allogeneic BMT\(^{111}\) and the dramatic effect of donor T-lymphocyte infusion after relapse following BMT,\(^{112}\) strongly support the importance of MHC-restricted GVL.

Unfortunately, a selective GVL effect (separated from GVHD) can be obtained very rarely in patients receiving allogeneic BMT. Moreover, although animal models indicate that autologous GVHD exists and could generate a significant antitumor effect, the high incidence of relapse in patients receiving autologous BMT demonstrates that autologous GVL is often clinically ineffective.\(^{113}\)

**Rationale for cytotoxic effectors’ expansion**

There are many important reasons to increase the number, the efficacy and the specificity of cytolytic effectors both in the allogeneic and in the autologous setting. The main clinical goals are the following:

1. to reduce the relapse-incidence after autologous BMT, which is still relevant in acute leukemia, lymphoma and breast cancer;
2. to cure diseases in which autologous BMT can only prolong the survival (multiple myeloma, CML, metastatic chemosensitive cancers);
3. to reduce the incidence of relapse after allogeneic BMT especially in patients transplanted with great tumor burden;
4. to accelerate the immune reconstitution after BMT, in order to reduce the morbidity and transplant-related-mortality caused by serious infections (e.g. CMV and systemic mycosis).

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Development and expansion of NK cells

NK cells belong to the naive portion of the immune system and begin to appear into PB early after allo and auto-BMT. These cells express the N-CAM homologous CD56 antigen, but lack T-cell receptor α/β complexes. They are also characterized by low affinity receptors for IgG (CD16)\textsuperscript{\textdagger}–\textsuperscript{\textdagger\textdagger} and their binding to malignant cells is mediated by CD18 molecule.\textsuperscript{177} The most important organ for NK differentiation is the BM. Several data suggest that a common precursor of T cells and NK cells does exist. Fetal NK cells express the TCR γ, δ, ε, ζ subunits while fetal B precursors do not express TCR subunits (Figure 3).

These bipotential T/NK precursors do not have TCR rearrangement and share CD34/CD33/CD7 antigens. They differentiate to NK in the presence of SCF, IL-7, IL-2 and stromal feeder cells. The first step of NK differentiation is stroma-dependent while the second step of NK maturation is stroma-independent and is strongly potentiated by the association of IL-2 with IL-7.\textsuperscript{178} In all studies IL-2 is required for NK cell differentiation from CD34\textsuperscript{+} cells. This finding suggests that a fraction of CD34\textsuperscript{+} cells expresses IL-2 receptors and that activated T-cells (as IL-2 source) should be detectable in the cellular milieu. However, T-cell deficient mice have normal NK cell development and humans lacking the γ-chain subunit of IL-2R lack NK activity. These unexpected observations have recently been supported by the demonstration that IL-15, produced by BM stromal cells, can directly induce CD34\textsuperscript{+} cells to differentiate into CD3\textsuperscript{−}/CD16\textsuperscript{−} NK cells in the absence of IL-2.\textsuperscript{179}

The last step of NK development, after the expression of CD16, is characterized by the appearance of the CD56 molecule. The intensity of CD56 expression directly correlates with the proliferative potential and the killing ability of NK cells.\textsuperscript{180}

There is clear evidence that mature NK elements have a clonally-distributed ability to recognize their target cell by class I MHC alleles and a precise correlation has been established between the expression of p58 receptors on NK cell surface and class I MHC alleles. These receptors transduce an inhibitory signal upon interaction with MHC class I antigens, to prevent NK cells from killing target cells expressing certain (self) HLA alleles.

These findings are consistent with a self-tolerance mechanism exerted by the NK population which can be disrupted as a consequence of tumor transformation or viral infection or any other events inducing (or masking) class I molecules.\textsuperscript{181,182}

After incubation with IL-2, NK cells become LAK cells capable of killing otherwise NK-resistant target cells. These (NK) activated cells express new markers such as CD25, MHC class II and fibronectin which can be useful for the evaluation of their functional state. The NK cell compartment is heterogeneous and distinct subsets have been characterized. The most informative functional differences are based on relative CD56 fluorescence: only CD56\textsuperscript{bright}, but not CD56\textsuperscript{dim} NK cells, express the high-affinity IL-2 receptor. As a consequence, they respond to low concentrations of IL-2 and expand 10 times more than CD56\textsuperscript{dim}. This subset seems to be significantly reduced in leukemic patients. A remarkable reduction of CD56\textsuperscript{bright} NK cells has been observed in CML patients coupled with a significant decrease of their spontaneous cytotoxicity against the K-562 line. However, this defect was corrected by 18 hours incubation with 1000 U/mL of recombinant IL-2.\textsuperscript{183} These data strongly suggest that during tumor progression, the NK compartment (and particularly the small fraction of NK-CD56\textsuperscript{bright} with high proliferative ability) is progressively suppressed even though the exogenous administration of IL-2 can partially reverse this phenomenon. The strong correlation between functional capacity of the NK compartment and tumor progression has often been reported as well as the efficacy of the administration of LAK cells plus IL-2 in restoring an anti-tumor response.\textsuperscript{184}

Along this line, more than 90% of patients with acute leukemia in complete remission do not show spontaneous cytotoxicity against autologous blast cells. However, ex vivo treatment with IL-2 restores cytolytic activity in 37.5% of these patients.\textsuperscript{185} The first attempts to generate and expand LAK activity either in vivo or in vitro (after ex vivo incubation with IL-2) have been clinically disappointing especially in patients autotransplanted for acute lymphoblastic leukemia (ALL).\textsuperscript{186} Nevertheless, there is now a renewed interest in the use of activated NK cells in hematologic malignancies, based on the optimization of different approaches:

- administration of IL-2 in vivo to expand functionally active CE in patients with low tumor-burden, in order to reach an optimal effector/target ratio;
- harvesting and culturing large amount of NK cells for additional ex vivo expansion/activation with IL-2. Expanded cells should be reinforced in the early phase after BMT;
- sequential combination of both techniques (Figure 4).\textsuperscript{187}

The systemic administration of IL-2 for in vivo expansion and activation of the NK compartment may theoretically have some advantages:

1. high number of CE precursors in the body;
2. possibility of activating CE residing in the tumor bulk;
3. more feasible and cheaper strategy than the generation and administration of LAK cells.

On the other hand, the main drawbacks of this approach are the high toxicity of systemic administration of IL-2 and the high variability of anti-tumor response.

Sources of cytotoxic effectors and modalities of NK cell expansion

Human NK progenitor cells can normally be found in the BM,\textsuperscript{127,128} and originate from CD34\textsuperscript{+} hematopoietic progenitors.\textsuperscript{129} So far, the generation of NK cells from CD34\textsuperscript{+} precursors has been described on a small scale.
High numbers of functionally active NK cells can be easily demonstrated in mobilized cells from patients receiving chemotherapy plus G-CSF. Silva et al. have shown a 5.4-fold expansion of NK cells from leukapheresis products incubated in the presence of IL-2 for 6-8 days without affecting the CD34+ cell content. However, decreased function of NK cells has recently been described in the PB of normal donors after G-CSF administration. Circulating NK progenitors showed a decreased killing capacity and diminished proliferative ability in response to IL-2, as compared to their unprimed BM counterpart.

Based on previously published LAK trials, it can be estimated that about 10^{10}-10^{11} activated NK cells are needed to stimulate an anti-tumor response. A 100-fold ex vivo expansion of these cells from a standard leukapheresis collection would, therefore, be required to obtain such a high number of effectors.

Beaujean et al. rein infused autologous BM cells incubated for 10 days with IL-2 in 5 ALL patients following a myeloablative treatment. This procedure resulted in an important loss of hemopoietic progenitors with delayed engraftment. Moreover, in spite of this attempt to induce an autologous GVL, all patients eventually relapsed.

Wong et al. compared the ability of IL-2 alone or combined with IL-7 or IL-12 to stimulate NK activity in BM or PB samples. They found that IL-2/IL-12-activated blood cells suppressed the growth of the leukemia cell line K-562 about eight-fold more efficiently than BM cells. They also found that cryopreservation and subsequent stimulation of BM and PB cells did not significantly decrease the activity of NK cells. Finally, the combination of IL-2 and IL-12 showed a synergistic effect on both BM and PB elements. Large-scale ex vivo expansion of NK cells for adoptive immunotherapy not only requires an optimal source of precursors, but also clinically approved materials and procedures. In this context, Miller et al. obtained a 21-fold expansion of NK cells using a 21-day large scale NK culture performed in gas-permeable bags. Pierson et al. observed a 352-fold expansion of NK cells after 33 days of incubation in a bioreactor. Their starting population was NK precursors enriched by negative panning with anti-CD5 and anti-CD8 antibodies. The activated NK population was highly purified (>90%) in CD56+/CD3− cells and maintained a powerful cytotoxicity against K-562 cells.

The use of a homogeneous NK cell fraction for cell therapy programs seems to be advantageous because activated NK cells have more specific lytic activity than heterogeneous LAK populations. However, creating such a fraction requires a first step of enrichment (e.g. by eliminating CD8+/CD5− cells) and long-term cultures carry the risk of fungal or bacterial contamination.
**Cytokine induced killer cells (CIK) expansion**

In 1986 Lanier described a subset of CD3+ T cells co-expressing the CD56 antigen which is a typical NK marker. A remarkable expansion of this cellular subset has recently been obtained by Schmidt et al. following a 16-day incubation in the presence of IFN, IL-1, IL-2 and a monoclonal antibody against CD3 as the mitogenic stimulus. The ability of CIK cells to deplete leukemic cells from CML marrow was then investigated by the same group. While standard LAK cells were, in most cases, unable to lyse CML cells, CIK cells were toxic to both autologous and allogeneic CML blasts without affecting normal hemopoietic progenitors.

**CTL expansion for adoptive therapy**

It is well known that the GVL effect can be transferred with donor-buffy-coat (BC) lymphocytes. The antitumor effect of the CTL contained in the BC has been shown to be more potent than that induced by NK cells, even though NK cells exert a GVL activity different from GVHD.

In a murine model, a single dose of 2 × 10^7 CTLs in tumor bearing mice (DBA/2) resulted in the eradication of primary cancer and metastases without causing severe GVHD. Unfortunately, this GVL effect cannot be easily separated from GVHD in humans. As a matter of fact, in clinical studies the beneficial effect of CTL administration is often offset by the severity of GVHD or marrow aplasia.

Although leukemic cells share common antigens with other tissues of the host, there is also the chance that distinct leukemic antigens may be recognized by specific allelogeneic CTL.

Leukemia-specific T-cell clones have been isolated from HLA-identical siblings and this finding may explain the high incidence of CR, without GVHD, in patients with CML relapsed after allo-BMT and treated with donor BC.

The subset of donor-lymphocytes involved in the GVL effect is not entirely defined. Both CD4+ and CD8+ GVL effectors have been described in animal models. Recent studies in man suggest a prominent role of CD8+ cells in acute leukemia and CD4+ cells in CML. The therapeutic index of this approach may be increased by treating the donor lymphocytes, previously activated with recipient PHA-stimulated blast, with anti-CD25 ricin-conjugated antibodies. This procedure gives origin to a CTL population which retains over 75% of its antileukemic activity with only 10% of the initial responsiveness against the non-leukemic cells of the recipient.

**Strategies for generation and expansion of specific CTL**

A very attractive system for generating and expanding CTL is based on the selection and isolation of tumor-specific peptides (e.g. those encoded by bcr-abl or PML-RARα fusion genes) and to presenting them to T-cells to stimulate a specific T-cell response. The responding T-clones can then be amplified and selected by limiting dilution techniques. Unfortunately, in many cases the tumor-specific peptide is not presented by leukemic cells making the generation of peptide-specific CTL useless. In this regard, the transfection in tumor cells of DNA sequences encoding for co-stimulatory molecules (B7-1) or cytokines such as GM-CSF has greatly enhanced the anti-tumor response of T-cells. Alternatively, the use of professional antigen presenting cells (APC), primed with tumor specific antigens (e.g. tumor specific idiotype in low-grade B-cell lymphoma) has proved to be effective for the generation of tumor-specific CTL clones capable of inducing a measurable anti-tumor response. Allo-CTL have also been used against EBV-related lymphoma developed in allograft recipients and HIV patients. EBV infection is controlled in normal individuals by specific CTL which lyse EBV-infected B-cells upon recognition of viral peptides presented on the cell membrane in association with MHC class I molecules.

EBV-specific CTL have been isolated from normal donor leucocytes and expanded ex vivo by Rooney et al. Following the reinfusion of 1.2 × 10^6 CTL/m² into an allograft recipient, the complete resolution of an EBV-related immunoblastic lymphoma was observed.

Ex vivo expanded CTL can also be used to restore CMV-specific responses in immunodeficient individuals receiving allogeneic BMT. Walter et al. treated 14 patients with infusions of CD8+ CTL directed to CMV proteins obtained from bone marrow donors. In this study, CMV-specific CTL were expanded by stimulation with anti-CD3 antibodies coupled with autologous CMV-infected fibroblasts in IL-2-containing culture. This approach to adoptive immunotherapy was well tolerated by the recipients and not associated with severe GVHD.

**Future directions**

Preliminary clinical data suggest that the efficacy of donor BC infusion for the treatment of leukemic relapse can be significantly improved by the administration of IL-2 in vivo after reinfusion and by a brief incubation of BC with IL-2 before the reinfusion. The antitumor efficacy of T-lymphocytes can also be enhanced by transfection of cytokine genes or new receptors. An interesting approach is represented by the binding of TCR to a specific anti-tumor antibody Fab fragment or the use of bispecific (anti-tumor and anti-CD3) antibodies capable of recruiting and expanding tumor-specific CTL at the tumor site. Recently, a significant autologous GVHD effect has been obtained by the addition of γ-IFN to cyclosporin-A in order to upregulate MHC class II molecules. Alternatively, GM-CSF seems to enhance the anti-tumor response by stimulating professional APC (see below).

In conclusion, there is clear evidence that CTL exert their cytotoxic effect through the recognition of minor HLA antigens. However, in some patients a very low frequency of specific antileukemic CTL, responsible for a GVL effect distinct from GVHD, have been isolated. Moreover, genetic approaches, such as the transduction...
of donor lymphocytes with a suicide gene, have been proposed to control GVHD occurring after CTL administration.  

**Ex vivo generation of human dendritic APC**

Clinical investigators are keenly interested in the role of APC in the initiation of immune responses because of the potential to exploit these cells for immunotherapy of cancer and viral diseases. Pioneer studies in mammals by Steinman et al. have demonstrated that the specialized system of APC is constituted by BM-derived dendritic cells (DC). DC are distinguished by their unique potency and ability to capture, process, and present antigens into peptide–HLA complexes to naive T lymphocytes and to deliver the co-stimulatory signals necessary for T lymphocyte activation and proliferation.

Here, we summarize the main experimental evidence supporting the working hypothesis that individuals vaccinated with DC expanded ex vivo and engineered to present tumor associated antigen(s) can mount tumor-specific humoral and cellular responses. This can lead to tumor regression as well as protective immunity against tumor growth in vivo.

**Identification of dendritic cells**

DC are leukocytes derived from hematopoietic stem cells along the myeloid differentiation pathway (Figure 5). The differentiation of DC is a stepwise process originating from myeloid progenitors in the BM, immature DC distribute via blood to tissues where they have the capacity to take up and to process antigens. As migratory DC, they transit through the lymph or blood to lymphoid organs, where they become mature DC, which lose antigen-processing ability and acquire superior antigen-presenting capacity for T lymphocytes. In humans, DC at different developmental stages circulate in PB and they are found in virtually all tissues of the body where, depending on the location, they are referred to as interstitial DC (heart, kidney, gut, and lung), Langerhans cells (skin, mucous membranes), interdigitating DC (thymic medulla, secondary lymphoid tissue); or veiled cells (lymph, blood). DC are regarded as distinct from monocytes/macrophages, although they share a common progenitor after the CFU-GM stage. However, this has been questioned as mixed colonies of dendritic cells and macrophages are generated in vitro from single CD34 hematopoietic progenitors more commonly than pure DC colonies.

DC can be distinguished from other APC by a) morphology; b) cell-surface membrane phenotype; and c) the strong capacity to present antigens to T cells, usually assessed in the allogeneic mixed leukocyte culture (reviewed in ref. #163).

Cutaneous DC, as well as most of the DC generated ex vivo from human CD34 progenitors cells express high levels of the surface membrane CD1a antigen (Figure 6). Although CD1a antigen can be found on cortical thymocytes and some B lymphocytes, its presence (noted by immunofluorescence and flow cytometry) is the most useful way to quantify the ex vivo generation of DC from early precursors. In addition to the CD1a antigen, DC express peculiarly high levels of class I and class II histocompatibility complex structures, co-stimulatory molecules for T-lymphocytes such as B7-1 (CD80) and B7-2 (CD86), and adhesion molecules such as ICAM-1 (CD54) and ICAM-3 (CD50) which are involved in DC-
dependent T-lymphocyte proliferation. DC lack monocyte/macrophage- and lymphocyte-lineage-restricted antigens with the exception of the CD4 antigen. As shown in Table 3, relevant co-stimulatory (B7-1 and B7-2) and adhesion molecules (ICAM-1) are expressed on all CD1a+ cells derived from CD34+ progenitors, but on fewer CD1a+ cells derived from monocytes. More recently, the CD83 cell surface antigen has been recognized as a valuable tool for detecting blood DC.

**Ex vivo expansion of dendritic cells**

Although DC circulate in the PB and are found in virtually all tissues of the body, it is difficult to obtain enough cells for ex vivo manipulation because of their scattered locations and low number in the blood where they account for approximately 0.1% of all leukocytes. For this reason, it has been of crucial interest to know that: a) TNF-α co-operates with GM-CSF to generate DC from CD34+ hematopoietic progenitors from BM, cord blood or PB; and b) IL-4 co-operates with GM-CSF in the development of DC from circulating monocytes. A detailed description of the methods utilized to obtain human DC from myeloid precursors has been recently reported. However, in evaluating these methods in view of a clinical trial, at least three issues should be taken into consideration:

a) the type of DC generated either from monocytes (monocyte-derived DCs) or from CD34+ hematopoietic progenitors (CD34+ derived DC);

b) the source of serum for DC growth in culture;

c) the combination of cytokines required for optimal ex vivo expansion of functional immunostimulatory DC.

Monocyte-derived DC are being employed in patients with advanced stage malignancies in phase I-II clinical trials. The trials are particularly aimed at evaluating toxicity and immune responses after subcutaneous administration following DC pulsing ex vivo with either melanoma tumor-associated peptides or with B-cell lymphoma and myeloma idiotype proteins from autologous serum. Early reports from clinical studies, in patients with melanoma who are HLA-A1 positive and whose malignant cells express the MAGE-1 gene, show that in vivo immunization with autologous monocyte-derived DC pulsed with MAGE-1 gene coded nonapeptide, is not toxic and can induce peptide-specific autologous melanoma reactive CD8+ cytotoxic T-lymphocyte responses in situ at the vaccination site and at distant tumor sites as well as in PB. From a technical point of view, in these studies the generation of DC from PB mononuclear cells is dependent on a culture medium necessarily containing GM-CSF without serum or with human pooled donor serum. Under these conditions the production of DC is quite scarce in comparison with that achieved with fetal calf serum, as reported in early studies. However, the presence of fetal calf serum in the culture medium induces undesired DC-mediated immune responses to xenogenic proteins as observed in murine and human preclinical studies. In this regard, experimental data suggest that CD34+ cell-derived DC can also be generated in the absence of serum if the culture medium containing GM-CSF and TNF-α is supplemented with TGF-β1.

Modalities for the large-scale procurement of functional DC from CD34+ hematopoietic progenitors in patients with cancer have been evaluated. It was found that mobilized PB progenitors currently utilized in phase III trials include a fraction of CD34+ DC precursors which give origin ex vivo to a progeny with the char-
acteristics of professional APC i.e., typical DC morphology and immunophenotype undistinguishable from cutaneous Langerhans cells and DC from cord blood and BM CD34+ cells. Most importantly, these ex vivo generated DC retained the capacity to process and present antigens to T lymphocytes as demonstrated by elicitation of HLA class II and class I-restricted activation of CD4+ and CD8+ autologous T lymphocytes in response to xenogenic antigens of fetal calf serum179 or melanoma tumor-associated antigen peptides,177,178 respectively. Quantification of progenitors of DC by limiting dilution analysis of CD34+ cells sorted from blood cell autografts showed that they are approximately 140-fold more numerous than in steady-state control autograft. To obtain this favorable result, blood cell autografts were collected at the time of maximal mobilization of CD34+ cells into PB as occurs after treatment with high-dose cyclophosphamide and cytokines.

In a systematic search for culture conditions capable of ameliorating the ex vivo generation of DC dendritic cells, a variety of exogenous stimuli have been evaluated as well as monocyte-derived versus CD34+ cell-derived DC.170,177,178 In this respect, it has been shown that GM-CSF plus TNF-α-dependent generation of DC from mobilized CD34+ cells is 2.5 fold enhanced by either FL or SCF, and 5-fold enhanced by a combination of these growth factors. In addition, autologous high-dose chemotherapy recovery phase serum rather than fetal calf serum or human donor pooled AB serum has been shown to be the optimal serum for the generation of DC. Regardless of the precise mechanism of action of FL and SCF in association with GM-CSF and TNF-α on the enhancement of DC differentiation and proliferation, these findings have provided new advantageous tools for the large-scale generation of DC from mobilized CD34+ cells in patients undergoing cancer treatment. In fact, the stimulation of CD34+ cells from an average blood cell autograft should permit the generation of a median of $0.6 \times 10^9$/kg DC from an average 65 kg individual, i.e., almost $40 \times 10^9$ DC. In contrast, differentiation of DC from monocytes in the presence of autologous high-dose chemotherapy recovery phase serum plus GM-CSF and IL-4 is not associated with a comparably high outgrowth of DC.177,178 These observations, together with the weaker expression of co-stimulatory molecules in monocyte-derived DC in comparison with CD34+ cell-derived DC177 may favor the utilization of the latter source of APC for the development of active immunization programs involving DC in humans. The comparative efficiency as APC of DC derived from monocytes versus CD34+ hematopoietic progenitors has recently been studied with DC isolated from blood of patients with melanoma. In particular, it has been shown that DC derived from G-CSF-mobilized CD34+ cells are more efficient than those derived from monocytes in inducing melanoma tumor-associated antigen peptide specific activation of autologous CD8+ cytotoxic T-lymphocytes. Interestingly, in the same experiments the latter cells were also capable of lysing a panel of melanoma cell lines sharing the same HLA class I alleles with the patients from whom CD8+ cytotoxic T-lymphocytes were generated with tumor-associated antigen peptide pulsed autologous DC.177,178 Moreover, CD34+ cells mobilized into PB by G-CSF were shown to be capable of generating a higher number of mature and fully functional DC than their BM counterparts.188 However, it should be pointed out that, at present, clinical studies utilizing DC as vehicles for anti-tumor vaccination have been carried out with monocyte-derived DC either freshly isolated from PB152 or cultured ex vivo.161,181-183 In addition, culture conditions which allow the large scale production of terminally differentiated and fully functional monocyte-derived DC have recently been described.189

**Dendritic cells for antitumor cell therapy**

An extensive review on the clinical use of DC is beyond the scope of this paper, however, a few remarks in this regard are needed.

The goal of vaccination is the induction of protective immunity. Originally, vaccinations were used in the setting of infectious diseases, but are now expanding to include the treatment of allergy, autoimmune diseases, and tumors. A rational approach to vaccination involves 3 steps: a) the identification of the protective effector mechanisms, b) the choice of an antigen that can induce a response in all individuals, and c) the use of an appropriate way to deliver the vaccine to induce the proper type of response.159-162

It has now been demonstrated that certain tumor cells are antigenic by expressing tumor-associated antigens that can be recognized by T lymphocytes in a syngeneic host. However, they are often poorly immunogenic, at least in part because they lack the cellular armamentarium for specific T-lymphocyte recognition, activation, and co-stimulation typical of APC especially DC.190,191 Different mechanisms may account for the ability of tumor cells to evade immune responses. Tumor cells may

<table>
<thead>
<tr>
<th>Table 3. Phenotype of CD1a+ dendritic cells generated from mobilized CD34+ progenitors or from monocytes.</th>
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<tbody>
<tr>
<td><strong>Antigens</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>CD14</td>
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<tr>
<td>CD80</td>
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<tr>
<td>CD86</td>
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<tr>
<td>CD54</td>
</tr>
<tr>
<td>MHC Class I</td>
</tr>
<tr>
<td>HLA-A202</td>
</tr>
<tr>
<td>MHC Class II</td>
</tr>
<tr>
<td>HLA-DR</td>
</tr>
<tr>
<td>HLA-DQ</td>
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Modified from Mortarini et al., Cancer Res, in press.
display low immunogenicity through low MHC and/or tumor-specific antigen expression or may downregulate Fas and constitutively express Fas-Ligand, which binds to Fas on cytotoxic T-lymphocytes, resulting in apoptosis of the latter.\textsuperscript{192} Furthermore, it has recently been shown that vascular endothelial growth factor produced by tumors inhibits the functional maturation of CD34\textsuperscript{+} cell-derived DC.\textsuperscript{193} When considering the use of the unique antigen-presenting capacity of DC to prime specific antitumor T-lymphocytes, this phenomenon should be taken into account, as it may result in poor recovery and function of DC directly recovered from the blood of cancer patients. In contrast, dendritic APC expanded ex vivo in the presence of cytokines and in the absence of inhibitory factors released by tumors would probably be functional.\textsuperscript{193-195} However, it should also be considered that published data demonstrate that whereas in vivo administration of DC loaded with low doses of tumor antigen enhances antitumor immunity, DC pulsed with high doses of antigen or high numbers of tumor antigen-pulsed DC may inhibit development of immunity.\textsuperscript{196} This finding supports the notion that stimulation of DC-mediated antigen presentation in vivo may act in a tolerogenic or immunogenic fashion depending on a variety of partially understood factors.\textsuperscript{197}

Basically, there are at least two approaches to tumor vaccination (Figure 7). The first is to identify a tumor-associated antigen to be used as a vaccine, the second is to increase the immunogenicity of tumor cells and let the immune system decide which antigen to target. Indeed, in experimental models with the appropriate manipulation exploiting the physiologic function of antigen-presenting DC, the immune system can be induced to mount responses that can kill tumor cells and also protect animals from subsequent challenge even with a poorly immunogenic tumor.\textsuperscript{198-203}

Given the richness of recently identified tumor-associated antigens and their corresponding peptide epitopes recognized by MHC-restricted CD8\textsuperscript{+} or CD4\textsuperscript{+} T lymphocytes (Table 4), investigators are currently evaluating the clinical efficacy of specific tumor-associated antigen-based vaccines for the treatment of various malignancies. Recently, in a cooperative clinical trial it was observed that partial tumor regressions can occur in HLA-A1\textsuperscript{+} patients with melanoma treated with a naked MAGE 3 peptide epitope vaccine even in the absence of any engineering of antigen-presenting cells or adjuvant cytokine(s).\textsuperscript{204} This clinical evidence induces the belief that the effectiveness of peptide-based vaccines is likely to benefit further from administration of appropriate cytokines\textsuperscript{156,205,206} or cellular adjuvants (e.g., DC) capable of promoting cellular immunity.

Among hematologic malignancies, CML is being intensively evaluated as a possible target of dendritic APC-based immunotherapy. It is well known that CML is characterized by a specific translocation of the c-abl oncogene (9q34) to the bcr region on chromosome 22 (22q11). Alternative recombination sites involving either the second or third exon of the bcr gene splicing to exon 2 of the abl gene yield two potential fusion gene transcripts, b2a2 and b3a2, respectively. The translated 210-kd bcr-abl fusion protein, which has abnormal tyrosine kinase activity, includes a new potentially antigenic sequence at the fusion site: a new amino acid is generated at the junctional site by the fusion event; in the b2a2 fusion a glutamic acid (E) is encoded, whereas in the b3a2 recombination event a lysine (K) is generated. Interestingly, a bcr-abl peptide from the b3a2 fusion region has been found to be immunogenic in mice.\textsuperscript{207} In humans, binding of b3a2 peptides to various HLA class I alleles\textsuperscript{208} and priming of CD8\textsuperscript{+} cytotoxic T-lymphocytes in vitro has been described although the capacity of these peptide-specific CD8\textsuperscript{+} T-lymphocytes to lyse CML cells has not been determined.\textsuperscript{209} In contrast, in a recent study it has been demonstrated that CD4\textsuperscript{+} T-lymphocytes can be identified that proliferate in an HLA class II restricted manner in response to a 11mer (GFKQSSKALQR) b3a2 peptide especially when the latter is presented by purified CMRF-44\textsuperscript{+} blood DC.\textsuperscript{210} In the same study the peptide-specific CD4\textsuperscript{+} T-lymphocytes were able to respond to the whole protein in crude extract from CML cells. Intriguingly, dendritic antigen-presenting cells in CML patients can be derived from the malignant clone and these malignant dendritic cells can induce antileukemic reactivity in autologous T lymphocytes without the necessity of additional exogenous antigens.\textsuperscript{211}

Although, the above observations cannot be extrapolated a priori to other malignancies carrying specific translocations and corresponding fusion genes and products,\textsuperscript{212} further investigations on the possible clin-

### Table 4. Tumor antigens capable of eliciting T-lymphocyte responses.

<table>
<thead>
<tr>
<th>Activated oncogene products</th>
<th>Tumor suppressor gene products</th>
<th>Reactivated embryonic gene products</th>
<th>Melanocyte differentiation antigens</th>
<th>Viral gene products</th>
<th>Idiotype epitopes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutated:</td>
<td>Position 12 point mutation of 21&lt;sup&gt;th&lt;/sup&gt;</td>
<td>bcr-abl (b3a2 peptide)</td>
<td>Tyrosinase protein</td>
<td>Human papilloma virus antigens (E6, E7)</td>
<td>Ig and TCR hypervariable regions</td>
</tr>
<tr>
<td>Rearranged:</td>
<td>HER-2/neu</td>
<td></td>
<td>Melan-A/MART1</td>
<td>EBV EBNA-1 gene products</td>
<td></td>
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<tr>
<td>Overexpressed:</td>
<td></td>
<td></td>
<td>gp100</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>gp75</td>
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clinical application of bcr–abl peptide(s) presented by autologous dendritic antigen–presenting cells are warranted.

The translation into the clinical setting of the above experimental hints favoring the use of DC pulsed ex vivo with synthetic tumor-associated antigen peptides for effective cell therapy in humans is likely to be hampered by: a) the limited availability of patients with HLA typing compatible for the utilized tumor-associated antigen peptide(s); b) the occurrence of independent mechanisms of tumor escape in vivo such as loss of expression of tumor-associated antigens or of HLA class I during tumor progression; and c) the short duration of the immune responses thus requiring annoying boost vaccinations. It has been suggested\(^2\) that these limitations may be overcome by transduction of genes encoding relevant proteins into DC or their progenitors so that DC could tailor peptides to their own HLA molecules thus obviating the need to synthesize tumor-specific peptides most of which have stringent HLA restrictions. A further advantage of the transduction approach may be the stable long-term expression of the antigen by the DC, which would allow its presentation to the immune system for longer periods without the concerns about the turnover of preformed peptide/HLA complexes in vivo after immunization.\(^2\)

Based on the above experimental body of evidence, a pioneer clinical trial has evaluated the ability of autologous Hodgkin’s lymphoma-specific idiotypic protein to stimulate host immunity when infused as a vaccine.\(^15\) In this study active immunotherapy of patients with B-cell lymphoma against idiotypic determinants led to anti-tumor immunity that correlated with improved clinical outcome in some patients.

Regardless of the type of cell manipulation (Figure 7) (ex vivo pulse with tumor-associated antigen peptides versus transduction with tumor associated antigen genes versus immunization with fusions of dendritic and carcinoma cells\(^2\)\(^1\)) that will be successful in clinical applications the necessity of methods of generating large numbers of functional DC is implicit for the evolution of such studies.

**Future directions**

Since DC have been shown to be intimately involved in the generation of CD4\(^+\) and CD8\(^+\) T-lymphocyte mediated tumor-specific immunity, it is attractive to speculate that vaccination with DC pulsed or engineered ex vivo to present tumor antigen(s) may be effective in generating tumor immunity in vivo. Among the recently prospected sources of DC, namely BM, neonatal cord blood, and adult PB, the last is certainly the richest and most accessible in all patients with cancer, although it remains to be confirmed whether functional differences will favor the utilization of monocyte- versus CD34\(^+\) cell-derived DC. Thus, in the clinical setting of adoptive immunotherapy for patients with malignancies, a therapeutic protocol could be envisioned involving the mobilization of CD34\(^+\) cells into PB with hematopoietic growth factors, with or without prior intensive chemotherapy. Thus, enrichment of hematopoietic progenitor cells could be followed by ex vivo generation of DC pulsed or engineered to present tumor antigen(s), to be reinfused as a potential secondary tumor-specific immunotherapy or vaccination. It remains to be established whether the latter effect could be further enhanced by in vivo adjuvant cytokines such as GM-CSF and/or FL, as occurs in murine models.

A potential advantage of ex vivo immune cell therapy over direct in vivo immune intervention, is the lack of functional inhibition that may occur in vivo. This hypothesis is based on a recently proposed mechanism of tumor escape/resistance from the host immune system, in which cancer cells produce a vascular endothelial growth factor that impairs antigen presentation required to induce specific antitumor immune responses in vivo.\(^19\)

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**Figure 7. Sources of tumor antigens for DC-based cancer vaccines.**
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All the authors equally contributed to the manuscript and they are listed in alphabetical order.

Disclosures
Conflict of interest. This review article was prepared by a group of experts designated by Haematologica and by representatives of two pharmaceutical companies, Amgen Italia SpA and Dompé Biotech SpA, both from Milan, Italy. This co-operation between a medical journal and pharmaceutical companies is based on the common aim of achieving optimal use of new therapeutic procedures in medical practice. In agreement with the Journal’s Conflict of Interest policy, the reader is given the following information. The preparation of this manuscript was supported by educational grants from the two companies. Dompé Biotech SpA sells G-CSF and rHuEpo in Italy, and Amgen Italia SpA has a stake in Dompé Biotech SpA.

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References


64. Peters SO, Kitterle ELW, Ramshaw HS, Quesenberry PJ. Ex vivo expansion of murine marrow cells with IL-3, IL-6, IL-11 and SCF leads to impaired engraftment in irradiated hosts. Blood 1996; 87:30-7.


132. Miller JS, Prosper F, MC Cullar V. Natural Killer cells are functionally abnormal and NK cell progenitors are diminished in granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cell collections. Blood 1997; 90:3098-105.


Clinical use of hematopoietic stem cells


210. Mannering SI, McKenzie JL, Fearnley DB, Hart DNJ. HLA-DR1-Restricted bcr-abl (b3a2)-specific CD4+ T lymphocytes respond to dendritic cells pulsed with b3a2 peptide and antigen-presenting cells exposed to b3a2 containing cell lysates. Blood 1997; 90:290-7.
Cell therapy: achievements and perspectives

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Review

Background and Objectives. Cell therapy can be considered as a strategy aimed at replacing, repairing, or enhancing the biological function of a damaged tissue or system by means of autologous or allogeneic cells. There have been major advances in this field in the last few years. This has prompted the Working Group on Hematopoietic Cells to examine the current utilization of this therapy in clinical hematology.

Evidence and Information Sources. The method employed for preparing this review was that of informal consensus development. Members of the Working Group met three times, and the participants at these meetings examined a list of problems previously prepared by the chairman. They discussed the single points in order to reach an agreement on different opinions and eventually approved the final manuscript. Some of the authors of the present review have been working in the field of cell therapy and have contributed original papers in peer-reviewed journals. In addition, the material examined in the present review includes articles and abstracts published in journals covered by the Science Citation Index and Medline.

State of the Art. Lymphokine-activated killer (LAK) and tumor-infiltrating lymphocytes (TIL) have been used since the ‘70s mainly in end-stage patients with solid tumors, but the clinical benefits of these treatments has not been clearly documented. TIL are more specific and potent cytotoxic effectors than LAK, but only in few patients (mainly in those with solid tumors such as melanoma and glioblastoma) can their clinical use be considered potentially useful. Adoptive immunotherapy with donor lymphocyte infusions has proved to be effective, particularly in patients with chronic myeloid leukemia, in restoring a state of hematologic remission after leukemia relapse occurring following an allograft. The infusion of donor T-cells can also have a role in the treatment of patients with Epstein-Barr virus (EBV)-induced post-transplant lymphoproliferative disorders. However, in this regard, generation and infusion of donor-derived, virus specific T-cell lines or clones represents a more sophisticated and safer approach for treatment of viral complications occurring in immunocompromized patients. Whereas too few clinical trials have been performed so far to draw any firm conclusion, based on animal studies dendritic cell-based immunotherapy holds promises of exerting an effective anti-tumor activity. Despite leukemic cells not being immunogenic, induction on their surface of co-stimulatory molecules or generation of leukemic dendritic cells may induce antileukemic cytotoxic T-cell responses. Tumor cells express a variety of antigens and can be genetically manipulated to become immunogenic. The main in vitro and in vivo functional characteristics of marrow mesenchymal stem cells (MSCs) with particular emphasis on their hematopoietic regulatory role are reviewed. In addition, prerequisites for clinical applications using culture-expanded mesenchymal cells are discussed.

Perspectives. The opportuneness of using LAK cells or activated natural killer (NK) cells in hematologic patients with low tumor burden (e.g. after stem cell transplantation) should be further explored. Moreover the role of new cytokines in enhancing the antineoplastic activity of NK cells and the infusion of selected NK in alternative to CTL for graft versus leukemia (GVL) disease (avoiding graft versus host disease (GvHD) seems very promising. Separation of GVL from GvHD through generation and infusion of leukemia-specific T-cell clones or lines is one of the most intriguing and promising fields of investigations for the future. Likewise, strategies devised to improve immune-reconstitution and restore specific anti-infectious functions through either induction of unresponsiveness to recipient alloantigens or removal of alloreactive donor T-cells might increase the applicability and success of hematopoietic stem cell transplantation. Cellular immunotherapy with DC must be standardized and several critical points, discussed in the chapter, have to be properly addressed with specific clinical studies. Stimulation of leukemic cells via CD40 receptor and transduction of tumor cells with co-stimulatory molecules and/or cytokines may be useful to prevent a tumor escaping immune surveillance. Tumor cells can be genetically modified to interact directly with dendritic cells in vivo or recombinant antigen can be delivered to dendritic cells using attenuated bacterial vectors for oral vaccination. MSCs represent an attractive therapeutic tool capable of playing a role in a wide range of clinical applications in the context of both cell and gene therapy strategies.

Key words: cell therapy

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The role of lymphoid cells in rejecting solid tumors transplanted into animal models was strongly suggested in the first decades of this century by J.B. Murphy (1926) who, nonetheless, did not demonstrate it formally. Following his revolutionary findings on the immunologic mechanisms of allogeneic skin tolerance and rejection, in 1958 P.B. Medawar coined the term "immunologically competent cell" to define a cell that is "fully qualified to undertake an immunological response". Forty years after Medawar's definition, the development of molecular and biological research has enormously improved our understanding of the complex regulatory mechanisms of proliferation, differentiation and function of the cells involved in the immune response. The concomitant evolution of biotechnology has also progressively given new opportunities to isolate and/or expand cell subsets, or to develop new molecules, in order to amplify or modify specific cell functions. Thus, the possibility of exploiting a specific cell function, in vivo or ex vivo, to obtain a therapeutic effect, such as an anti-tumor cytotoxic activity, or complete immune reconstitution, is part of the definition of cell therapy that is herein reviewed.

In a general context, cell therapy can be considered as a strategy aimed at replacing, repairing, or enhancing the biological function of a damaged tissue or system by means of autologous or allogeneic cells. For instance, in the hematopoietic system cell therapy may include: a) removal or enrichment of various cell populations; b) expansion of hematopoietic cell subsets; c) expansion or activation of lymphocytes for immunotherapy; and d) genetic modification of lymphoid or hematopoietic cells, when these cells are intended to engraft permanently or transiently in the recipient and/or be used in the treatment of a disease.

This review contains extensive considerations on the clinical use of lymphocytes and/or natural killer (NK) cells as a strategic weapon in preventing or curing the neoplastic relapse after chemotherapy and/or hematopoietic stem cell transplantation, the infusion of T-cell clones or lines able to restore a specific anti-viral activity, the in vivo and ex vivo potential use of dendritic cells to generate a tumor-specific cytotoxic activity, and the innovative use of donor stromal cells in conjunction with stem cell transplantation. Even tumor cells engineered to express cytokine or co-stimulatory molecules and representing the entire antigenic repertoire of a certain neoplasm can be used as a cancer vaccine. On the other hand, a broad definition of cell therapy at this time should include autologous and allogeneic transplants of purified hematopoietic stem cells, which, however, have been extensively reviewed in previously published reports.

**Tumor escape from immune surveillance**

Although several mechanisms allowing tumor cells to escape the host immune protection have been recently described, it is conceivable that others remain still undiscovered. However, tumor cells often fail to induce specific immune responses because of their inability to function as competent antigen presenting cells (APC). Professional APC, in fact, are fully capable of delivering two signals to T cells: the first is antigen (Ag) specific and is mediated by the interaction of MHC molecules carrying antigenic peptides with the T-cell receptor (TCR), and the second signal, or co-stimulatory signal, is not Ag-specific and is principally mediated by members of the B7 family, namely B7-1 (CD80) and B7-2 (CD86), via their T-cell receptors CD28 and CTLA-4, and/or by CD40 via CD40L binding.

The lack of a suitable tumor-associated antigen (TAA), or defective antigen processing, or production of immunologic inhibitors, or lack of co-stimulatory signaling by tumor cells, as other mechanisms, can all contribute to prevent or abrogate an anti-tumor immune response. Moreover, neoplastic cells within the same tumor may show different reactivity with monoclonal antibodies (mAbs), cytotoxic T-lymphocyte (CTL) clones and lymphokine-activated killer (LAK) or tumor infiltrating lymphocyte (TIL) populations. Furthermore, despite many tumors having TAA and potentially being capable of stimulating T cells, in some cases they fail to induce an adequate CTL frequency *in vitro*. In other cases the antigen loss can be one of the mechanisms for escaping immune protection. Private TAA often result from mutated gene products and are potentially useful for developing tumor vaccines. These Ags, however, can be down-regulated or modified by point mutations, inducing a consistent reduction or the abrogation of peptide-binding by specific CTLs. Another critical issue for preventing immune responses is the absence, or the down-regulation of MHC molecules on neoplastic cells, as shown in animal models, or in human lung cancer.

The pivotal role of B7 molecules in the immune response has been demonstrated in a variety of experimental models showing that after TCR signaling, binding of CD28 induces T-cell interleukin-2 (IL-2) secretion, proliferation and effector function, whereas presentation of the antigen in the absence of co-stimuli induces T-cell unresponsiveness either by anergy or clonal deletion. Therefore, since most neoplastic cells lack co-stimulatory molecules, it is likely that they can deliver the first signal through the MHC:TCR binding, but not the second one, thus driving host T-cells to tolerate the tumor. Potential strategies to prevent or to reverse T-cell tolerance by CD28 or CD40L stimulation, or IL-2 receptor triggering, are under investigation.

Further mechanisms impairing immunologic responses include the suppression of cytotoxic activity by the release of soluble factors or by direct cell–contact. In fact, tumor cells may secrete cytokines, such as MIP-1α, or TGF-β, or IL-10, that may be capable of inhibiting T cell activity. Alternatively, tumor cells may induce T-cell apoptotic clonal deletion by increasing Fas:Fas-L ligation. A schematic example of the main defects described in the tumor cell: T cell interaction is shown in Figure 1.

Finally, since normal lymphocytes can bind to vascular endothelial cells through adhesion receptors, such as
L-selectin or α/β integrins, and then by rolling out they can reach tissues, lack of adhesion receptors on tumor vessels might prevent lymphocytic infiltration and contact with neoplastic cells. In this case even the best strategies aimed at modifying the immunogenicity of tumor cells may not be successful at overcoming the lack of an antitumor immune surveillance.

### Lymphokine-activated killer and tumor-infiltrating lymphocytes: past and present

**Natural killer cells and lymphokine-activated killer phenomenon**

Since 1970 NK cells have been recognized as a functionally distinct subset of cytotoxic effectors (Table 1). NK cells from rodent or from human peripheral blood kill a wide range of tumor cells and virus-transformed cells without the need for prior sensitization.

In 1975 Heberman et al. described a phenomenon of normal unstimulated lymphoid cells lysing cultured tumor-cell lines in a short in vitro assay. This cytolytic activity was subsequently shown to be neither MHC restricted nor mediated by the T-cell receptor complex. Such ability to eliminate tumor cells, but not normal tissues suggests that NK cells are not only involved in the control of cancer, but also that their presence and state of activation are important in the outcome of the disease and finally in the treatment of tumors.

Mature NK have a clonally-distributed ability to recognize their target cell by class I MHC alleles. Karre et al. demonstrated in a murine model that leukemia cell

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<table>
<thead>
<tr>
<th>Effector type</th>
<th>CTLs</th>
<th>TILs</th>
<th>NK cells</th>
<th>LAK cells</th>
<th>CIKs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>Peripheral blood lymphocytes</td>
<td>Metastatic lymph nodes</td>
<td>Peripheral blood and bone marrow</td>
<td>NK cells and CTL activated by IL-2</td>
<td>Subset of T-lymphocytes activated by cytokines</td>
</tr>
<tr>
<td>Culture conditions:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor stimulation need of IL-2 for response</td>
<td>Yes</td>
<td>None</td>
<td></td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Duration of culture target cells in vitro</td>
<td>6 weeks</td>
<td>4 weeks</td>
<td>2-3 weeks</td>
<td>2-5 days</td>
<td>None</td>
</tr>
<tr>
<td>Specificity</td>
<td>MHC restricted to allogenic cells</td>
<td>Restricted to autologous tumor (MHC and/or tumor associated)</td>
<td>Restricted lysis of virus-infected cells, autologous tumor cells, allogenic tumor cells</td>
<td>Antibody-dependent cell-mediated cytolysis (ADCC) specificity</td>
<td>None: lyse a wide spectrum of tumor cells including cells that are resistant to NK; cytotactic activity superior to LAK; lyse whether CML autologous or allogenic blasts but do not lyse normal hematopoietic progenitors</td>
</tr>
<tr>
<td>Effector phenotype</td>
<td>-CD3+/4+, C03+/8+/-CD3+/8+/16+</td>
<td>CD03+/8+/56+</td>
<td>CD03+/CD016+/CD056+</td>
<td>CD056+</td>
<td>CD3+/56+</td>
</tr>
</tbody>
</table>

**Table 1. Characteristics of cytotoxic effectors useful for adoptive immunotherapy of cancer.**

**Figure 1. Main mechanisms for tumor escape of immune surveillance.**

*CTL: cytotoxic T-lymphocytes; TIL: tumor infiltrating lymphocytes; NK: natural killer; LAK: lymphokine-activated killer; CIK: cytokine-activated killer.*

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lines lacking certain MHC class I molecules were killed by NK cells, while parental H-2 bearing line were not. In humans both NK and a subset of cytotoxic T-lymphocytes express receptors for MHC HLA class I molecules which exert an inhibitory effect on cell-mediated cytotoxicity. These surface molecules, belonging to the immunoglobulin superfamily, have been termed killer-cell inhibitory receptors (KIRs). Two distinct KIR families have been described: a) KIRs with IG-like domains, recognizing HLA-A, B and C alleles; and b) the CD94/NKG2A subtype, with a lectin domain, recognizing peptides related to the HLA-E class I system. The interaction between KIRs and the corresponding MHC class I antigens prevent NK from killing target cells expressing self HLA alleles. In addition some NK also express receptors that induce lysis of target cells expressing foreign HLA class I alleles.

These findings explain the mechanism of self-tolerance in the NK population, which can be disrupted as a consequence of tumor transformation or viral infection or any other events inducing a loss or a substantial modification of class I molecules. These transformed cells can easily escape detection by T-lymphocytes by down regulating MHC antigens, but are normally destroyed by autologous NK cells.

The NK cell compartment is heterogeneous and distinct NK subsets have been characterized. The most informative functional differences are based on relative CD56 fluorescence: only CD56<sup>bright</sup>, but not CD56<sup>dull</sup> NK, express the high-affinity IL-2 receptor, and respond to the low IL-2 concentration. They also expand 10 times more than CD56<sup>dull</sup>. NK precursors differentiate into immature NK in presence of SCF, IL-7, IL-2 and bone marrow stromal cells producing IL-15. This last cytokine can directly induce CD34<sup>+</sup> cells to differentiate into NK cells in the absence of IL-2. The second step of NK maturation is stroma-independent and is characterized by the appearance of CD56 molecules: the intensity of CD56 expression reflects the proliferative potential and the killing ability of the NK.

The effects of IL-2 on NK precursors appears to be stage-specific, confirming that, while mature NK precursors readily respond to IL-2, more immature progenitors need complete mixtures of cytokines and stromal cells. NK cells, after incubation with IL-2, become lymphokine-activated killer cells: LAK cells kill NK-resistant cell targets (e.g. Daudi cell line) and a wide spectrum of different fresh tumor cells in both autologous and allogeneic settings, while fresh normal tissues are resistant to LAK-mediated lysis.

Although some tissue-resident lymphocytes may have spontaneous LAK activity, normal blood mononuclear cells (MNC) do not show any LAK activity, which can be acquired only after incubation with interleukin-2. These NK activated cells express new markers such as CD25, MHC class II antigens and fibronectin. LAK activity can be generated not only in peripheral blood MNC, but also in the thymus, spleen, bone marrow and in MNC from lymph nodes. Many experimental data suggest that most LAK precursors are present in the null lymphocyte population.

In humans LAK activity was much more evident in the MNC population after depletion of macrophages, T and B-cells. Residual MNC were CD16<sup>+</sup> and did not show T-cell markers.

**LAK cells: experimental observations and clinical trials**

In animal models the combined administration of IL-2 and LAK has proved to be more efficacious than either component alone. In murine models the administration of high-dose IL-2 alone or in conjunction with LAK cells induced the regression of lung, liver and subdermal metastases. The antitumor effect correlated both with the IL-2 dose and the number of LAK cells administered; finally at different doses of IL-2, the concomitant administration of LAK cells resulted in increased reduction in established metastases.

LAK cells are capable of inhibiting acute myeloid leukemia (AML) progenitor growth, and leukemia incidence is higher in people with deficiency of NK cells. In the large majority of patients at diagnosis or in relapse blasts appear resistant to lysis by autologous LAK cells. Moreover, about 90% of patients with acute leukemia in complete remission do not show spontaneous cytotoxicity against autologous blast cells, but ex vivo treatment with IL-2 restores cytolytic activity in 37.5% of these patients. In a population of 42 patients with AML in complete remission, LAK cytotoxicity against autologous leukemic blasts was not significantly different from LAK of normal subjects. However, multivariate analysis for prognostic factors showed that patients whose LAK had more lytic activity on leukemic blasts had significantly less risk of relapse than patients with poor LAK activity.

In the first National Cancer Institute trial endstage cancer patients received high-dose bolus IL-2 therapy for 3 to 5 days. Lymphocytes harvested during the systemic treatment with IL-2 were cultured in the presence of IL-2 for 2 to 4 days, in order to expand the LAK cell number; autologous LAK cells were then rein fused into patients in combination with the high-dose intravenous bolus IL-2 administration. Of 72 patients with renal cancer who were treated, 33% obtained an overall response, 8 with complete response (CR) and 17 with partial response (PR); of 48 patients with metastatic melanoma 21% responded with 4 CR and 6 PR; responses were also observed in patients with colorectal carcinoma and non Hodgkin's lymphoma. The ILWG used the same strategy, obtaining an overall response rate of 19% in patients with melanoma and 16% in those with renal carcinoma. After these initial trials the original schema of the National Cancer Institute was modified with the use of IL-2 in continuous infusion rather than bolus injection in order to reduce the systemic toxicity.

The first randomized study, comparing IL-2 alone to IL-2 plus LAK cells, was published by McCabe. This trial included patients with either renal carcinoma or
melanoma; no significant difference in response rate between the two groups was reported. A second randomized study at the National Cancer Institute followed these pioneering experiences, comparing IL-2 alone to IL-2/LAK cells;\textsuperscript{44} 181 patients were enrolled in this study (90 in the IL-2 plus LAK arm and 91 in the IL-2 alone). A total of 10 CR were observed in the IL-2/LAK arm as compared to only 3 in the IL-2 alone arm. The overall response rates were similar, but there was a survival trend ($p=0.07$) in favor of the IL-2/LAK arm: the actuarial survival for patients receiving IL-2/LAK was 31\% compared to 17\% for those receiving IL-2 alone. Toxicity was virtually equivalent in both arms and the majority of toxic effects were due to IL-2 administration, while the only complication associated with LAK therapy was transient hepatitis A, due to contamination of the culture medium.

A third randomized trial, comparing IL-2 alone versus IL-2/LAK therapy was published in 1995.\textsuperscript{45} In this study only patients with advanced renal carcinoma were treated and IL-2 was administered as a continuous infusion rather than bolus injection. Seventy-one patients entered (36 vs. 35) this trial and only 6\% overall obtained a major response, with a median survival of 13 months; the difference between the two groups was not significant. Therefore it may be concluded that LAK cells did not improve the activity of IL-2 in patients with advanced renal carcinoma.

The last randomized trial published was conducted in 174 primary lung carcinoma patients after surgery, comparing the adjuvant treatment with IL-2 plus LAK (for two years) with conventional treatment.\textsuperscript{46} The 5- and 9-year survival rates were significantly superior in patients receiving IL-2/LAK therapy, but no comparison was planned between IL-2 alone and IL-2/LAK therapy. The impressive results obtained in terms of overall survival also in non-curative cases after surgery (OS: 52\% at 5 years) should probably be interpreted as due to fact that in this study patients received the immunotherapy after consistent tumor debulking.

Other clinical trials (non-randomized) were conducted with IL-2 with or without LAK cells, and the overall response rate was similar for both the immunotherapy modalities.\textsuperscript{47,48} The detailed review of other (non-ran-

Table 2. Clinical trials with LAK cells.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Patients</th>
<th>Kind of tumor</th>
<th>IL-2 (dose and schedule)</th>
<th>LAK cells</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosenberg</td>
<td>1987</td>
<td>157</td>
<td>Melanoma</td>
<td>Randomize: IL-2 vs. IL-2+LAK</td>
<td>CR: 2.2% vs 7.5% PR: 10.9% vs 14.2% mR 2.2% vs 9.4%</td>
<td></td>
</tr>
<tr>
<td>West</td>
<td>1987</td>
<td>40</td>
<td>Miscellaneous</td>
<td>1-7x10(^6) U/m(^2)/day CI</td>
<td>CR+PR: 22-28%</td>
<td></td>
</tr>
<tr>
<td>Yoshida</td>
<td>1986</td>
<td>23</td>
<td>Brain tumor</td>
<td>Direct injection of LAK into recurrent tumor cavity + IL-2 (50-400 U); multiple treat</td>
<td>Regression: 26%</td>
<td></td>
</tr>
<tr>
<td>Fisher</td>
<td>1988</td>
<td>29(x)</td>
<td>Renal carcinoma</td>
<td>12.9 MIU/kg (median 10 doses) 7x10(^9) cells</td>
<td>OR: 16%</td>
<td></td>
</tr>
<tr>
<td>West</td>
<td>1989</td>
<td>30</td>
<td>Renal carcinoma</td>
<td>3x10(^6) U/m(^2)/day CI</td>
<td>NR</td>
<td>22-28%</td>
</tr>
<tr>
<td>Dutcher</td>
<td>1989</td>
<td>32</td>
<td>Renal carcinoma</td>
<td>100,000 U/kg q8h</td>
<td>8.9x10(^9)</td>
<td>CR+PR: 19%</td>
</tr>
<tr>
<td>Paciucci</td>
<td>1989</td>
<td>24</td>
<td>Miscellaneous</td>
<td>1-9x10(^6) U/m(^2)/day CI</td>
<td>5.6x10(^9)</td>
<td>CR+PR: 20.8%</td>
</tr>
<tr>
<td>Neqrier</td>
<td>1989</td>
<td>51</td>
<td>Renal carcinoma</td>
<td>3x10(^6) U/m(^2)/day CI</td>
<td>1.2x10(^9)</td>
<td>CR+PR: 27%</td>
</tr>
<tr>
<td>Stahel</td>
<td>1989</td>
<td>23</td>
<td>Miscellaneous</td>
<td>3x10(^6) U/kg q8h</td>
<td>5.1x10(^9)</td>
<td>CR+PR: 17%</td>
</tr>
<tr>
<td>Rosenberg</td>
<td>1993</td>
<td>181</td>
<td>Metastatic cancer</td>
<td>Randomized: IL-2 vs. IL-2+LAK</td>
<td>CR: 5% vs 11.76% PR: 15.2% vs 16.5% mS (5 yrs): 17% vs 31% (p2=0.089)</td>
<td></td>
</tr>
<tr>
<td>Bajorin</td>
<td>1994</td>
<td>49</td>
<td>Renal carcinoma</td>
<td>Randomized: IL-2 (3 MU/m(^2)) vs. IL-2+LAK (7x10(^9))</td>
<td>No difference</td>
<td></td>
</tr>
<tr>
<td>Keilholz</td>
<td>1994</td>
<td>9</td>
<td>Liver metastatic carcinoma</td>
<td>IL-2 CI into the splenic artery or intravenous infusion</td>
<td>LAK transfer into the portal vein or the hepatic artery</td>
<td>CR+PR: 33%</td>
</tr>
<tr>
<td>Murray Law</td>
<td>1995</td>
<td>66</td>
<td>Renal carcinoma</td>
<td>Randomized: IL-2 (3x10(^6) U/m(^2)/day) vs. IL-2+LAK (NR)</td>
<td>CR+PR: 9% vs 3% (p=0.61)</td>
<td></td>
</tr>
<tr>
<td>Kimura</td>
<td>1997</td>
<td>82,788</td>
<td>Resected lung carcinoma</td>
<td>Randomized: IL-2+LAK vs. Standard therapy (1-5x10(^9) cell)</td>
<td>OS (5 yrs): 54.4% vs 52% OS (9 yrs): 33.4% vs 24.2%</td>
<td></td>
</tr>
</tbody>
</table>
domized) experiences using these two different immunotherapies suggests that LAK cell reinfusion slightly increased the number of CR and the duration of response, especially in patients with metastatic melanoma (Table 2).

In hematological malignancies the first attempts to generate and expand LAK activity by using IL-2 in vivo were clinically disappointing especially in patients autotransplanted for ALL; after transplantation patients were randomly assigned to treatment with systemic IL-2 (without LAK cell administration) or no treatment, but the disease-free survival was similar in the two arms. The use of LAK cells has also been proposed after autologous transplantation for hematological malignancies, but the very small series of patients reported does not allow any definitive conclusion to be drawn about its clinical benefit. Beaujean et al. rein fused, after myeloablative therapy, BM incubated with IL-2 into 5 ALL patients, observing a very marked delay of the engraftment and the recurrence of disease in all patients. Recently there has been a report of 61 women with breast cancer autotransplanted with IL-2 activated PBPC and treated with low dose IL-2 starting from PBPC reinfusion, without graft failures or major toxicity; there are no data concerning the outcome of patients and this experience only confirms the feasibility of the approach.

In a very preliminary experience a sustained major cytogenetic response to immunotherapy with GM-CSF+IL-2 and LAK infusion was observed in chronic myeloid leukemia (CML) patients after autologous transplantation. However, a renewed interest in this approach has led to new research pursuing different directions:

- a. selection of patients with low tumor-burden and with significant in vitro LAK activity against autologous tumor cells, in order to reach an optimal effector/target ratio;
- b. harvest of large amounts of NK cells (for additional ex vivo expansion/activation with IL-2) to be reinfused in the early phase after BMT;
- c. direct activation of leukapheresis, after priming with chemotherapy followed by cytokines, in order to reinfuse, after HDT, a product richer in cytotoxic effectors and probably less contaminated;
- d. identification and selection of more efficient NK progenitors (e.g. adherent NK) by eliminating undesired accessory cells which could inhibit their killing and proliferative ability;
- e. generation and expansion of other CE subsets with more powerful activity against autologous tumor cells, e.g. cytokine-induced killer cells (CIK);
- f. use of other cytokines in association with IL-2, in order to potentiate the activity and/or improve the selectivity of activated peripheral blood MNC.

**Tumor infiltrating lymphocytes**

The disappointing results of adoptive immunotherapy with blood-derived LAK cells led to a search for more specific CE cells. Tumor infiltrating lymphocytes (TIL) are T-lymphocytes with unique tumor activity that infiltrate some tumors and can be expanded by long-term culture with IL-2 at low-intermediate concentrations. In murine models TIL have exhibited a stronger anti-tumor effect than LAK cells on a per-cell basis; in humans TIL have been isolated with variable frequency from different solid tumors and very often (about 30% of cases) from patients with melanoma. Phenotypic analysis showed that TIL consisted mainly of CD4+ cells in colon, breast and urothelial tumors, while in melanoma CD8+ cells are prevalent. CD3+ CD16+ NK cells have also been isolated from several tumors, confirming the large heterogeneity of tumor infiltrates. The mechanism of the antitumor action of TIL is unknown; there is some evidence that these cells secrete cytokotixins and cytokotins which are capable of killing tumor cells and recruiting other CE.

**Experimental models and clinical trials**

Mice carrying spontaneous metastases, treated with IL-2 plus tumor-derived T-cells, obtained from splenocytes after mixed lymphocyte-tumor cultures, had a better survival than those treated with LAK cells; previous tumor debulking (with chemotherapy and/or radiotherapy) was needed to maximize the efficacy of TIL-therapy.

Unfortunately large amounts of TIL can be collected very rarely, and the large scale expansion of this population is crucial in order to obtain relevant clinical responses; this step of ex vivo manipulation is not always successful, because the need for prolonged culture of TIL (from 6 to 8 weeks with IL-2) may abrogate the selectivity against the tumor; moreover only a small fraction of the readministered human TIL is able to concentrate in the tumor sites.

Wong et al. showed in a mouse model that TIL preferentially localize in the liver and lungs. In contrast trafficking studies employing TIL radiolabeled with In111, have shown that TIL do traffic to tumor sites, this homing property should produce high concentrations of TIL, and probably their permanence, in the area of a tumor.

Human TIL transfected in vitro with the neomycin-resistance gene and reinfused intravenously, have been detected by polymerase chain reaction (PCR) techniques from 6 to 60 days in patients affected by metastatic melanoma. Aebersold et al. observed a strong correlation between the tested tumor cytotoxicity in vitro and the in vivo response, in a small cohort of patients with metastatic melanoma. A similar relationship was observed in a murine model in which the in vivo therapeutic effect of TIL correlated with secretion of IFNγ and tumor necrosis factor (TNFα). In order to increase their specificity and potency, TIL have been engineered with genes encoding cytokotins or cytokotins such as TNF or IFN-γ or IL-2. However, some experimental observations suggest that these high concentrations of cytokotins can cause systemic toxicity and in some cases could even make the tumor more aggressive.
In addition to their potential therapeutic use as cytolytic effectors, the ability of some TIL to recognize unique antigens on tumor cells has made the study of the biologic characteristics of these antigens more feasible. Melanomas from different patients who share MHC antigens are often cross-recognized by allogeneic TIL, as could be expected for an MHC-restricted T-cell response; the presence of shared antigens in different patients with melanoma suggests the possibility of using these antigens in an active immunization program for this disease.

When adoptively transferred into patients, TIL showed significant therapeutic efficacy in patients with advanced melanoma, but not in renal carcinoma patients. In a phase II trial patients with malignant melanoma were treated with IL-2 and TIL following chemotherapy: Kradin et al. treated some patients with a combination of chemotherapy, IL-2 and TIL, obtaining 23% of responses in those affected by melanoma and 29% in those with renal carcinoma, but none in patients with non-small cell lung carcinoma. A summary of most clinically relevant clinical trials with TIL is given in Table 3.

The lack of important clinical trials with TIL is probably due to the difficulties in finding TIL at diagnosis and especially because the techniques for TIL priming and expansion are time-consuming and not completely standardized. TIL therapy is still young, but its very interesting potential has not yet been thoroughly investigated.

### New approaches with LAK or TIL cells

**Allogeneic setting.** Whereas it is widely accepted that graft-versus-host disease (GvHD) is initiated by donor T cells recognizing foreign host antigens, other factors including toxicity of conditioning regimens and cytokine dysregulation are involved in the pathogenesis of GvHD. Data from murine experiments show that NK cells play an active role both in GvHD and in graft-versus-leukemia (GVL) events: in a recently published model 100% of SCID mice bearing human leukemic cells, and transplanted with NK+ T-cells, developed clinical GVL associated with relevant chronic GvHD, NK-transplanted animals showed the same degree of protection from leukemia, experiencing only mild-moderate acute GvHD without chronic GvHD. These data suggest that in order to optimize the GVL effect while minimizing the severity of acute GvHD, donor grafts

---

**Table 3. Clinical trials with LAK cells and IL-2.**

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Patients</th>
<th>Kind of tumor</th>
<th>IL-2 (dose and schedule)</th>
<th>TIL cells</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosenberg</td>
<td>1988</td>
<td>20</td>
<td>metastatic melanoma</td>
<td>1×10^6 U/kg every 8h; CPM 25 mg/kg</td>
<td>20.5×10^6 cell</td>
<td>Regress: 60%</td>
</tr>
<tr>
<td>Kradin</td>
<td>1989</td>
<td>38</td>
<td>miscellaneous</td>
<td>1-3×10^6 U/m² Cl 24h</td>
<td></td>
<td>OR: 26%</td>
</tr>
<tr>
<td>Rosenberg</td>
<td>1990</td>
<td>5</td>
<td>metastatic melanoma</td>
<td></td>
<td>TIL gene modified</td>
<td></td>
</tr>
<tr>
<td>Aoki</td>
<td>1991</td>
<td>10</td>
<td>advanced or recurrent ovarian cancer</td>
<td>TIL after single Cl CPM</td>
<td>OR: 70%</td>
<td>Longterm: 57%</td>
</tr>
<tr>
<td>Dillman</td>
<td>1991</td>
<td>21v</td>
<td>metastatic melanoma</td>
<td>10^4 cell</td>
<td>OR: 24%</td>
<td>expensive, difficult</td>
</tr>
<tr>
<td>Arienti</td>
<td>1993</td>
<td>12v</td>
<td>metastatic melanoma</td>
<td>130×10^6 U/m²/day Cl</td>
<td>6.8×10^6 cell</td>
<td>RR: 33%</td>
</tr>
<tr>
<td>Belldegrun</td>
<td>1993</td>
<td>10v</td>
<td>metastatic renal cell carcinoma</td>
<td>2×10^6 U/m²/day in 96h (IL-2)</td>
<td>TIL</td>
<td>OR: 30%</td>
</tr>
<tr>
<td>Schwartz-entruber</td>
<td>1994</td>
<td>41</td>
<td>melanoma</td>
<td>IL-2</td>
<td>TIL</td>
<td>OR/PR: 21.9%</td>
</tr>
<tr>
<td>Pockaj</td>
<td>1994</td>
<td>38</td>
<td>metastatic melanoma</td>
<td>7.2×10^6 U/kg every 8h</td>
<td>1.3-2.2×10^11 cell and CPM 25 mg/kg</td>
<td>OR: 38.5%</td>
</tr>
<tr>
<td>Chang</td>
<td>1997</td>
<td>20v</td>
<td>advanced melanoma and renal cell cancer</td>
<td>IL-2</td>
<td>anti-CD3 vaccine primed lymph node cells activated</td>
<td>OR: 33.3% PR: 9.1%</td>
</tr>
<tr>
<td>Curti</td>
<td>1998</td>
<td></td>
<td>solid tumor and NHL</td>
<td>9×10^6 U/m²/day x 7 days Cl</td>
<td>T CD4+ cell+ anti CD3</td>
<td>some tumor regression</td>
</tr>
<tr>
<td>Ridolfi</td>
<td>1998</td>
<td>32</td>
<td>miscellaneous</td>
<td>12-6 MIU/ day (West's schedule)</td>
<td>5.8×10^9 TIL</td>
<td>no response in patients with advanced cancer</td>
</tr>
</tbody>
</table>

ev: evaluable; PR: partial response; OR: overall response.
should be manipulated by adding a moderate dose of T-cells in the early phase and using purified NK cells in the late phase after transplantation.

Preliminary data suggest that in normal donors, after G-CSF mobilization, NK progenitors have decreased killing capacity and diminished proliferative activity in response to IL-2, compared to the unprimed bone marrow counterpart. In contrast, after an HLA incompatible transplant, a progressive expansion of NK and CTL with NK like function (CD3+CD56+) has been observed; recipients received the T-cell depleted graft without developing GVHD, but in most cases a significant GV effect could be demonstrated both in vitro and in vivo; these data support the critical role of CTL KIR in this particular subset of transplanted patients.

Concerning the expanding role of cord blood transplantation, even though the content of NK in this source seems normal, the decreased IL-12 production by cord blood MNC, reducing IFN-γ stimulation, may contribute to reduce NK and LAK cytotoxicity; these data suggest one possible explanation for cord blood immaturity and their clinical implications such as decreased GVHD and GVL, which could be enhanced by IL-12 administration.

**Autologous setting.** Considering the impressive results observed in the allogeneic setting using donor-buffy coat lymphocytes for treatment of relapse, CML seems an attractive field for testing the efficacy of adoptive immunotherapy in the autologous setting too; some experimental data support this hypothesis. The MNC of patients with CML contain a population of benign NK cells which can be expanded and activated by IL-2, generating a CE population capable of killing both NK-sensitive and NK-resistant tumor targets. Both number and functional activity of activated NK (ANK) in CML patients decrease with the progression of the disease. In vitro data show that autologous ANK inhibit both committed and very early Philadelphia positive progenitors in a MHC-unrestricted manner. In these experiments CML progenitor cell killing by autologous and allogeneic ANK (after T-cell depletion) was comparable. Finally the CML blast killing was not dependent of soluble factors because it was abrogated by a transwell membrane, but was mediated by cell-to-cell contact being significantly blocked by anti-integrin antibodies.

In 1986 Lanier and Phillips described a subset of CD3+ T cells co-expressing the CD56 antigen which is a typical NK marker (CIK). More recently Schmidt-Wolf et al. obtained large expansion of this subset in a 16-day liquid culture containing IFN-γ, IL-1, IL-2 and a monoclonal antibody against CD3 as the mitogenic stimulus. The same group tested the ability of this population to purge bone marrow in patients with CML; they found that while standard LAK cells were in most cases unable to lyse CML cells, CIK cells were able to lyse both autologous and allogeneic CML blasts, without affecting normal hematopoietic progenitors. Recently it has been reported that CIK administration in SCID mice bearing human CML induced the disappearance of Ph+ cells in the spleen of 12/14 animals.

Another interesting potential application of autologous LAK is the treatment of EBV-related lymphomas arising in organ-transplanted patients; a preliminary description of four complete responses after treatment with autologous peripheral MNC incubated with IL-2 seems very promising. Recently in thyroid cancer patients Katsumoto et al. generated cytotoxic CD4+ lymphocytes from TIL after non-specific in vitro stimulation with OK-432 (which induces severe local inflammation in the draining lymph nodes) and low-dose IL-2, obtaining large amounts of cytotoxic CD4+ (Th1) cells, producing high levels of IFN-γ and TNF-β in the supernatants. These CE lysed a wide spectrum of tumor cell lines; anti-TCR antibodies did not inhibit their killing activity, which was in favor of a non-MHC restricted lysis, while antibodies anti-ICAM-1 completely inhibited the activity.

Tsurushima et al. induced autologous CTLs directly from peripheral blood MNC by preparing a co-culture of minced tissue fragments of glioblastoma multiforme with a mixture of cytokines (IL-1, 2, 4, 6 and IFN-γ) for 2 weeks. At the end of culture the population contained mainly CD4+ and CD8+ lymphocytes able to kill 82 to 100% of the glioblastoma cells while normal LAK cells killed only 33%.

Finally, in follicular lymphomas freshly isolated TIL, normally lacking tumor-specific cytotoxicity, were stimulated with lymphoma cells, in the presence of IL-2 and CD40 ligand; these T-TIL were capable of proliferating in response to follicular lymphoma cells; moreover TIL could be further expanded in the presence of IL-4, IL-7 and IFN-γ.

**The potential role of new cytokines**

Several cytokines affect CTL and NK response: first of all IL-2 which expands the precursor pool of alloreactive CTL; IL-15 (produced by monocytes) mimics IL-2 action by inducing IFN-γ production, T-cell memory activation and CTL proliferation. IL-12 shares certain functional properties with IL-2, but using a different, IL-2 independent pathway. In addition IL-12 enhances the lytic activity of human peripheral blood MNC against a wide spectrum of tumors. Recently it has been observed that the combination of IL-2 and IL-12 is capable of inducing lysis of blasts resistant to IL-2-activated effectors, even in the autologous setting.

Therefore the association of IL-2 plus IL-12 could potentially become an important tool to increase the antitumor efficacy both ex vivo, by generating large amounts of CIK, and in vivo, by systemic administration.

GM-CSF is a cytokine capable of inducing a pleiotropic immunostimulatory effect and also increases the immunogenicity of tumors; in a model for ex vivo expansion of LAK cells from leukaphereses in order to obtain contemporaneously a decontaminated harvest and a large amount of CE to reinfuse after myeloablative therapy, the association GM-CSF + IL-2 obtained a 5-fold expansion of the NK compartment while sparing the clongogenic potential of hematopoietic progenitors.
Biodistribution and targeting of LAK and TIL

At present adoptive immunotherapy with LAK or IL-2 activated TIL has had limited success in patients with advanced cancer. Although a well-defined mechanism remains to be established, numerous in vitro findings and in vivo data suggest that the cancer-specific cytotoxicity of CE is obtained in multiple steps; a prerequisite, however, is optimal delivery of CE to the target tissues while minimizing systemic cytotoxicity. Two major areas currently requiring investigation are the survival and localization of adoptively transferred CE in the tumor-bearing host, and the detailed mechanism of tumor regression. The major goals in this area concern the optimal administration of systemic cytokines together with CE, and (finally) the ways to enhance localization and transcapillary migration of the infused cells.

Experimental evidence together with theoretical considerations based on CE functions indicate that the ability of adoptive immunotherapy to eradicate an established tumor is quantitatively determined by the initial tumor burden, growth pattern, and the magnitude of immunologic response generated by CE and other accessory cells at the site of the tumor. Thus, to achieve tumor eradication and minimize systemic toxicity, the explanation of the mechanisms underlying lymphocyte biodistribution and the factors governing effector cell uptake in tumor sites is critical, but unfortunately data about CE biodistribution in humans are scarce.

Although a physiologically based kinetic modeling approach has been applied to the pharmacokinetics of drugs and antibodies, there has been no effort to extend this approach to cell biodistribution, probably because of its complexity.

One interesting attempt to apply this method to adoptive immunotherapy has, however, recently been published. The importance of lymphocyte infiltration from surrounding normal tissues into tumor tissue was found to depend on lymphocyte migration rate, tumor size, and host organ.

It is likely that therapy with CE has not been as effective as originally promised, in part because of the very low CE concentration in the systemic circulation; this was mainly due to lung entrapment. Reducing this phenomenon by decreasing the attachment rate or adhesion site density in the lung by 50%, the tumor uptake could be increased by 40% for TIL to 60% for adherent NK cells.

Theoretical models indicate that intra-arterial administration has a dramatic advantage over intravenous delivery, with more than a 1,000-fold higher CE accumulation in the tumor site. Indeed experiments in murine models show that it is possible to eliminate liver metastasis by loco-regional administration of human IL-2 ANK or by systemic adoptive transfer.

Finally the differences in biodistribution between different lymphocyte populations, mainly due to the different attachment rates in the tumor and the lung, should be carefully considered. ANK cells are more easily trapped than CTL in lung vessels due to their larger diameter and greater rigidity. A greater accumulation of TIL was expected in the spleen as a result of their stronger adhesion at this site through the lymphocyte homing receptor. Although this model has limitations related to the sensitivity of analysis of parameters such as adhesion-site density, lymphocyte attachment and arrest rate, it could be considered a useful basis for designing new experimental models to increase the concentration and recirculation of CE in tumor sites, reducing effector cell rigidity or blocking adhesion molecules.

The so-called antibody-dependent cellular cytotoxicity (ADCC) could be mediated by cells expressing Fcy receptor II and Fcy receptor III (e.g. NK cells and CD3+CD16+ cells). This kind of cytotoxicity, even though exhibited by non MHC-restricted cells, cannot be considered aspecific and is also exhibited by monocytes.

LAK cells are extremely potent mediators of ADCC and thus the use of LAK plus IL-2 in combination with monoclonal antibodies will probably become a powerful tool for treating some immunogenic tumors. This approach has been tested in patients with colorectal cancer, but could be also proposed for treating some immunogenic hematologic malignancies such as follicular lymphoma or multiple myeloma.

Donor lymphocyte infusion for treatment of leukemia relapse and as a means for accelerating immunologic reconstitution in patients given transplantation of hematopoietic progenitors

Manipulation of the immune system after hematopoietic stem cell transplantation (HSCT) to reverse leukemia relapse or to reduce its incidence remains one of the most fascinating, even though difficult, challenges for successful cure of patients with hematologic malignancies. In fact, over the last 10-15 years, evidence has emerged from clinical transplantations to suggest that the anti-leukemia effect of allogeneic HSCT cannot merely be ascribed to the myeloablative therapy employed during the preparative regimen, donor lymphocytes playing a pivotal role in the eradication of malignant cells. Adoptive immunotherapy with donor lymphocyte infusion (DLI) in patients relapsing after HSCT has provided one of the most effective demonstrations of the importance of the graft-versus-leukemia effect in the cure of patients with hematologic malignancies.

Even though DLI may sometimes be burdened by complications that endanger the patient’s life, mainly myelo-suppression and GvHD, in individuals with CML experiencing relapse in chronic phase after an allograft approximately 70% complete remissions can be obtained with this treatment. Most of these remissions are sustained over time, this proving the capacity of DLI to eradicate clonogenic leukemia cells or control their regrowth. DLI has also been extensively employed to reverse relapse in patients with acute leukemia, non-Hodgkin’s lymphoma and multiple myeloma. However,
the response rate of patients with other hematologic malignancies, especially acute leukemia, is significantly lower.\textsuperscript{115,116} In fact, only 20–30\% of patients with AML achieve a hematologic remission after DLI and the value for patients with ALL is even lower. Patients with acute leukemia experiencing recurrence following an allograft have a higher probability of response with DLI if treated after having achieved a state of complete remission with chemotherapy, that is in a condition characterized by a limited tumor burden.\textsuperscript{117}

The most important factor predicting response to DLI in patients with CML is the type of relapse. In fact, as already mentioned, patients suffering from cytogenetic relapse or hematologic relapse in chronic phase have a high probability of response to DLI, while patients with more advanced disease (accelerated phase or blast crisis) respond less frequently (20–25\% of cases).\textsuperscript{112–116} Relapse occurring in the first 1–2 years after allograft,\textsuperscript{115} little or no acute and chronic GvHD after transplantation or removal of T-lymphocytes before HSCT\textsuperscript{116} are also associated with a higher probability of benefitting from DLI. In patients with CML responding to DLI, the median time to obtain hematologic remission has been reported to be about 6–8 weeks,\textsuperscript{115} whereas a longer time (in the order of 11 months) is needed for molecular remission, this documenting that clearance of leukemia cells is a dynamic, progressive phenomenon.\textsuperscript{118} The number of T-cells to be infused and the best schedule of DLI for optimal response without concurrent development of severe GvHD are still to be conclusively established since they depend on several variables, such as degree of HLA-compatibility between donor and recipient, original disorder, and type of relapse.\textsuperscript{119} Some authors have claimed that infusion of no more than $1\times10^8$ donor-derived T-cells per kg of recipient body weight or CD8-depleted lymphocytes can induce a state of remission and substantially prevent GvHD occurrence.\textsuperscript{119} However, recently, the Hammersmith Hospital group reported that the response in CML patients relapsing after HSCT and given graded increments of donor lymphocytes seems to be less sustained over time than that observed after infusion of a larger number (i.e. $>1\times10^9$/kg of recipient body weight) of T-cells (Dazzi F, personal communication, 1999). Support to the importance of the number of cells infused is also given by the results of Lokhorst et al.,\textsuperscript{120} who observed that, in multiple myeloma, patients given more than $1\times10^9$ T-cells/kg had the highest probability of benefitting from DLI. In some of these patients, the response was complete with disappearance of myeloma proteins.

The two major complications occurring after DLI are myelosuppression and GvHD. Myelosuppression is experienced by approximately 50\% of the patients treated with DLI for CML in hematologic relapse, while it occurs much less frequently in patients with cytogenetic recurrence,\textsuperscript{112} this indicating that such a complication is observed in situations characterized by a predominance of host-type hematopoiesis. Therefore, myelosuppression can be explained by a direct effect of the transfused donor lymphocytes on hematopoietic cells of the recipient, similarly to that observed in transfusion-associated GvHD. The majority of patients experiencing myelosuppression after DLI recover a normal blood cell count spontaneously: nevertheless, myelosuppression may be fatal in approximately 10\% of patients, with death being caused by infection or bleeding.\textsuperscript{115,116} Infusion of a huge number of donor-derived peripheral blood hematopoietic progenitors, mobilized through hematopoietic growth factors such as granulocyte colony-stimulating factor (G-CSF), can alleviate the problem of pancytopenia in some selected cases, hastening the recovery of neutrophil and platelet counts.

Grade II–IV acute GvHD develops in almost half of patients given DLI,\textsuperscript{115,116} the highest incidence being observed when the donor is an unrelated volunteer.\textsuperscript{121} Incidence and severity of GvHD after DLI does not appear to correlate with GvHD after the original transplant and it may occur with a high incidence since donor lymphocyte therapy involves the infusion of large numbers of T-cells, whose immunocompetence is not usually modulated by cyclosporin A and/or methotrexate. Even though GvHD occurring after DLI is well-correlated with disease response as proved by the observation that most patients obtaining a hematologic remission after this treatment developed acute and/or chronic GvHD, GvHD may not be sufficient to induce GVL. Moreover, some patients not experiencing GvHD after DLI achieve hematologic remission, this indicating the existence of a GVL effect separate from development of GvHD.\textsuperscript{113,116,117,122} GVL effect occurring after HSCT and DLI is considered to be mediated by HLA-unrestricted NK or LAK cells or by T-lymphocytes that recognize leukemia cells in an HLA-restricted fashion.\textsuperscript{123,124} In particular, when patient and donor are HLA-identical, it is believed that recipient non-MHC-encoded minor histocompatibility antigens (mHAg) are recognized by donor CTL. While widely distributed mHAg account for the GVL effect associated to GvHD, tissue restricted or leukemia-specific antigens can elicit a specific GVL reaction\textsuperscript{108–112} and it has been demonstrated that both CD4\+ and CD8\+ CTL recognizing mHAg in a classical MHC-restricted fashion can be generated in vitro.\textsuperscript{124,125} In particular, mHAg-specific CD8\+ CTL can display strong lysis of mature leukemia cells, as well as suppress, together with CD4\+ mHAg-specific CTL, the growth of clonogenic leukemia precursor cells.\textsuperscript{126,127} Production of cytokines (such as γ-interferon and tumour necrosis factor α) able to induce the apoptotic death of leukemia cells can also contribute to the GVL effect.\textsuperscript{128,129} This said, it is not surprising that several efforts have been directed towards the identification of strategies capable of selecting and/or amplifying specific GVL response, not associated with development of GvHD. Since it has been documented in humans that CTL directed against allogeneic leukemic blasts can be detected in the peripheral blood of healthy donors\textsuperscript{130} and that CTL specifically reactive towards recipient leukemic blasts can emerge and persist over time in children given allogeneic HSCT\textsuperscript{131} a possible intriguing approach is that of generating and
expanding clones or cell lines that are leukemia-reactive. The first elegant demonstration of the feasibility and efficacy of this sophisticated strategy has been recently reported by Falkenburg et al.,12 who, through the infusion of donor-derived in vitro cultured CTL specifically recognizing leukemia progenitor cells, induced a complete hematologic and cytogenetic response in a patient with CML who had relapsed after an allograft and was resistant to DLI treatment.

A diverse, but equally elegant, approach proposed to abrogate the DLI-associated GvHD and its relevant morbidity and mortality is the infusion of thymidine kinase gene-transduced DLI followed by treatment of the recipient with ganciclovir if GvHD occurs.132 In a study reported by Bonini et al.,133 this strategy proved to be able to control GvHD in 3 patients experiencing this complication after DLI; two of them, who had achieved a complete hematologic remission before ganciclovir administration, remained in full remission after disappearance of the transduced lymphocytes. If confirmed in a larger number of patients with a longer follow-up, genetic manipulation of donor lymphocytes, through the transfer of a suicide gene for specific and selective elimination of effector cells responsible for GvHD, could demonstrate the possibility of separating GvHD from GVL effect, thus sparing the anti-leukemia activity of DLI.

One of the most important, still unsolved problem of DLI is that concerning the much lower efficacy of GVL in patients with acute leukemia than in those with CML. An immediate explanation for this observation may be that the more rapid growth kinetics of blast cells, which occurs in patients with acute leukemia during the lag period between leukocyte infusion and GV development, may hamper the immune-mediated effect played by donor lymphocytes in controlling disease progression. In fact, response to DLI occurs after weeks and hence the exponential expansion of leukemia cells in vivo may exceed the immune response.112,129,124 The more encouraging results obtained when DLI is used as consolidation therapy for patients who have obtained a complete remission after chemotherapy provide support for this interpretation. However, other hypotheses, involving different intrinsic susceptibility of acute leukemia to adoptive immunotherapy must be considered. In particular, since patients with ALL have the lowest chance both of responding to DLI and of benefitting from the GVL effect after bone marrow transplantation,134 a peculiar resistance of lymphoid leukemia to immunotherapy cannot be excluded.

As peptides differentially expressed within the hematopoietic system can trigger and act as a target of the GVL reaction,112,124 it could be hypothesized that, for example, the presence of these antigens on myeloid blasts, but not on lymphoid leukemia cells accounts for the low response of ALL to donor lymphocytes. The reported demonstration of CTL response directed towards peptides derived from proteinase 3, which is expressed by myeloid cells (including blast cells),135 is a typical example of the possible differential susceptibility to the immune-mediated anti-leukemia effect of different types of hematologic malignancies.

Several other possibilities exist to explain why acute leukemia (and in particular ALL) can escape the GVL effect. For example, leukemia cells may have defective expression of HLA-class I or II molecules on their surface such that they do not present antigens or, alternatively, the mechanisms of antigen processing and transport may be impaired.112,129 Moreover, leukemia blasts may produce cytokines (such as transforming growth factor β, IL-10) capable of suppressing T-cell activation, expansion and effector function or may express on their cell surface molecules, such as FAS ligand, able to mediate T-cell apoptosis.112,129 One of the most interesting fields of investigation for explaining why in some patients a sustained anti-leukemia response in vivo fails to be induced is that of co-stimulatory molecules. As previously described, full activation of T-cells requires two distinct but synergistic signals.136 In fact, in the absence of co-stimulatory signals, a T cell encountering an antigen becomes unresponsive to the appropriate stimulation (anergic)137 or undergoes programmed cell death (apoptosis).138 Leukemia cells lacking these co-stimulatory molecules have a poor capacity of inducing a T-cell specific immune response and induction of CD80 and CD86, by signalling through the CD40 molecule, is able to restore T-cell co-stimulation via CD28 and to generate both allogeneic and autologous CTL, which could contribute to inducing or maintaining a state of hematologic remission.139,140

Some clinical strategies have been devised to improve the efficacy of adoptive immunotherapy in patients with acute leukemia. An approach for ameliorating the efficacy of DLI which has produced interesting results is that recently reported by Slavin et al.,106 who documented that the success rate of this adoptive immune therapy may be increased in patients with both acute and chronic leukemia by activation of donor peripheral blood lymphocytes with IL-2 both in vivo and/or in vitro. In particular, a relevant proportion of patients who had not responded to DLI were induced into remission only after in vivo administration of IL-2 or in vitro activation of donor lymphocytes. If further confirmed, the results obtained make it possible to hypothesize that this strategy could be employed as first-line treatment of patients with acute leukemia relapsing after an allograft, since ALL and to a lesser extent AML patients do not greatly benefit from DLI alone. Another reasonable attempt for improving the response to DLI in patients with acute leukemia is to use this adoptive immunotherapy in individuals with minimal residual disease, as determined by cytogenetic investigations or sensitive molecular tools, that is in conditions characterized by a limited tumor burden, in which the GVL effect has demonstrated its greatest efficacy.

Unmanipulated DLI may also provide a means of compensatory T-cell depletion for the prevention of leukemia recurrence in patients given a T-cell depleted marrow transplantation from a relative. This approach has been
recently proposed and studies enrolling larger cohorts of patients are necessary to define whether this strategy can be useful to prevent the increased risk of relapse associated with the removal of donor T-cells. However, the main indication of adoptive infusion of donor immune cells to accelerate immune reconstruction after HSCT is transplants from HLA-disparate family donors. Infusion of a high number of T-cell depleted, peripheral blood hematopoietic progenitors from these donors has been demonstrated to be associated with a high chance (>95%) of donor hematopoietic engraftment. The significant delay in immune reconstitution, due mainly to removal of mature T cells from donor marrow and HLA disparity between donor and recipient, remains the major problem of HSCT from HLA-disparate donors. In fact, it is responsible for the dramatic incidence of leukemia relapse and life-threatening viral and fungal infections observed after this type of HSCT. A possible strategy to improve the process of immune recovery is to infuse donor T-lymphocytes selectively rendered non-reactive towards alloantigens of the recipient, but maintaining the capacity to generate an immune response against viruses, fungi and leukemia cells. In this regard, as previously mentioned, the manipulation of co-stimulatory molecules is an extremely promising field of investigation, since the absence of a second signal induces anergy rather than activation of T lymphocytes. Drugs and monoclonal antibodies blocking co-stimulatory pathways have been demonstrated to be able to prevent T-cell activation in response to alloantigens and to induce a state of anergy. In particular, it was recently documented that the combination of monoclonal antibodies blocking CD80/CD86 molecules and cyclosporin A was able to generate a state of selective in vitro unresponsiveness of T cells towards allo-antigens, not reversed by adding IL–2. Since the induction of this state of unresponsiveness was associated with the maintenance of in vitro capacity to respond toward virus antigens and leukemia cells, the relevance of this approach is evident for strategies of donor T-cell add-back after T-cell depleted transplant of hematopoietic progenitors from HLA-partially matched donors aimed at accelerating the process of immune reconstitution.

A different, but equally promising, method of deletion of unwanted alloresponses is based on the elimination of alloreactive T-cells after specific activation through their killing or fluorescence-activated cell sorting, while sparing T cells with other functions. In a human pre-clinical study, it was demonstrated that allospecific T-cell depletion by using an immunotoxin directed against the p55 chain of IL–2 receptor, was feasible and specific. The spared T-cells were still able to proliferate against third-party cells, Candida and cytomegalovirus antigens, as well as to kill both leukemia blasts and autologous EBV-B lymphoblastoid cell lines. Moreover, in vivo studies in a murine animal model showed that this particular T-cell depletion was efficient, at least partially, in preventing both graft rejection and GvHD in a complete haplotype mismatched combination.

Finally a brief mention should be made of the generation and infusion of T cells with suppressive and regulatory activity. A particular subset of these cells called Tr1 has recently been described by Groux et al., who in an animal model demonstrated the ability of this population to prevent, through their activity on naive cells, the occurrence of ovo-albumin induced inflammatory bowel disease. Whether these cells will have a role in promoting a true state of tolerance in transplant of hematopoietic progenitors or solid organs (in which the immune response to alloantigens is mainly sustained by memory cells) remains to be proved in specific pre-clinical and clinical studies currently underway.

Adoptive immunotherapy for the treatment of viral infections in immunocompromised patients

Prevention or treatment of viral infections in immunocompromised patients through the infusion of specific T-cell lines or clones is one of the most sophisticated examples of adoptive immunotherapy approaches. In fact, it implies the elaboration of true cellular-engineering strategies able to generate, select and expand lymphocyte subsets, which display a specific function. The first study in humans to evaluate the efficacy of adoptively transferred T-cell clones for reconstitution of specific immunity was performed in recipients of allogeneic HSCT at risk of developing human cytomegalovirus (HCMV) infection and/or disease. Even though preemptive therapy of HCMV infection based on monitoring of antigenemia and prophylaxis of seropositive HSCT recipients using antiviral drugs (i.e. ganciclovir and foscarnet) have significantly reduced the number of patients experiencing HCMV disease, this viral infection still represents a major life-threatening complication of stem cell allograft. The capacity to recover from a severe HCMV infection in transplanted patients is directly correlated with the ability of the host to generate virus-specific class I HLA-restricted CD8+ cytotoxic cells and during the first 100 days after HSCT approximately 50% of patients are persistently deficient in CD8+ cytotoxic T-lymphocytes specific for HCMV. It is not surprising that, to evaluate the efficacy of adoptive immunotherapy in this viral infection, HCMV-specific CD8+ T-cell clones of donor origin were generated and infused in HSCT recipients. These cells, generated through a highly complex expansion strategy using irradiated donor-origin skin fibroblasts infected with a strain of HCMV, proved to be efficient in the prophylaxis against HCMV infections that can complicate allogeneic HSCT. Moreover, the cloning strategy allowed selection of T cells which lacked significant alloreactive capacity and, thus, did not cause clinically relevant GVHD or toxicity. These clones, directed towards either pp65 or pp150 (two abundant viral tegument proteins presented for recognition by cytotoxic T-lymphocytes), restored HCMV-spe-
pecific cytotoxicity, which persisted for several weeks. In fact, through a PCR technique able to detect the Vα and Vβ T-cell receptor rearrangements specific for the donor clones, it was possible to prove the donor origin of these cells formally and to document the persistence of the adoptively transferred HCMV-specific T-cells for at least 12 weeks. Unfortunately, these clones persisted in the circulation at high levels only in patients experiencing an endogenous recovery of CD4+ virus-specific cells. By contrast, in patient lacking this spontaneous recovery of HCMV-specific CD4+ lymphocyte, the donor-origin, adoptively transferred cytotoxic T-cell activity progressively declined and eventually disappeared. This observation emphasizes the importance of CD4+ lymphocytes in promoting sustained restoration of antigen-specific immunity and suggests that the use of polyclonal T-cell lines containing both CD4+ and CD8+ cells could be preferable to the infusion of cytotoxic T-cell clones.

In this regard, the use of T-cell lines for prevention and/or treatment of Epstein-Barr virus-induced lymphoproliferative disorders (LPD) has represented a further, equally sophisticated, evolution of the approaches of adoptive immunotherapy for the restoration of virus-specific immunity. EBV-LPD have emerged as a significant complication for both HSCT and solid organ transplant recipients. In the former cohort, the use of HLA-partially matched family and unrelated donors, as well as selective procedures of T-cell depletion sparing B-lymphocytes, are risk factors for the development of EBV-LPD. In HSCT recipients these disorders are of donor origin and usually present in the first 4–6 months after transplantation, whereas in patients given a solid organ allograft they usually develop from the recipient B-lymphocytes months to years after transplantation. High levels of EBV-DNA in blood and in vitro spontaneous growth of EBV-lymphoblastoid cell lines predict development of these lymphoproliferative disorders. They often present as high-grade diffuse large cell B-cell lymphomas, which are oligoclonal or monoclonal and express the full array of EBV antigens including EBNA-1 through EBNA-6 and the latency membrane proteins LMP-1 and LMP-2. The lymphomas which develop in immunocompromised hosts not only invade the hematopoietic system, but also the lung, nasopharynx and central nervous system. The therapeutic approaches proposed to date (i.e. discontinuation of immunosuppression, α-IFN, antiviral agents and cytotoxic chemotherapy) have been applied with varying success, but overall unsatisfactory results; moreover, graft rejection, GvHD and toxicity are frequent complications of these strategies, and mortality rate due to EBV-LPD remains high.

Normal EBV seropositive individuals have a high frequency of circulating virus-specific cytotoxic T-lymphocytes precursors, which control outgrowth of EBV-infected B-cells. Since EBV-LPD in immunocompromised hosts appears to stem from a deficiency of virus-specific cytotoxic activity, it is reasonable to hypothesize that an adoptive immunotherapy approach with donor-derived T-lymphocytes could be able to prevent unchecked lymphoproliferation and eradicate established disease. In 1994, the Sloan Kettering group first demonstrated that, through the infusion of unselected peripheral blood mononuclear cells from a donor, 5 patients given HSCT with post-transplant EBV-LPD obtained remission of the disease. However, this treatment was associated with development of clinically relevant GvHD and 2 patients of inflammatory-mediated lung damage, leading to respiratory failure.

A further refinement of this approach was achieved by Rooney and colleagues, who generated EBV-specific T-cell lines from donor lymphocytes and infused them as prophylaxis against EBV-LPD in patients given T-cell depleted HSCT from HLA-disparate family or unrelated donors, and, thus, considered at high risk for this disease. The infusion of these polyclonal T-cell lines proved to be safe and effective in the prevention of EBV-LPD. Moreover, these cytotoxic cells may also have a role in the treatment of established disease. The most recent update of this experience confirms that the infusion of EBV-specific T-cell lines is highly effective for the prevention of EBV-LPD, since none of 39 patients given a T-cell depleted allograft and treated with this adoptive immunotherapy developed the disease, as compared to 7 out 61 transplanted patients not receiving the prophylactic treatment. Gene marking studies have shown the persistence of these donor-derived EBV-specific cell cytotoxic lines in patient’s peripheral blood for months after infusion and their re-appearance after periods of apparent non-identifiability during episodes of viral reactivation, this further stressing the importance of helper T-cell function in the persistence of transferred CD8+ cells.

The profound immunosuppression necessary for graft survival carries a well-recognized predisposition to the development of viral complications, in particular EBV-LPD, also in recipients of solid organ transplantation. An immunotherapy approach to EBV-LPD using autologous in vitro generated EBV-specific cytotoxic lines could be an appealing strategy in this cohort of patients. Support for this hypothesis is given by the recently described, although not unexpected, possibility of generating, from pre-transplantation blood samples of EBV-seropositive solid organ transplant recipients, virus-specific T-cell lines which are effective in controlling EBV replication post-transplantation. However, generation and storage of cytotoxic lines for each patient undergoing solid organ transplantation requires enormous, unavailable levels of funding, laboratory facilities and workforce. A more rational strategy is to generate, expand and infuse autologous EBV-specific cytotoxic lines from the peripheral blood of organ transplant patients presenting increased EBV-DNA levels after transplantation, which, as previously mentioned, are a risk factor for EBV-LPD development. The feasibility of generating autologous EBV-specific cytotoxic lines from the peripheral blood of organ transplant patients receiving in vivo immunosuppression for prevention of graft rejection has been recently
proven. Moreover, these cytotoxic T-lymphocytes were demonstrated to be able to display EBV-specific killing in vivo, as proved by prompt viral DNA clearance, without augmenting the probability of graft rejection. A peculiar problem, fortunately not particularly common, is that of EBV-seronegative patients, who develop primary EBV infection after solid organ transplantation. In fact, in these patients, in vitro generation of virus-specific T-cell lines able to control EBV-driven B-cell proliferation can be particularly complicated, time-consuming and sometimes unsuccessful.

Autologous EBV-specific cytotoxic lines with demonstrated anti-viral activity in vitro and in vivo may also have a role in the treatment of other EBV-associated primary malignancies: for example, 40-50% of patients with Hodgkin’s disease tumor cells are EBV-antigen positive and may therefore be suitable targets for virus-specific cytotoxic lymphocytes. A recently reported study provides further support for this possibility, documenting that, although more complicated than in normal donors, generation of EBV-specific cytotoxic lines is feasible in a relevant proportion of patients with EBV-positive Hodgkin’s disease. These lines retained their potent antiviral effects in vivo and persisted for more than 13 weeks in patients with relapsed Hodgkin’s disease. Whether this approach of adoptive immunotherapy will become an adjunctive treatment option for patients failing to gain benefit from conventional chemotherapy remains to be proved in prospective clinical trials.

Finally, it should be mentioned that adoptive transfer of cytotoxic T-cell response could be of value also in the prevention or treatment of other viral infections that cause morbidity and mortality in immunocompromised patients. In this regard, preclinical studies are underway to establish systems for generating cytotoxic T-cell responses to adeno virus.

Genetically engineered donor lymphocyte infusion for treatment of leukemia relapse and as a means of accelerating immunologic reconstitution in patients given transplantation of hematopoietic progenitors

Tumor recurrence is the major cause of treatment failure of autologous bone marrow transplantation. Indeed, the rate of tumor relapse is lower when transplantation is performed between matched unrelated or mismatched family member donor and recipients. It is now established that the curative potential of allo-BMT is represented by the additional effect of high dose chemo-radiotherapy in addition to the presence of allogeneic T-lymphocytes that are responsible for the GVHD. However, the therapeutic impact of allogeneic BMT is limited by the inevitable occurrence of GVHD. Severe GVHD can be circumvented by the in vitro removal of T-lymphocytes from the BMT. However, recipients of depleted marrow have delayed immune recovery, and increased incidences of viral infections and tumor relapse.

Recent studies have shown the clinical efficacy of the adoptive transfer of immune effectors specific for viral antigens in patients who underwent BMT. In this context gene transfer of a marker gene provides a means of evaluating the survival, homing and efficacy of the infused cells.

In marrow–transplanted recipients, lymphoproliferative disorders associated with EBV, a human herpes virus that normally replicates in epithelial cells of the oropharyngeal tract, occurs in 5–30% of the treated patients. EBV-LPD are usually malignant B-cell lymphomas of donor origin, which may be either polyclonal or monoclonal. The latter have a rapidly progressive, fulminating and fatal course. The transformed B cells express virus-encoded latent cycle nuclear antigens, latent membrane proteins, and a number of cell adhesion molecules. Most of these viral proteins are recognized as antigens by the immune system of a normal individual. In the normal host, in fact, EBV-induced lymphoid proliferation is controlled by EBV-specific and MHC-restricted T-lymphocytes, MHC-unrestricted effectors and by antibodies directed toward specific viral antigens. Since a limited number of specific cytotoxic T-lymphocytes is required for controlling EBV-transformed B-lymphocytes in normal individuals, the administration of donor lymphocytes for the occurrence of EBV-LPD in recipients of T-cell depleted bone marrow transplantation could control this severe complication by providing the patient with donor immunity against EBV. Successful regression of the disease, documented histologically and by full clinical remission, has been achieved by the infusion of unmanipulated donor leukocytes. However, acute or mild chronic GvHD developed in all the patients who responded to the treatment.

To prevent GvHD, Brenner’s group has evaluated the use of EBV-specific CTL rather than unmanipulated T cells. Donor derived EBV-specific CTL have been generated in vitro by stimulation with irradiated donor-derived EBV-infected lymphoblastoid cell lines (LCL). The polyclonal effector populations were predominantly CD8+ with a varying number of CD4 and showed specific cytotoxic activity toward the EBV-infected target cells. In order to investigate the long-lasting survival of the injected cells, the anti-EBV effectors were marked with the neo-gene before administration.

Neo-marked cells were detected in circulation for at least 10 weeks after the injections. Moreover, the infusions allowed the establishment of a population of CTL precursors that could be activated to proliferate by in vivo or in vitro challenge with the virus. The authors showed that EBV-specific CTL lines expressing the neo-marker, could be derived from patient’s peripheral blood lymphocytes (PBL) for up to 18 months, by in vitro restimulation with the autologous EBV-lines.

These findings support a more widespread use of antigen-specific CTL in the treatment of infections and cancer. Their use may extend in the near future to other dis-
eases which express well-known antigens that could serve as target of CTL therapy (e.g. Hodgkin’s disease and nasopharyngeal carcinoma).

The adoptive transfer of in vitro stimulated effectors achieves clinical results without causing the appearance of GvHD. However, the application of this strategy to a large number of allo-BMT treated patients, especially in prophylaxis protocols has some limitations related to the in vitro manipulation necessary for the generation of specific effectors (e.g. availability of donor-EBV lines; in vitro stimulation and expansion of antigen-specific effectors). An alternative approach was proposed in 1994 by the S. Raffaele Hospital group.\textsuperscript{185,186} Their protocol was aimed at maintaining the potential of the infusion of polyclonal cell lines while providing a specific means to control acute GvHD. To this aim they transduced donor lymphocytes by a retroviral vector containing a suicide gene for in vivo selective elimination of the infused lymphocytes.

It was previously shown that introduction of a gene encoding for a susceptibility factor, a so-called suicide gene, makes transduced cells sensitive to a drug not ordinarily toxic.\textsuperscript{187} A series of retroviral vectors carrying a suicide gene for ganciclovir-mediated in vivo selective elimination of the infused lymphocytes was designed. The vectors carried either an HSV-thymidine kinase-neo (Tk-neo) fusion gene, coding for a chimeric protein for both negative and positive selection, or the HSV-Tk gene alone.\textsuperscript{189}

A crucial prerequisite for the application of this strategy in the clinical context is the transduction of all infused donor lymphocytes. For this purpose, the designed retroviral vectors also carried a gene encoding a modified (non-functional) cell surface marker not expressed on human lymphocytes. Positive immunoselection of the transduced cells\textsuperscript{190} by the use of the cell surface marker resulted in virtually 100% gene-modified lymphocytes. Based upon the preclinical data described above, a clinical protocol was developed\textsuperscript{191} for the use of donor lymphocytes transduced by the SFCMM-2 retroviral vector for transfer and expression of two genes: the HSV-Tk gene that confers to the transduced PBL in vivo sensitivity to the drug ganciclovir, for in vivo specific elimination of cells potentially responsible for GvHD; and a modified (non-functional) form of the low affinity receptor for the nerve growth factor gene (ΔNGF receptor), for in vitro selection of transduced cells and for in vivo follow-up of the infused donor lymphocytes.

Increasing doses (beginning at $1\times10^6$/kg) of donor PBL were infused into several patients affected by hematologic malignancies who developed severe complications following a T-cell depleted BMT from HLA-identical related donors. After the infusion, the transduced lymphocytes could be detected in the blood of patients by cytofluorimetric and PCR analyses. In particular one patient affected by an EBV-LPD, showed a progressive increase in the number (up to 13.4% of the total PBL) of infused marked lymphocytes that was accompanied by a complete clinical response. However, signs of acute GvHD, confirmed by skin biopsy, were observed approximately four weeks after the infusion of the transduced-donor lymphocytes. The intravenous (i.v.) administration of two doses of ganciclovir (10 mg/kg/day) quickly resulted in elimination of marked donor PBL, and near resolution of all clinical and biochemical signs of acute GvHD.\textsuperscript{187}

As mentioned before, when comparable preparative regimens are employed, the rate of tumor recurrences after autologous BMT is significantly higher than the rate observed after allogeneic BMT. GvHD develops in 50–70% of patients undergoing allogeneic BMT. The effectors of such response are thought to be mature donor lymphocytes from the marrow graft that respond to the foreign major and/or minor histocompatibility antigens of the recipient and also recognize and destroy the tumor cells. In fact, patients who underwent mature T-cell-depleted allogeneic BMT have a lower rate of GvHD but also a higher rate of leukemia relapses.\textsuperscript{178,179} The infusion of donor lymphocytes, early after T-cell-depleted allogeneic BMT, increases the incidence of GvHD without improving the control of leukemia.\textsuperscript{191} However, a delayed transfusion of donor lymphocytes, when graft tolerance is established, seems to be more effective in preventing and treating tumor relapses.

Indeed the delayed administration of donor lymphocytes has recently become a new tool for treating leukemic relapse after BMT. Patients affected by post-BMT recurrence of chronic myelogenous leukemia, acute leukemia, lymphoma, and multiple myeloma could achieve complete remission after the infusion of donor leukocytes without requiring cytoreductive chemotherapy or radiotherapy.\textsuperscript{106,192–194} even though the response rate of patients with acute leukemia, non-Hodgkin’s lymphoma and multiple myeloma is significantly lower than that of patients affected by chronic myelogenous leukemia. Although the delay in the administration of T lymphocytes is expected to reduce the risk of GvHD, this risk is still present at higher doses of donor T-cells.\textsuperscript{116} Therefore, as described above, a clinical protocol was developed, for the use of donor lymphocytes transduced by the SFCMM-2 retroviral vectors\textsuperscript{116} for transfer and expression of the HSV-Tk gene, and the cell surface marker ΔNGF receptor, for in vitro selection of 100% transduced cells and for in vivo follow-up of the infused donor lymphocytes.\textsuperscript{195}

In a phase I–II study, eight patients affected by hematologic malignancies who developed severe complications following an allogeneic T-cell depleted BMT, received escalating doses of donor PBL transduced by the described retroviral vector.\textsuperscript{132} After gene transfer, transduced cells were selected for the expression of the cell surface marker ΔNGFr by the use of specific immunobeads and the proportion of transduced cells was assessed by cytofluorimetric analysis.\textsuperscript{130} In this study, we made the following observations: 1) transduced cells survived long-term in vivo and were detectable by cytofluorimetric analysis and PCR in high proportions (up to
13.4% of circulating PBL) and long-term (up to 6 months); 2) three patients showed complete response, three patients had partial response, one progressed with no response, and one patient could not be evaluated; 3) three patients developed GvHD that required ganciclovir treatment; 4) ganciclovir-mediated elimination of transduced cells resulted in near resolution of all clinical and biochemical signs of acute GvHD. Data from this study\textsuperscript{133} indicate that genetically modified cells maintain their in vivo potential to develop both anti-tumor and GvHD effect, and may represent a new potent tool for exploiting anti-tumor and anti-host immunity, while providing a specific means for eliminating acute GvHD, in the absence of any immunosuppressive drug.

A potential limitation of the clinical approaches described could be the development of a specific immune response against vector-encoded proteins, which might allow the selective elimination of the transduced cells by the host immune system. For some gene products, such as the hygromycin-thymidine kinase (Hy-Tk) fusion protein, a specific immune response, able to eliminate large numbers of transduced cells in less than 48 hours, has been described in HIV-patients.\textsuperscript{195}

We observed that immune recognition and killing of cells transduced by retroviral vectors is a more general phenomenon related to the foreign nature of the proteins expressed by the injected cells. Indeed, cells expressing the widely used marker gene neo and the HSV-Tk gene are targets of a strong immune response, while the endogenous proteins (e.g. the cell surface marker \(\Delta\)LNGFr) are not recognized, even if ectopically expressed in a context which is otherwise extremely immunogenic.\textsuperscript{196} The relative immunogenicity detected for the three vector-encoded components (none by \(\Delta\)LNGFr, low by HSV-Tk, high by neo) clearly outlined the modifications of this type of gene therapy. Since neo is the only component not strictly necessary for the strategy and can be efficaciously replaced by the surface marker for all in vitro handling and selection,\textsuperscript{189,197} the immunogenicity of the new neo-less vectors should be reduced.

The clinical results obtained with gene modified donor lymphocytes, for the treatment of hematologic relapses and EBV-lymphoproliferative disorders, suggest the potential use of this approach.\textsuperscript{133} The transfer of a suicide gene, that allows selective and specific elimination of effector cells of GvHD may allow full advantage to be taken of the beneficial effect of allogeneic lymphocytes with the possibility of eliminating all unwanted effects of GvHD in the absence of toxic side effects. A large scale application of this strategy will increase the number of patients who could potentially benefit from allogeneic BMT by allowing the use of less compatible marrow donors.

With regard to the immune recovery associated with the genetically-engineered donor lymphocytes, our group has recently obtained in vitro data demonstrating that genetically-engineered donor T-cells maintain a normal TCR \(\text{V}\beta\) immune repertoire and retain antigen-specific lytic activity against an allogeneic target or an autologous EBV cell line at cytotoxic T-cell precursor frequencies comparable to unmodified lymphocytes. In the light of this in vitro evidence, and our previous clinical application,\textsuperscript{133} a clinical trial, based on the prophylactic infusion of \(1 \times 10^7/kg\) HSV-Tk transduced T-cells six weeks after T-cell-depleted bone marrow transplantation, was developed. In the first five treated patients we documented the presence of various proportions of transduced cells in the peripheral blood. In particular, genetically-engineered donor lymphocytes were responsible for antiviral immune reconstitution in one patient. CD3\(^+\) lymphocytes began to appear in the circulation of this patient two weeks after the infusion of HSV-Tk T-cells. All the CD3\(^+\) lymphocytes were genetically engineered as demonstrated by the expression of the cell surface marker \(\Delta\)LNGFr. These cells retained a polyclonal TCR repertoire and were probably responsible for the clearance of a persistent CMV antigenemia. Indeed, the CMV antigenemia dropped below levels which could be detected by PCR shortly after the appearance of circulating genetically-engineered CD3\(^+\) T cells in the absence of any antiviral drug therapy.\textsuperscript{198} These data, if confirmed in a larger number of patients with longer follow-up, suggest that in addition to the anti-tumor activity, the infusion of genetically-engineered donor lymphocytes may play a role in restoring immunity against opportunistic infections early after allogeneic BMT.

**Dendritic cells as natural adjuvants in cancer immunotherapy**

Among professional antigen presenting cells (APC), dendritic cells (DC) are specialized in capturing and processing antigens into peptide fragments that bind to major histocompatibility complex molecules. DC are the most potent stimulators of T-cell responses and they are unique in that they stimulate not only memory but also naive T-lymphocytes. Thus, DC appear critical (\textit{nature adjuvants}) for the induction of B- and T-cell-mediated immune responses. Recent evidence in experimental models supports the role of DC for immunization strategies aimed at stimulating specific anti-tumor immunity.

In this section we will briefly review:

1. the biological characterization of DC;
2. different strategies for \textit{ex vivo} generation of DC;
3. methods for the efficient delivery of tumor-associated antigens (TAA) to DC;
4. the use of DC for cellular immunotherapy.

**Biological characterization of dendritic cells**

DC are widely distributed in the body and are particularly abundant in tissues that interface the environment (i.e. Langerhans cells in the skin and mucous membranes) and in lymphoid organs (interdigitating DC) where they act as \textit{sentinels} for incoming pathogens.
Inflammatory signals such as TNF-α and IL-1β as well as bacteria, bacterial products (LPS) and viruses induce migration of antigen-loaded DC from the peripheral tissues to secondary lymphoid organs. During migration, DC mature and upregulate MHC, adhesion and co-stimulatory molecules, thus strongly augmenting their ability to prime T-cells.\(^{199-204}\)

The functional activity of DC derives from a number of properties of these cells (Figure 2). Their dendritic shape, along with the high level of expression of certain adhesion molecules and integrins (LFA-3, ICAM-1, ICAM-3), increases the area of contact with the effector cells of the immune system.\(^ {205}\) DC strongly express the HLA class II molecules -RD, -DQ and -DP and co-stimulatory molecules (CD80, CD86 and CD40) which activate their ligands on T-cells (CD28, CTLA-4 and CD40L), thus providing the second signal strictly necessary to induce a proliferative response, rather than tolerance, upon antigen recognition.\(^ {199}\) In addition, DC produce a number of cytokines including IL-12 which promotes a cytotoxic immune response by inducing the differentiation of TH0 cells to IFN-γ and IL-2-producing TH1 cells.\(^ {206,207}\) It has recently been demonstrated that upon Ag recognition, T-helper cells activate DC via CD40-CD40L interaction and activated DC are then able to trigger a cytotoxic response from T-killer cells.\(^ {208-210}\)

However, DC are present in peripheral tissues in an immature state unable to prime T-cells. At this stage of differentiation, they can very efficiently take up soluble antigens, particles and micro-organisms by phagocytosis, macropinocytosis or by the macrophage mannose receptor, Fcγ and Fcε receptors,\(^ {211}\) but they lack all the accessory signals for T-cell activation. Antigen uptake induces DC to maturation by up-regulating MHC and co-stimulatory molecules as well as DC-associated Ag (e.g. CD83 and p55) whereas the capacity to capture and process Ag is lost. However, full activation of DC is dependent upon the contact with T-cells by the CD40-CD40L interaction which induces the production of IL-12. Thus, the key functions of DC (antigen uptake, T-cell stimulation) are strictly segregated to subsequent stages of differentiation (Figure 3). It is noteworthy that IL-10\(^ {212}\) and vascular endothelial growth factor (VEGF), secreted by cancer cells,\(^ {213}\) prevent the maturation of DC thus inhibiting the efficient priming of T-cells.

**Different strategies for the generation of DC ex vivo**

Circulating CD14+ monocytes represent the most readily available source of DC if incubated with appropriate cytokines such as GM-CSF, IL-4 and TNF-α.\(^ {214,215}\) Moreover, DC precursors have been isolated within the CD34+ cell fraction in bone marrow, cord blood and steady state or mobilized peripheral blood.\(^ {216-221}\) Also in this case the differentiation of CD34+ cells into fully functional DC is strictly dependent upon stimulation with certain cytokines such as GM-CSF, TNF-α, SCF, FLT3-L and IL-4.

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**Figure 2.** Phenotypic and functional characteristics of dendritic cells. Modified from ref. #199 (Bancherau and Steinman, Nature, 1998).
An extensive review of the different types of human DC and their ex vivo generation is beyond the scope of this chapter. However, in view of the clinical use of DC a few critical points should be stressed. GM-CSF and IL-4 induce the differentiation of non-proliferating CD14+ monocytes to immature DC with a low level of expression of CD83 and p55 Ag and are largely incapable of priming naive T-cells. These immature DC are not fully differentiated and revert to an adherent state if the cytokines are removed from the culture medium. The addition of inflammatory cytokines such as TNF-α, IL-1β or PGE2 for 1-2 days to the medium containing GM-CSF and IL-4 promotes the maturation of DC and increases the ability of stimulating T-cells. A potential bias toward the clinical use of this culture system is the requirement of fetal calf serum (FCS), a xenogenic protein that is contraindicated for human use. An innovative culture system for the generation of mature and functional DC from circulating monocytes that uses FCS-free conditions has recently been described. In this system, adherent peripheral blood (PB) cells are cultured for 6-7 days with GM-CSF and IL-4 in the presence of FCS, which is then washed out, and subsequently exposed to macrophage-conditioned medium (Mo-CM) and 1-5% autologous plasma for 1-3 days. Mo-CM is very efficient in inducing the terminal maturation of DC and is prepared by growing T-cell-depleted PB cells on immunoglobulin (Ig)-coated Petri dishes for 24 hours.

Taken together, these findings lead to the conclusion that immature DC generated from CD14+ cells in the presence of GM-CSF and IL-4 are well equipped for capturing and processing soluble TAA. However, they do require a further maturation stimulus (Mo-CM, TNF-α) to exert their stimulatory effect on T-cells. Immature DC are the ideal targets for genetic manipulation using viral or bacterial vectors which infect non-replicating cells (see below). In this case, the modified pathogens can induce by themselves the full maturation of DC. In alternative, mature DC could be used in vaccination protocols involving TA peptides as DC also prime T-cells to foreign Ag that bind directly to MHC molecules without prior processing.

As reported above, CD34+ cells can be induced to differentiate into fully functional DC which resemble cutaneous Langherans cells. The issue of the large scale production of DC from CD34+ precursors has been discussed in detail elsewhere. However, very recently the phenotypic and functional characteristics of DC derived from CD34+ cells mobilized into PB or from BM progenitors have been formally compared. The published results indicate that G-CSF mobilizes DC precursors (CFU-DC) with an increased frequency and a higher proliferative capacity than their BM counterparts. This finding translates into a higher number of mature DC generated in liquid culture. Despite pre-treatment with G-CSF, these cells maintain the same functional capacity of stimulating allogeneic T-cells as BM-derived DC. CD34+ cell-derived DC are also capable of processing and presenting soluble Ag to autologous T-cells for both primary and secondary immune responses. The potential clinical usefulness of autologous serum in place of FCS was also confirmed in the same study. Of note, IL-4 was shown to be capable of modulating DC differentiation from bipotent CD34+ cells during the later stages of the culture as previously demonstrated for monocyte-derived DC. Thus, mobilized CD34+ cells may represent the optimal source for the generation of DC for cancer immunotherapy rather than BM precursors. Very recent data indicate the mobilization of large numbers of DC precursors by GM-CSF and FLT-3L. However, it remains to be

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Figure 3. Functional properties of dendritic cells at different stages of differentiation. Pathogens or inflammatory cytokines induce the maturation of dendritic cells which become activated upon interaction with T-cells via CD40-CD40L.
established whether circulating CD34+ elements are an equivalent source of DC to CD14+ monocytes. In this view, it has recently been demonstrated that CD34+ cell-derived DC are more efficient than monocyte-derived DC, from the same patients, in stimulating a specific CTL response to Melan-A/Mart-1 peptides.

**Delivery of TAA to DC**

Several methods for the efficient delivery of TAA to DC have been described so far (Figure 4). Their rationale is based on the finding that tumor cells are often poorly immunogenic due to the lack of T-cell recognition, activation and co-stimulation typical of professional APC. To this end, Gong et al. fused murine DC with the carcinoma cell line MC38 to provide tumor cells with the functional characteristics of DC. The fusion cells showed all the phenotypic features of DC and were shown to be capable of preventing tumor growth when the mice were challenged with the cell line. Moreover, treatment with fusion cells induced the rejection of pulmonary metastases.

Several TA peptides which are presented to T-cells in association with HLA class I molecules have been recently identified and proved to be useful in stimulating an autologous CTL response in vitro and in vivo. However, pulsing DC with peptides may not be optimal for clinical application because of the strict MHC restriction of the immune response and their limited stability. In addition, pulsing with peptides may not induce a T-cell response directed toward tumor cells expressing the relevant Ag. Although DC can be loaded with a cocktail of peptides from different Ag derived from the same type of cancer (see below), this vaccination approach is likely to limit patient selection on the basis of HLA phenotype. An attractive alternative is the use of unfractionated tumor-derived proteins, when available (see below), apoptotic cells or tumor lysates. In the last case the obvious disadvantage is the possibility of inducing immune responses against self-Ag expressed in tissues other than tumor cells.

A further possibility is the transduction of DC with expression vectors encoding for TAA genes (Figure 4). DC can be engineered by different means which differ in their capacity of targeting quiescent cells, stable integration in the genome, infection efficiency and stimulation of anti-tumor immunity (Figure 5). Retrovirally-transduced DC constitutively express the relevant sequence and are potent stimulators of a specific T-cell response. However, retroviral vectors have a relatively low efficiency of transduction, they can only infect actively replicating cells and carry the theoretical risk of oncogenic transformation of target cells. Conversely, adenoviruses infect both quiescent and proliferating cells and do not integrate into DNA. Moreover, supernatants with a high titer of the virus can be easily obtained. Recently, DC have been transduced with an adenovirus combined with cationic liposomes showing an infection efficiency close to 100%. The major limitation to the clinical use of adenoviruses is their high immunogenicity which induces the production of neutralizing antibodies and the rapid development of CTL directed at infected cells.

Vaccinia virus vectors are not oncogenic, do not integrate into genome and can be manipulated to carry large fragments of heterologous DNA. However, these viruses are toxic for target cells and the viability of DC is approximately 50%. Nonetheless, antigen-specific inhibition of tumor growth has been observed in murine models using vaccinia vectors encoding for CEA and Mucin-1. Two phase I clinical trials have been conducted to assess the safety of vaccinia virus vectors engineered to express HPV and CEA genes and to assess their capacity of stimulating an immune response. More recently, maturation of DC with neo-biosynthesis, translocation and stabilization of MHC molecules on the cell surface and efficient induction of both CD4 and CD8 T-cell activation has been induced by infection with bacterial vectors. As a result, a model Ag (ovalbumin) expressed on the surface of recombinant Streptococcus
Gordonii, is processed and presented on MHC class I molecules 10^6 times more efficiently than soluble OVA protein. Therefore, bacterial vectors are potentially useful means of delivering exogenous Ag to DC for stimulating a tumor-specific CTL response. A different approach has been taken by Boczkowsky et al.\textsuperscript{241} who transfected DC with the total RNA extracted from tumor cells and combined it with cationic lipid to enhance the infection efficiency. Similarly to the use of tumor lysates, this strategy can be applied in those situations in which a tumor-specific antigenic marker is lacking; the major concern is the increased risk of autoimmune reactivity.

**DC for cellular immunotherapy**

The central role of DC in stimulating a tumor-specific immune response is well established \textit{in vitro} and \textit{in vivo} in animal models.\textsuperscript{232,241-246} Whereas murine DC pulsed with TA-proteins or peptides or transduced with TAA genes have induced both the rejection of challenge tumor cells and the regression of established cancers, it remains to be determined which of the several strategies proposed for cellular immunotherapy is the most efficient. It may well be that different tumors require different approaches.

In humans, initial studies were performed in patients with melanoma using DC pulsed with MAGE peptide.\textsuperscript{247,248} The infusion of loaded DC induced the migration of MAGE-specific CTL to the site of injection and increased the frequency of circulating tumor-specific CTL. More recently, Nestle \textit{et al.}\textsuperscript{249} have treated advance stage melanoma patients with intranodal injection of peptides or tumor lysates-pulsed DC according to the HLA profile of the patient. The authors reported the stimulation of a peptide-specific T-cell response in all cases. Moreover, in 5/16 patients an objective clinical response was observed. In this study, DC were generated \textit{ex vivo} from monocyte precursors in the presence of IL-4 and GM-CSF and directly injected into an inguinal lymph node to reach T-cell rich areas.

Tumor-specific peptides (fragments of prostate specific antigen, PSA) have also been used to pulse autologous DC in prostate cancer patients refractory to hormone-therapy.\textsuperscript{250} Seven out of 51 patients showed a partial response while none of the patients in the control group, injected with peptides alone, showed any clinical benefit. In B-cell malignancies, the patient-specific idioype (Id) gene sequence and its protein product represent the optimal targets for vaccination strategies as previously shown in murine models\textsuperscript{251,252} and humans.\textsuperscript{253} Hsu \textit{et al.}\textsuperscript{254} have reported on the treatment of 4 patients with low-grade non-Hodgkin's lymphoma (NHL), resistant to conventional chemotherapy or who had relapsed, with DC pulsed with the Id as soluble antigen. A tumor-specific T-cell-response was observed in all cases coupled, in one case, with the regression of tumor burden. At the time of writing, 16 patients have been treated and a tumor-specific cellular response has been found in 8 individuals (R. Levy, personal communication). The same strategy of targeting the Id has been proposed by the same group for inducing a T-cell immune response in multiple myeloma patients.\textsuperscript{255}

In contrast to the strategy used by Nestle \textit{et al.}\textsuperscript{249} in this preliminary trial DC were freshly isolated from the PB by subsequent enrichment steps and were reinfused intravenously. Although a much larger number of DC were injected in NHL patients compared to melanoma patients (3-20×10^6 DC vs. 1×10^6), this approach raises concerns about both the efficacy of uncultured PB DC of efficiently
stimulating T-cells and the capacity of Ld-loaded APC to reach secondary lymphoid organs to prime T-cells, escaping the entrapment of the pulmonary apparatus.

**Future directions**

The few clinical data available so far have barely provided the proof of principle that autologous DC generated ex vivo and reinfused into cancer patients are effective in stimulating an anti-tumor immune response. This is the result of the complexity of the interplay between different cellular populations involved in tumor immunity. In addition, cellular immunotherapy with DC has yet to be standardized. As mentioned above, crucial issues such as 1) the choice of the most suitable TAA to stimulate an immune response; 2) the use of soluble proteins-peptides or DC engineered with expression vectors; 3) the optimal source for the generation of DC and the number of APC needed to promote a clinical effect; and 4) the most effective route of administration of DC, are points which still need to be solved. At this stage, relying for the most part on animal studies, we can only conclude that DC-based immunotherapy holds promises of exerting a potent anti-tumor effect in humans.

**Oral vaccination by in vivo targeting of DC**

A simple approach to targeting APC in vivo is to use attenuated bacterial vectors, such as those commonly developed to control infectious diseases. They usually enter the host through the oral route and selectively replicate within macrophages and DC. *Listeria monocytogenes* is a promising vaccine carrier that naturally infects APC, and may deliver immunogens to both MHC-I and II pathways of antigen processing and presentation. Furthermore, this bacterium may constitute a per se excellent danger signal for the immune system, since it stimulates the innate immune response to produce cytokines (e.g. IL-12) and mediators (e.g. nitric oxide) that enhance antigen presentation. In addition, it promotes a TH1-type cellular response, which is mainly associated with the eradication of tumors and intracellular parasites. Most of these features are also shared by *Salmonella typhimurium*-based carriers.

The ideal vaccine carrier should maintain its immunogenicity intact, being attenuated enough to allow its use in humans. However, the safety profile of a vaccine destined for human use also requires the absolute stability of the mutant phenotype, which can only be guaranteed by the generation of chromosomal deletion mutants. Furthermore, the release of recombinant microorganisms under uncontrolled conditions makes the lack of antibiotic resistance markers essential. Mutation of genes involved in bacterial spread and survival are the best targets for attenuation.

The recent progress in *Listeria* and *Salmonella* genetic manipulation and the availability of suitable *in vitro* and *in vivo* models, make these microorganisms very attractive vaccine delivery systems.

For example, attenuated *Listeria monocytogenes* carrier strains expressing the β-galactosidase (β-gal) model antigen can prevent outgrowth of an experimental tumor in BALB/c mice by inducing a specific immune response against the β-gal TAA. Similarly, a live attenuated AroA- auxotrophic mutant of *Salmonella typhimurium* (SL7207) has been used as a carrier for the pCMVββ vector that contains the β-gal gene under the control of the immediate early promoter of cytomegalo virus (CMV). After a primary immunization and three orally administered boosts at 15-day intervals, a *Salmonella*-based vaccine induced both cell-mediated and systemic humoral responses to β-gal. These experiments suggested that insertion of a plasmid containing an expression cassette into a *Salmonella*-carrier allowed DNA immunization and specific targeting of antigen expression to APC, *in vivo*, through oral immunization. To prove that the transgene was actually expressed by APC as a function of a eukaryotic promoter the green fluorescent protein (GFP) was placed under the control of either the eukaryotic CMV or a prokaryotic promoter and spleen cells from treated mice were analyzed by cytofluorometric analysis.

GFP was detectable in both macrophages and DC, but not in other splenocytes, of mice treated with *Salmonella* containing the CMV-plasmid, 28 days after the first vaccine administration, whereas it was undetectable in spleen cells of mice receiving the *Salmonella* containing the constitutive prokaryotic promoter which directs GFP synthesis only within the carrier. GFP expression in DC highlights the possibility of loading DC without the need for ex vivo manipulations and opens up the possibility of administering a cancer vaccine orally. Oral vaccination is viewed as an easier and more acceptable strategy for patients especially in a phase in which they are disease-free.

**Leukemic cells as antigen presenting cells**

Tumors may escape immune detection and killing through a variety of mechanisms affecting the capacity of either presenting tumor antigens or fully activating T-cells. In particular, tumor cells are likely to prevent a clinically evident cytotoxic T-cell response because of the absence of a specific antigenic tumor peptide, or because they lack HLA molecules, or co-stimulatory molecules on their surface. In this last case the patient's T-cells might become anergic and tolerate tumor cells. Alternatively, neoplastic antigens may induce a clonal deletion of thymocytes, or tumor cells expressing Fas molecule may be responsible for an apoptotic T-cell deletion through Fas:FasL interaction. So far, different immunologic strategies aimed at overcoming these defects by inducing or improving the antigen presenting function of tumor cells have been demonstrated in experimental models, and the hypothesis that leukemic cells may become efficient APC by changing their phenotype or by differentiating into DC-like cells has been tested. A first example was shown in B-cell neoplasms since it is well known that normal B-cells may present antigen to T-cells and that cognate interactions between B- and T-cells may induce either a T-cell prolif-
Dendritic cells can be generated from CML marrow progenitors in the presence of GM-CSF, TNF-α and IL-4, and after 7-10 days of expansion, they develop DC phenotypic and functional characteristics. The authors hypothesize that at the time of initial transformation, clonogenic pre-B acute leukemia cells may not express CD86 but induce a T-cell anergy that could not be reversed by following expression of CD86 on a blast cell fraction; second, they suggest that marrow microenvironment may play a role in modulating T-cell immunity by secreting negative regulators, as previously shown in experimental models. However, after co-stimulation by either B7 transfectants or professional APC, autologous antileukemic cytotoxic marrow T cells can be generated upon contact with CD40-stimulated pre-B acute leukemia cells.

All these data on B-cell neoplasms strongly suggest that poor tumor immunogenicity may depend on both the quality and the quantity of accessory molecules required for T-cell stimulation. However, future therapeutic strategies aimed at stimulating the CD40 receptor, or at directly transducing B7 molecules on chronic or acute leukemia B-cells will facilitate the ex vivo expansion of specific anti-tumor cytotoxic T-cells. Normal myeloid CD34+ progenitors include a small subset of APC that are committed precursors of the macrophage/dendritic lineage. In fact, both marrow and peripheral blood CD34+ cells, and circulating monocytes can be utilized to obtain large numbers of dendritic cells in vitro. Due to the relevance of co-stimulatory molecules on tumor cells for the generation of anti-tumor immune responses, the hypothesis of whether even acute or chronic myelogenous leukemic cells might differentiate into dendritic cells in vitro and become immunogenic has been addressed by several groups. Alternatively, transduction of co-stimulatory molecules on leukemic myeloblasts has been attempted in experimental models to generate specific cytotoxic responses. Both these approaches require that TAA are expressed and exposed on HLA molecules, and it is likely that genetic alterations, such as chromosomal translocations, might result in the appearance of pathologic peptides, specific for each acute or chronic leukemia and potentially immunogenic. Chronic myelogenous leukemia may represent an optimal candidate for antitumor vaccine strategies since several reports have shown that the bcr-abl fusion protein can bind to defined HLA class I and class II molecules and also that dendritic cells generated in vitro from CML patients still carry the t(9;22). In this latter study, in fact, CML cells that were incubated with GM-CSF, IL-4 and TNF-α developed DC phenotypic and functional characteristics inducing autologous cytotoxic T-cells capable of directly lysing leukemic cells and of inhibiting CML colony growth in vitro. Further studies suggested that CML DC-stimulated anti-leukemic T-cell reactivity is due to an oligoclonal T-cell response and develops in an HLA-restricted manner. Dendritic cells can be generated even from CD34+ CML marrow progenitors in the presence of GM-CSF, TNF-α and IL-4, and after 7-10 days of culture they are Ph+, express high levels of HLA molecules and co-stimulatory receptors and induce a T-cell proliferation 10-30 fold higher than unprocessed marrow cells. Nonetheless, it is likely that different culture systems may be required for efficient in vitro generation of DC when using CML-CD34+ cells rather than normal progenitors, since the former show a lower DC clonogenic activity but both their expansion and their differentiation can be significantly improved by prolonging the duration of culture in the presence of specific growth factors.

When a neoplastic event affects undifferentiated or more mature progenitors of the granulocytic and/or macrophage lineage an AML develops, and we can distinguish different subtypes of AML on the basis of morphologic and phenotypic characteristics. The identification of AML cells with some phenotypic affinities to DC, such as the expression of the CD1a marker, or deriving from a monocytic/dendritic cell progenitor, has been attempted in the past. Indeed in this latter study, cells from an AML, FAB M2 patient were shown to differentiate into terminal DC with potent alloantigen presenting capacity after in vitro culture with GM-CSF, TNF-α, SCF and IL-6. Similar results were achieved by culturing freshly isolated AML cells with GM-CSF, IL-4 and IL-13 for 7 days. Alternatively, restoration of anti-tumor immune control can be attempted by identifying peptides, such as...
PR-1 derived from proteinase 3,\textsuperscript{135} that could be capable of inducing HLA-restricted cytotoxic T-lymphocytes to lyse fresh leukemic cells, or by engineering leukemic cells to induce either the expression of co-stimulatory molecules or the production of cytokines. The role of B7-1 in developing protective immunity was initially tested in a mouse model in which the injection of a myeloid cell line transfected with the bcr/abl gene was rapidly lethal, while prolonged survival was observed only in mice that received the cell line co-transfected with the B7-1 gene.\textsuperscript{295} Moreover, the same model was used to test the role of both B7-1 and B7-2, suggesting that B7-1 may be more effective than B7-2 in obtaining an efficient \textit{in vivo} anti-leukemic response.\textsuperscript{296} The potential advantage of B7-transduced blasts was confirmed by using primary AML cells instead of a cell line; a CD8\textsuperscript{+} T-cell dependent and B7:CD28-mediated anti-leukemia activity was documented.\textsuperscript{297} A recent study compared the \textit{in vitro} immunogenic activity of human AML cells cultured with GM-CSF, IL-4 and TNF-\textalpha, or transfected with CD80.\textsuperscript{298} Both these approaches resulted in an enhanced T-cell response in a mismatched primary MLR, however, only B7-1 transduced AML cells stimulated a strong immune response of T-cells from an HLA identical bone marrow donor, and generated leukemia reactive CD4\textsuperscript{+} T-cell lines and clones. Interestingly, this model allowed the authors to observe CD80\textsuperscript{+} AML-mediated T-cell responses that can be directed against the patient’s minor histocompatibility antigens or tumor-specific antigens.

Although B7-1 and B7-2-engineered tumor cells could play a pivotal role in anti-leukemia immunotherapy strategies, there is evidence that transduction of other receptors\textsuperscript{299} or cytokines\textsuperscript{300-303} might, at least, co-operate with B7 molecules in the antigen-presenting capacity of neoplastic cells.

### Genetically modified cells as vaccine for the active immunotherapy of cancer

Non-specific approaches to cancer immunotherapy probably date back to the beginning of the 18\textsuperscript{th} century and originated from the observation of sporadic, spontaneous remission of tumors in patients who suffered severe bacterial infection. This observation prompted Dr. William B. Coley to begin, in 1891, to treat patients with soft tissue sarcoma with a mixture of Gram positive and negative bacteria: Coley’s toxins.

This empirical approach was enforced by Shear’s discovery that endotoxins were active components responsible for tumor hemorrhagic necrosis. Furthermore, the finding that bacillus Calmette-Guérin (BCG) increased resistance to tumor transplants in mice led to clinical application of BCG which, together with \textit{Streptococcus} derived OK-432, is a strategy used to this day.

The anti-tumor effects obtained by treatment with BCG and derivatives are largely dependent on indiscriminate necrosis of tissues containing mycobacterium (the Koch phenomenon). The discovery of cytokines explained most of the phenomena induced by microbial products and cytokines were then used with the initial hope of copying the positive effects of such bacterial products while avoid-

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**Figure 6.**

**Active immunization for cancer:** strategies are based on whether tumor antigens have been molecularly defined.
ing the negative ones.

More recently, the discovery of Th1 and Th2 distinct pathways of T-cell maturation helped to explain protective and non-protective BCG-induced cell-mediated immune reactions in tuberculosis, phenomena that have correlates with protection against cancer. In the presence of a Th1 deflected immune response, the effect of TNF-α is not that of large necrosis which is, rather, the characteristic of inflamed tissues of a Th2 type of response, in this case extremely sensitive to TNF-α.  

Cytokines deflecting the immune response to a Th1 or Th2 type of response may drive the type of immune response to cancer cells, escaping the simple definition of Th1 promoting and Th2 inhibiting anti-tumor immunity. Rather, a strong Th1 as well as a strong Th2 response may induce tumor destruction and immune memory with the same efficacy although through different mechanisms (see below). Moreover, genetic background may influence the ability to mount a Th1 or Th2 response, as shown in murine models.

Microbial products have mainly local effects which may be reproduced and improved by local injection of recombinant cytokines. Experiments in non-tumor systems have shown that IL-2 offsets antigen recognition and overcomes tolerance. Thus cytokines could be used not only to stimulate tumor destruction but also to impair tolerance and activate effective and specific immune recognition of TAA.

Identification and cloning of the long elusive TAA, especially from human melanomas, pointed tumor immunotherapy to a general systemic response and, thus, the use of cytokines shifted from that of being responsible for local tumor debulking to that of being an aid to triggering and boosting the immune response to TAA.

In addition to antigens triggering the T-cell-receptor (TCR) of T-lymphocytes, optimal T-cell response also requires co-stimulatory molecules, as detailed above.

Cytokines, co-stimulatory molecules and several cloned TAA are now available: how can we use them to provide an effective immunotherapeutic approach to cancer patients?

Two major strategies are envisaged (see Figure 6): one, already described, takes advantage of antigen availability in the forms of genes, proteins or peptides and of the standardized methods of obtaining DC from peripheral blood in large quantities to be loaded with the antigen and reinfused in vivo; the other strategy still considers the tumor cells representative of the entire antigenic repertoire of a certain neoplasia; such cells, genetically modified to produce cytokines and/or co-stimulatory genes, could be injected into patients as a cellular vaccine. In the latter case a pool of cell lines derived from different patients with the same type of tumor could increase the antigenic repertoire and avoid immunoselection that certain antigens may have encountered in some patients. Unmatched MHA are not a problem in terms of antigen presentation since injected cells are destroyed and represented by host APC. Moreover if different sets of alloantigens are selected from different pools, the risk of repeated alloimmunization during booster vaccination would probably be avoided. The background and prospectives of genetically modified tumor cell vaccines are presented below.

**Cytokines at the tumor site**

In initial studies recombinant cytokines were injected at the tumor site or cytokine genes were inserted into somatic cells to be injected at the tumor site. All these studies collectively established that most of the cytokines accumulated at the tumor site were able to induce tumor destruction and the reaction they induced was sometimes strong enough to eradicate a tumor antigenically unrelated to the cytokine-releasing cells. The obtained tumor debulking was often followed by a systemic tumor-specific immune memory. It should be underlined, however, that tumor debulking may occur through non-specific immune reactions or so fast as to prevent efficient T-cell priming, this being reminiscent of the dichotomy described for BCG: indiscriminate necrosis versus protective immunity.

**Engineered tumor cell vaccines**

Engineering of tumor cells with the gene of a particular cytokine is an efficient way of ensuring that this cytokine will be durably present at the tumor site. Repeated local injections would, of course, have the same effect. Bolus administration, however, does not provide a constant supply of cytokine. Its effects are much less evident than those achieved by the injection of engineered tumor cells that can ensure the provision of antigen and continued local accumulation of the cytokine until a physiologic or a pharmacologic threshold is reached, and the biological activity of the cytokine can begin.

The immunogenicity that tumor cells can acquire upon cytokine-gene transduction may stem from recruitment by released cytokines, of particular repertoires of inflammatory cells, whose differing abilities to influence TAA presentation and secrete secondary cytokines may shape both immunogenicity and deflection of the ensuing immune memory towards a Th1 or Th2 type of response. A cytokine may be simultaneously involved in tumor rejection, leukocyte recruitment and activation of memory mechanisms.

Many experimental studies have been performed in mice over the last seven years and cytokine genes from IL-1 to IL-18 have been tested. Most of those studies described whether a certain cytokine gene, upon transduction, can inhibit tumor growth in vivo; some also described whether the cytokine induced protective immunization against challenge by parental cells whereas only a few studies described efficacy in a therapeutic setting. It is clear that the way cytokines modify tumor oncogenicity, immunogenicity and curative effect is not only dependent on the cytokine employed but also on the tumor model utilized. The immune mechanisms responsible for inhibition of tumor growth may not be the same as those required for immune memory or those necessary for eradication of an established tumor.
Translation of animal studies into a clinical setting faces a substantial difference, that is the fast growth of transplanted tumors and therefore the short time window in which immunization can be performed before the animal's death. In murine models, the so-called *established tumor* is a tumor that has been injected one to three days before the beginning of vaccination. This contrasts with phase I/II clinical studies in which enrolled patients have advanced disease. Clear evidence of therapeutic effects is not expected in these patients, therefore tumors with antigens whose genes have been cloned and are recognized by CTL should be used to allow, at least, an immunologic follow-up that could prove the effect of vaccination. This confines the choice to those carrying the MAGE, GAGE and BAGE family genes and to melanomas, which also express antigens of the melanocyte lineage, such as tyrosinase, gp100 and MART-1/Melan-A. The choice is further restricted by the difficulty of obtaining cells and cell lines from tumors that are not melanomas to be transduced and then employed for immunologic evaluation. Melanoma is thus the tumor most frequently chosen for vaccination studies.

Nevertheless, vaccination with cytokine-transduced, freshly isolated cells, which should retain the tumor-antigen repertoire, could be a way of generating tumor-specific T-lymphocyte lines and clones with which to identify antigens expressed by tumors other than melanomas.

In a few cases only, the antigens associated with the murine tumors employed in pre-clinical studies were characterized; the majority of studies designed to discover the immunologic mechanisms associated with tumor rejection utilized proteins not classifiable as tumor-associated antigens, such as β-galactosidase and influenza nucleoprotein. Most of these animal studies were carried out in the syngeneic system, that in humans corresponds to the autologous situation, in which a tumor cell line was both the cell vaccine and the tumor to be cured. Autologous application is actually difficult, since it requires tumor cell cultures from every patient for both gene transduction and immunologic follow-up. Each patient's cell vaccine should then be checked for safety, and a great variability in terms of cytokine production other than adhesion molecules and antigenic phenotypes may exist between cell vaccines. The use of allogeneic cell lines, on the other hand, has the advantage of employing vaccines well-characterized in terms of tumor antigen, MHC and adhesion molecules, as well as the constant amount of cytokine released; these parameters in combination may provide a standard reagent for clinical studies.

Both syngeneic and allogeneic tumor cells expressing a common TAA are processed by host APC such that TAA derived peptides are presented in association with host MHC in either case. Nevertheless, in most clinical protocols the expression of the MHC class I allele, which presents TAA derived peptide(s), on the immunizing tumor cells is preferred. If cross-priming occurs efficiently, this should not be necessary, but it is still unclear whether vaccination with transduced tumor cells actually primes the host or boosts already present activated T-lymphocytes. This observation indicates that co-stimulatory molecules, such as B7, in addition to cytokines may be transduced in cell vaccines in order to amplify the boosting effect, since is not clear whether B7 transduced cells prime the host directly.

Clinical vaccination protocols using IL-2 or IL-4 gene-transduced allogeneic melanoma cells have been performed at the Istituto Nazionale Tumori in Milan, Italy. An HLA-A2 melanoma cell line expressing Melan-A/MART-1, tyrosinase, gp100 and MAGE-3 has been transduced and irradiated before the treatment of advanced HLA-A2+ melanoma patients. In the first protocol, patients were injected subcutaneously on days 1, 13, and 26 with IL-2 gene-transduced and irradiated melanoma cells at doses of 5 (3 patients) and 15 (4 patients) × 10⁸ cells. Mixed lymphocyte-tumor cultures (MLTC) and limiting dilution analyses were performed to compare pre- and post-vaccination PBL. While MLTC revealed an increased but MHC-unrestricted cytotoxicity, in two cases the frequencies of melanoma-specific CTL precursors were clearly augmented by vaccination. In one patient, HLA class II-restricted effectors were found to be involved in the recognition of autologous tumor. Which antigen(s) was involved in the recognition by PBL of vaccinated patients remains unclear. In 3 out of 5 cases studied, pre- and post-vaccination PBL could not recognize any melanoma peptide tested or known to be restricted by HLA-A2 allele. Among other possible explanations, this might be due to a tumor associated antigenic repertoire that exceeds the limited number of antigens whose genes have been cloned so far.

This indicates that vaccination with cell lines is advantageous because the cell lines stimulate the host with the entire repertoire of known and unknown antigens. In the allogeneic system it is then easy to rotate the transduced cell line within the protocols and so maximize the chances that a relevant tumor antigen is present in the vaccine. Some antigens, in fact, may be negatively selected and lost in one patient-derived line, but not in others. In addition, selection of allogeneic cell lines displaying various MHC reduces the interference of repeated boosting with strong alloantigens. Indeed vaccination with a pool of three melanoma cell lines commenced before the cloning of known melanoma associated antigens, resulted in increased survival correlated with the level of antibody against the GM2 ganglioside, indicating possible involvement of a humoral response; correlation with the CTL response was not investigated.

Going back to animal studies in which vaccination therapy with cytokine-transduced tumor cells was successful, it should be underlined that it was not clear which of the measured immune responses was responsible for the therapeutic effect since, generally, induction of cytotoxic T-lymphocytes was, per se, insufficient to produce a cure. In keeping with this statement, vaccination of 13 evaluable patients with MAGE-3.A1 peptide resulted in 3 clinical regressions, although no CTL
precursors were found in the PBL of these responders.\textsuperscript{310} Refined animal studies performed to identify which immune responses correlate with the therapeutic activity indicated that both T- and B-cells should be properly activated.\textsuperscript{311, 312}

These observations may suggest that while a patient could be immunized against a tumor, the immunity thus induced might be insufficient to fight the established tumor growing within its own stroma. The combination of poor immune function and large tumor burden makes patients with advanced disease dubious predictors of clinical response.

The general idea is that cytokine engineered tumor cells should be used as vaccines in minimal disease settings.\textsuperscript{313} A new form of treatment would thus be available for combination with conventional management of patients after surgical removal of their tumor, patients with minimal residual disease, or patients expected to manifest tumor recurrence after a significantly apparent disease-free interval. When compared with conventional forms of management, vaccination is a soft, non-invasive treatment, unlikely to cause particular distress or side-effects, and could be administered after resection of a primary tumor when recurrence is expected.

Use of mesenchymal cells for treatment of neoplastic and non-neoplastic disorders

In addition to hematopoietic stem cells which can differentiate to produce progenitors committed to terminal maturation,\textsuperscript{314} human bone marrow also contains stem cells of non-hematopoietic tissues which are currently referred to as mesenchymal stem cells (MSC), because of their ability to differentiate into cells that can roughly be defined as mesenchymal, or as marrow stromal cells because they appear to arise from the complex array of supporting structures found in marrow.\textsuperscript{315} Stromal cells of the narrow microenvironment include fibroblasts, endothelial cells, reticular cells, adipocytes, osteoblasts and macrophages, the last, although of hematopoietic origin, being considered functional components of the regulatory stroma.\textsuperscript{316} The heterogeneous populations of mesenchymal cells and their associated biosynthetic products have the unique capacity to regulate hematopoiesis.\textsuperscript{317}

Environmental components can modify the proliferative and differentiative behavior of hematopoietic cells by means of (i) cell-to-cell interactions, (ii) interactions of cells with extracellular matrix molecules, and (iii) interactions of cells with soluble growth regulatory molecules.\textsuperscript{318} All these regulatory modalities participate in stromal cell-mediated regulation of hematopoiesis. In fact, marrow stromal cells provide the physical framework within which hematopoiesis occurs, play a role in directing the processes by synthesizing, sequestering or presenting growth-stimulatory and growth-inhibitory factors, and also produce numerous extracellular matrix proteins and express a broad repertoire of adhesion molecules that serve to mediate specific interactions with hematopoietic stem/progenitor cells of both myeloid and lymphoid origin.\textsuperscript{319} Although growth factors play key roles in stem/progenitor cell proliferation and differentiation it seems improbable that hematopoiesis is regulated only by a random mix of growth factors and responsive cells. Rather, it is likely that regulatory molecules and localization phenomena within marrow stroma are required to sustain and regulate the function of the hematopoietic system.\textsuperscript{320}

Although it is commonly accepted that stem cells are capable of homing to the marrow and docking at specific sites, the exact role of microenvironmental cells, adhesion molecules and extracellular matrix molecules in regulating the localization and spatial organization of hematopoietic stem cells in the marrow and driving myeloid and lymphoid regeneration following stem cell transplantation remains a matter of hypothesis.\textsuperscript{321} Studies in animals demonstrated that stem and progenitor cells have different distributions across the femoral marrow cavity of mice, thus suggesting that marrow stroma is organized into functionally discrete environments, such as primary microenvironmental and secondary microenvironmental areas, allowing distinct differentiation patterns of hematopoietic stem cells.\textsuperscript{322} The stem cell niche hypothesis, proposed by Schofield\textsuperscript{323} suggested that certain microenvironmental cells of the marrow stroma could maintain the stem cells in a primitive, quiescent state. Another mechanism supporting the concept of specialized microenvironmental areas is stroma-mediated, compartmentalized growth factor production. Growth factor produced locally by stromal cells may bind to the extracellular matrix and be presented to immobilized target cells which recognize each growth factor through specific receptors.\textsuperscript{320} This mechanism may provide the opportunity for localizing distinct growth factors at relatively high concentrations to discrete sites. As yet, relatively little is known of the nature of the factor(s) produced by different stromal cell types which modulate lineage development. However, a growing body of evidence suggests that marrow stroma is involved not only in regulating myeloid cell growth, but also in T- and B-cell lymphopoietic development.\textsuperscript{324-327} Distinct adhesion molecules and cytokines are known to regulate stroma-dependent T- and B-lymphopoiesis,\textsuperscript{328} suggesting that marrow stroma may function as a site of T- as well as B-cell lymphopoiesis.

The existence of self-renewing MSC is supported by several in vitro and in vivo data.\textsuperscript{330} At the functional level, MSC residing within marrow microenvironment, establish marrow stroma both in vitro and in vivo and have multilineage differentiation capacity, being capable of generating progenitors with restricted development potential which include fibroblast, osteoblast, adipocyte, chondrocyte and myoblast progenitors (Figure 7).\textsuperscript{331-333} Putative stromal cell progenitors have been identified in human marrow by their ability to generate colonies of fibroblast-like cells originating from single clonogenic progenitors termed fibroblast colony-forming units (CFU-F).\textsuperscript{334} These progenitors, which belong to the osteogenic
stromal lineage, play a central role in establishing the marrow microenvironment both in vitro and in vivo.\textsuperscript{335-337} Under appropriate culture conditions and supplementation with specific stimuli, a proportion of marrow CFU-F can be induced to either adipogenesis\textsuperscript{333} or osteoblastogenesis.\textsuperscript{338} Studies involving ectopic transplantation of individual fibroblastic clones grown in vitro from mouse marrow beneath the renal capsule of syngeneic hosts demonstrated that approximately 15% produced a marrow organ containing the full spectrum of stromal cell types of hematopoietic microenvironment, thus suggesting that CFU-F have multilineage differentiation capacity and supporting the stromal stem cell hypothesis.\textsuperscript{339} Based on these findings, CFU-F can be identified as multipotent stromal progenitors rather than lineage-restricted fibroblast progenitors.

CFU-F can be enriched from adult bone marrow by means of the STRO-1 monoclonal antibody that identifies essentially all assayable marrow CFU-F.\textsuperscript{340} STRO-1\textsuperscript{+} cells do not express the CD34 antigen and fail to generate hematopoietic progenitors, thus facilitating a clean separation between hematopoietic and stromal progenitors.\textsuperscript{341} Flow-sorted STRO-1\textsuperscript{+} cells grown under long-term culture conditions generate adherent stromal layers consisting of fibroblasts, osteoblasts, smooth muscle cells and adipocytes.\textsuperscript{340} These stromal layers are capable of supporting hematopoiesis in long-term cultures initiated with CD34\textsuperscript{+} cells. In addition to STRO-1, other monoclonal antibodies, such as SH-2, have been described which specifically detect mesenchymal progenitors.\textsuperscript{342}

\textit{In vivo} data generated in animal models support the functional regulatory role of the marrow microenvironment. In the fetal sheep model of \textit{in utero} stem cell transplantation, co-transplantation of stem cells with marrow stromal cells has been shown to improve levels of donor cell engraftment.\textsuperscript{343} In the NOD/SCID mouse model of \textit{in utero} stem cell transplantation, fetal stem cells have a nine times greater engraftment potential but this advantage is abrogated if the recipients are irradiated prior to transplant, indicating that the marrow microenvironment is important in driving myeloid and lymphoid engraftment.\textsuperscript{344}

The importance of stromal cells in hematopoiesis has also been demonstrated by several studies in humans. Despite normal peripheral blood counts, levels of primitive and committed progenitors in the bone marrow of patients who have received allogeneic stem cell transplantation remain subnormal for many years.\textsuperscript{345} Furthermore, cultured stromal cells from patients who have received allogeneic stem cell transplant (SCT) show significant impairment in their ability to support the growth of hematopoietic progenitors from normal marrow.\textsuperscript{346} Decreased CFU-GM production and defective stroma production have been demonstrated following autologous SCT\textsuperscript{347} as well as after induction chemotherapy.\textsuperscript{348}

The role of marrow stroma in hematopoietic regulation and the peculiar functional characteristics of stromal cells raise the possibility that the delivery of \textit{ex vivo} expanded marrow MSC into a hematopoietically-compromised marrow might promote hematopoiesis. Bone marrow stromal cells are a quiescent, non-cycling population with low cell turn-over, as demonstrated by the resistance to irradiation. Based on these characteristics, methods have been developed which allow for gene delivery into stromal cells.\textsuperscript{349} Since stromal cells are metabolically active they also provide a suitable means of secreting therapeautic proteins, including coagulation factors or adenosine deaminase.\textsuperscript{350} Recent data showing that MSC suppress allogeneic T-cell responses \textit{in vitro} suggest a role for stromal cells in modulating allogeneic
transplant rejection and graft-versus-host disease.\textsuperscript{351}

It must be emphasized that because of the limited knowledge of MSC biology, clinical applications of stromal cells, although exciting, essentially remain a matter of hypothesis to be carefully tested in the appropriate clinical setting. Essential prerequisites for clinical applications using culture-expanded mesenchymal cells as a supplement for hematopoietic SCT are (i) the possibility of isolating mesenchymal progenitors and manipulating their growth under defined \textit{in vitro} culture conditions\textsuperscript{352} and (ii) the demonstration of the possibility of efficiently introducing cultured stromal cells back into patients.

Studies in rodents and dogs have clearly demonstrated that if sufficient stromal cells are reinfused, they not only seed the bone marrow but also enhance hematopoietic recovery.\textsuperscript{353-357} Although demonstrated in several mouse models, the transplantability of marrow stromal elements remains a controversial issue in humans.\textsuperscript{358, 359} The majority of data so far generated in recipients of HLA-identical marrow transplants has failed to demonstrate any contribution of donor cells to marrow stroma regeneration.\textsuperscript{358} Although many factors may affect the transplantability of stromal elements, the low frequency of stromal progenitors in conventional marrow harvests may explain the failure of mesenchymal cell transplantation in humans.

Indeed, during the last decade, SCT methodology has changed substantially, particularly as a result of the increasing use of peripheral blood transplants. The existence of a circulating stromal progenitor has been demonstrated by using a NOD/SCID model and this is extremely relevant to stromal cell therapy.\textsuperscript{360} By using the X-linked human androgen receptor (HUMARA) gene and fluorescent \textit{in situ} hybridization analysis for the Y chromosome, the transplantability of stromal progenitors in a proportion of recipients of haploidentical HLA-mismatched T-cell-depleted allografts reinfused with a combination of bone marrow and mobilized peripheral blood cells has recently been demonstrated (Carlo-Stella and Tabilio, \textit{unpublished observations}, 1999). Taken together, these findings allow the hypothesis that MSC are transplantable in man provided that an adequate, but as yet unidentified, number of CFU-F is reinfused. In addition, these data allow the planning of clinical studies using culture-expanded, gene-marked mesenchymal cells in order to investigate a number of issues, including (i) dose of marrow stromal progenitors necessary to achieve a transplant; (ii) duration of post-transplant marrow stromal cell function; (iii) role of stromal cells in myeloid, B- and T-lymphoid reconstitution following SCT.

A limited number of clinical trials using \textit{ex vivo} generated MSC are currently underway. So far, the only published phase I clinical trial using MSC reported that the systemic infusion of autologous MSC appears to be well tolerated.\textsuperscript{361} MSC can be explored as vehicles for both cell therapy and gene therapy (Table 4). MSC could be used to replace marrow microenvironment damaged by high-dose chemotherapy in order to either improve hematopoietic recovery from myeloablative chemotherapy or to treat late graft failures or delayed platelet engraftment. Based on their functional characteristics, MSC are attractive vehicles for gene therapy in that they are expected not to be lost through differentiation as rapidly as hematopoietic progenitors. Examples of diseases in which stromal cell-mediated gene therapy might be appropriate include factor VIII and factor IX deficiencies and the various lysosomal storage diseases. Interestingly, compared to skin fibroblasts or leukocytes, marrow-derived mesenchymal cells produce significantly higher levels of α-iduronidase, an enzyme involved in type II mucopolysaccharidoses (Danesino and Carlo-Stella, \textit{unpublished data}). In addition, stromal cells might also be transduced with cDNA of various hematopoietic growth factors or cytokines. This approach might allow high levels of compartmentalized growth factor production and might be used (i) to stimulate hematopoiesis in patients with congenital or acquired hematopoietic defects, (ii) to improve B- and T-cell recovery following allogeneic SCT, (iii) to accelerate myeloid reconstitution in recipients of cord blood transplants.

In conclusion, MSC appear to be an attractive therapeutic tool capable of playing a role in a wide range of clinical applications in the context of both cell and gene therapy strategies. However, a number of fundamental questions about MSC still need to be resolved before they can be used for safe and effective cell and gene therapy.

\begin{table}[h]
\centering
\caption{Potential clinical applications of mesenchymal stem cells.}
\begin{tabular}{l}
\textbullet \ Replacement of chemotherapy-damaged stroma \\
\textbullet \ Enhancement of myeloid recovery following hematopoietic stem cell transplantation \\
\textbullet \ Enhancement of T- and B-cell reconstitution following allogeneic stem cell transplantation \\
\textbullet \ Compartmentalized growth factor/cytokine production \\
\textbullet \ Modulation of GvHD \\
\textbullet \ Delivery of exogenous gene products \\
\end{tabular}
\end{table}

\section*{Conclusions}

Although most of the new therapeutic approaches of cell therapy are experimental and have not yet been validated by phase III clinical trials, they appear to hold a high therapeutic potential. Separation of GVL from GvHD through generation and infusion of leukemia-specific T-cell clones or lines is one of the most intriguing and promising fields of investigations for the future. Likewise, strategies devised to improve immune reconstitution and restore specific anti-infectious functions through either induction of unresponsiveness to recipient alloantigens or removal of alloreactive donor T-cells...
might increase the applicability and success of hematopoietic stem cell transplantation. Cellular immunotherapy with DC must be standardized and several critical points, discussed in this review article must be properly addressed with specific clinical studies. Stimulation of leukemic cells via CD40 receptors and transduction of tumor cells with co-stimulatory molecules and/or cytokines may be useful in preventing tumor escape from immune surveillance. Tumor cells can be genetically modified to interact directly with dendritic cells in vivo or recombinant antigens can be delivered to dendritic cells using attenuated bacterial vectors by oral vaccination. MSC represent an attractive therapeutic tool capable of playing a role in a wide range of clinical applications in the context of both cell and gene therapy strategies.

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All authors gave substantial contributions to analysis and interpretation of literature data and drafting the article or revising it critically. CB was primarily responsible for the section on genetically engineered donor lymphocyte infusion for treatment of leukemia relapse, CCS for the section on mesenchymal cells, MPC for the sections on oral vaccination and engineered tumor vaccines; RML for the section on dendritic cells DR. FL was primarily responsible for sections on adoptive immunotherapy, AO for the sections on LAK and TIL and DR for the section on tumor escape from immune surveillance and on leukemic cells as APC. The authors are listed in alphabetical order.

Disclosures

Conflict of interest. This review article was prepared by request from Haematologica. The authors were a group of experts and representatives of two pharmaceutical companies, Amgen Italia SpA and Dompé Biotec SpA, both from Milan, Italy. This co-operation between a medical journal and pharmaceutical companies is based on the common aim of achieving optimal use of new therapeutic procedures in medical practice. In agreement with the Journal’s Conflict of Interest policy, the reader is given the following information. The preparation of this manuscript was supported by educational grants from the two companies. Dompé Biotec SpA sells G-CSF and rHuEpo in Italy, and Amgen Italia SpA has a stake in Dompé Biotec SpA.

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References


30. Pierson BA, Miller JS. CD56+bright and CD56dim natural killer cells in patients with chronic myelogenous leukemia progressively decrease in number, respond less to stimuli that recruit clonogen natural killer cells, and exhibit decreased proliferation on a per cell basis. Blood 1996; 88:2279-87.


81. Miller JS, Prosper F, McCullar V. Natural killer cells are functionally abnormal and NK cell progenitors are diminished in granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cell collections. Blood 1997; 90:3098-105.


therapy with donor peripheral blood cells and recombinant human interleukin-2 to treat leukaemia relapse after allogeneic bone marrow transplantation. Blood 1996; 87:2195-204.


264. Townsend SE, Allison JP. Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. Science 1993; 259:368-70.
285. Li Y, Hellstrom KE, Newby SA, Chen L. Costimulation by CD48 and Blc-1 induces immunity against poorly
301. Zilocchi C, Stoppacciaro A, Chiiodoni C, Parenza M, 
Terrazzini N, Colombo MP. Interferon gamma-inde
dependent rejection of interleukin 12-transduced carci
noma cells requires CD4+ T cells and granulo
302. Zitvogel L, Robbins PD, Storkus WJ, et al. Interleukin-
12 and B7.1 co-stimulation cooperate in the induc
tion of effective antitumor immunity and therapy of established tumors. Eur J Immunol 1996; 26:1335-
41.
303. Dunussi-Joannopoulos K, Dranoff G, Weinstein HJ, 
Ferrara JL, Bierer BE, Croop JM. Gene immunothera
py in murine acute myeloid leukemia: granulocyte-
macrophage colony-stimulating factor tumor cell vacci
305. Boon T, van der Bruggen P. Human tumor antigens recog
nized by T lymphocytes. J Exp Med 1996; 183: 
725-30.
induced tumor immunogenicity: from exogenous cytokines to gene therapy. J Immunother 1993; 14: 
253-7.
307. Huang YC, Columbeck P, Ahmadzadeh M, Jaffee E, 
Pardoll D, Levitsky H. Role of bone-marrow derived 
cells in presenting MHC class I-restricted tumor antige
T cell response in melanoma patients vaccinated with 
interleukin-2 gene-transduced allogeneic melanoma 
of survival in metastatic melanoma after active specific 
immunotherapy with a new polyclonal melanoma vac
310. Marchand M, Weynants P, Rankin E. Tumor regres
sion responses in melanoma patients treated with a 
63:883-5.
by IL-12-producing tumor cell vaccines but not IgG1 
induced by IL-4 vaccine are associated with the eradica
58:5812-7.
312. Rodolfo M, Zilocchi C, Cappetti B, Parmigiani G, 
Melani C, Colombo MP. Eradication of experimental 
metastases by IL-12-transduced tumor vaccine is asso-
ciated with GM-CSF secreted by tumor immunity com-
pared with B7 family and other cytokine vaccines. 
313. Bennet JH, Joyner CJ, Owen ME. Adipocyte 
cells cultured from marrow have osteogenic potential. 
314. Scott MA, Gordon MY. In search of the haemopoiet-
315. Gronthos S, Simmons PJ. The biology and applica
tion of human bone marrow stromal cell precursors. 
316. Dexter TM. Regulation of haemopoietic cell growth and 
development: experimental and clinical studies. 
317. Morrison SJ, Shah NM, Anderson DJ. Regulatory 
mechanisms in stem cell biology. Cell 1997; 88:287-
98.
318. Trentin JJ. Influence of hematopoietic organ stroma 
(hematopoietic inductive microenvironments) on stem cell differentiation. In: Gordon AS, ed. Regula
tion of hematopoiesis. vol 1. New York: Appleton, 
319. Simmons PJ, Zannettino A, Gronthos S, Levesley D. 
Potential adhesion mechanisms for localisation of 
haemopoietic progenitors to bone marrow stroma. 
320. Gordon MY, Riley GP, Watt SM, Greaves MF. Com-
partmentalization of a haemopoietic growth factor 
(GM-CSF) by glycosaminoglycans in the bone mar
321. Carlo-Stella C, Tabilio A. Stem cells and stem cell 
322. Gordon MY. Physiological mechanisms in BMT and 
haemopoiesis - revisited. Bone Marrow Transplant 
323. Schofield R. The relationship between the haemopoie-
tic stem cell and the spleen colony-forming cell: a 
324. McGinness K, Quesniaux V, Hitzler J, Paige C. Human 
B-lymphopoiesis is supported by bone marrow-
325. Landreth KS, Dorshkind K. Pre-B cell generation 
potentiated by soluble factors from a bone marrow 
326. Kierney PC, Dorshkind K. B lymphocyte precursors 
and myeloid progenitors survive in diffusion chamber 
cultures but B cell differentiation requires close asso-
327. Touw I, Lowenberg B. Production of T lymphocyte 
colony-forming units from precursors in human long-
RA. Generation of purified stromal cell cultures that 
support lymphoid and myeloid precursors. J Immunol 
Methods 1986; 89:37-47.
329. Barda-Saad M, Rozenzajn LA, Glowerson A, Zhang 
AS, Zipori D. Selective adhesion of immature thymo-
cytes to bone marrow stromal cells: relevance to T cell 
330. Prockop DJ. Marrow stromal cells as stem cells for 
331. Owen ME, Cave J, Joyner CJ. Clonal analysis in vitro of 
osteogenic differentiation of CFU-F. J Cell Sci 1987; 
87:731-9.
332. Owen ME, Friedenstein AJ. Stromal stem cells: mar-
rrow-derived osteogenic precursors. CIBA Found Symp 
333. Bennet JH, Joyner CJ, Triffitt JT, Owen ME. Adipocyte 
cells cultured from marrow have osteogenic potential. 
acterization of human bone marrow fibroblast 
colony-forming cells (CFU-F) and their progeny. 
335. Friedenstein AJ, Chaiakhian RK, Lakinsky KS. The 
development of fibroblast colonies in monolayer cul-
tures of guinea-pig bone marrow and spleen cells. Cell 
bone and cartilage by marrow stromal cells in diffu-
sion chambers in vivo. Clin Orthop 1980; 151:294-
307.
Stromal cells responsible for transferring the microen-
vironment of the haemopoietic tissues. Cloning in vit-
ro and retransplantation in vivo. Transplantation 
1974; 17:331-40.


Antitumor vaccination: where we stand

Background and Objectives. Vaccination is an effective medical procedure of preventive medicine based on the induction of a long-lasting immunologic memory characterized by mechanisms endowed with high destructive potential and specificity. In the last few years, identification of tumor-associated antigens (TAA) has prompted the development of different strategies for antitumor vaccination, aimed at inducing specific immune memory that may eliminate residual tumor cells and protect recipients from relapses. In this review characterization of TAA, different potential means of vaccination in experimental models and preliminary data from clinical trials in humans have been examined by the Working Group on Hematopoietic Cells.

Evidence and Information Sources. The method employed for preparing this review was that of informal consensus development. Members of the Working Group met four times and discussed the single points, previously assigned by the chairman, in order to achieve an agreement on different opinions and approve the final manuscript. Some of the authors of the present review have been working in the field of antitumor immunotherapy and have contributed original papers to peer-reviewed journals. In addition, the material examined in the present review includes articles and abstracts published in journals covered by the Science Citation Index and Medline.

State of the art. The cellular basis of antitumor immune memory consists in the generation and extended persistence of expanded populations of T- and B-lymphocytes that specifically recognize and react against TAA. The efficacy of the memory can be modulated by compounds, called "adjuvants", such as certain bacterial products and mineral oils, cytokines, chemokines, by monoclonal antibodies triggering co-stimulatory receptors. Strategies that have been shown in preclinical models to be efficient in protecting from tumor engraftment, or in preventing a tumor relapse, include vaccine by means of soluble proteins or peptides, recombinant viruses or bacteria as TAA vectors, DNA injection, tumor cells genetically modified to express co-stimulatory molecules and/or cytokines. The use of professional antigen-presenting cells, namely dendritic cells, either pulsed with TAA or transduced with tumor-specific genes, provides a useful alternative for inducing antitumor cytotoxic activity. Some of these approaches have been tested in phase I/II clinical trials in hematologic malignancies, such as lymphoproliferative diseases or chronic myeloid leukemia, and in solid tumors, such as melanoma, colon cancer, prostate cancer and renal cell carcinoma. Different types of vaccines, use of adjuvants, timing of vaccination as well as selection of patients eligible for this procedure are discussed in this review.

Perspectives. Experimental models demonstrate the possibility of curing cancer through the active induction of a specific immune response to TAA. However, while preclinical research has identified several possible targets and strategies for tumor vaccination the clinical scenario is far more complex for a number of possible reasons. Since experimental data suggest that vaccination is more likely to be effective on small tumor burden, such as a minimal residual disease after conventional treatments, or tumors at an early stage of disease, better selection of patients will allow more reliable clinical results to be obtained. Moreover, a poor correlation is frequently observed between the ability of TAA to induce a T-cell response in vitro and clinical responses. Controversial findings may also be due to the techniques used for monitoring the immune status. Therefore, the development of reliable assays for efficient monitoring of the state of immunization of cancer patients against TAA is an important goal that will markedly improve the progress of antitumor vaccines. Finally, given the promising results, identification of new or mutated genes involved in neoplastic events might provide the opportunity to vaccinate susceptible subjects against their foreseeable cancer in the next future.

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Key words: antitumor vaccination.
Antitumor vaccines: the meaning

As illustrated in a previous paper of this series, many strategies are being used to try to cure cancer, each one based on different theoretical and experimental grounds. Active immunotherapy strategies elicit specific or non-specific antitumor reactions by stimulating the patient’s immune system. Alternatively, lymphocytes collected from patients are stimulated in vitro and re-injected into the patient (adoptive immunotherapy).

Lastly, passive immunotherapy consists in the administration of antitumor antibodies to the patient. However, dealing with such a dramatic issue as is cancer, emotional empiricism spurs the adoption of distinct strategies or their mixing in an apprehensive pursuit of efficacy. Indeed, emotional empiricism has been and still is a deadly sin of tumor immunology. In the long run, only rational considerations lead to clinical progress.

The issue

Vaccination is an effective medical procedure characterized by being a) predominantly a maneuver of preventive medicine; b) based on the induction of a long-lasting immunologic memory that is c) characterized by mechanisms endowed with high destructive potential and specificity. It rests on an artificial encounter of a sham pathogen with the immune system. The sham pathogen elicits a strong host reaction and leaves a persistent memory of this first artificial fight. If the real pathogen enters the immunized organism it becomes the target of a much stronger and precise reaction than that put up by a non-immunized organism. If the pathogen had a small possibility of escaping the reaction of a naive immune system, very seldom would it evade a memory reaction. The cellular basis of immune memory consists in the extended persistence of expanded populations of T- and B- lymphocytes that specifically recognize and react against the pathogen. Memory lymphocytes are also experienced veterans, able to detect a pathogen promptly and fight effectively against it.

There is a large universe of sham pathogens that are used for vaccination, named antigens or immunogens. A killed or inactivated pathogen, or a non-pathogenic organism sharing critical molecules with the real thing can be a good immunogen. Memory can also be induced by a protein from the pathogen. In addition, a virus engineered with the DNA coding for a protein of the pathogen or even the mere naked DNA induces an effective memory.

The efficacy of the memory is modulated by numerous compounds, called adjuvants and danger signals. These provide additional activation signals, recruit reactive leukocytes at the immunization site and delay antigen catabolism. Bacterial products and mineral oils are typical conventional adjuvants. Cytokines and chemokines also act as adjuvants. As will be discussed in detail, their use allows the induction of selective mechanisms of immune memory.

Antitumor vaccination has a defined goal: to provoke specific recognition of tumor-associated antigens (TAA) in order to elicit a persistent immune memory. Many experimental data have shown that following immunization, the growth of tumor cells expressing the same TAA as the vaccine can be impaired. In patients, the immune memory elicited by vaccines is sometimes fast and strong enough to hamper the growth of their tumor.

A brief history

Interest in antitumor vaccination arose around 1900 when a series of microbial vaccines proved to be effective. The idea was straightforward: to apply the same intervention to tumor, «if ... it is possible to protect small laboratory animals in an easy and safe way against infectious and highly aggressive neoplastic specimens, then it will be possible to do the same for human patients». These words of 1897 by Paul Ehrlich ignited a series of studies with transplantable mouse tumors. However the underlying issue turned out to be more complex than had first been presumed. More than one century was required to elucidate its molecular and genetic features.

The first outcome was not a progress in anti-tumor vaccination, but instead the definition of a few rules of allograft rejection. Transplanted tumors were rejected by immunized host not because they expressed a particular TAA but because they were from histoincompatible mice and displayed normal allogeneic histocompatibility antigens. Later studies with syngeneic mouse strains showed the feasibility of immunizing a mouse against a subsequent tumor challenge. However, the suspicion that residual unnoticed histocompatibility differences were involved in these vaccination-rejection studies was not ruled out until experiments by George Klein in 1960. Carcinoma was induced by methylcholanthrene in syngeneic mice. The carcinoma was then surgically removed, and its cells were cultured in vitro and used to repeatedly immunize the mouse in which the tumor had arisen. Finally the immunized mouse and a few control syngeneic mice were challenged with the carcinoma cells of the original tumor maintained in vitro. While these cells gave rise to a carcinoma in control mice, they were rejected by the immunized mouse in which the carcinoma had arisen originally. This evidence of the possibility of immunizing against a lethal dose of own tumor was of seminal importance and had notable consequences. A very large series of subsequent studies established a few basic foundations of tumor vaccination.

Looking back at tumor immunology over the last 20-30 years, the importance of models in influencing immunologic beliefs is strikingly evident. Inappropriate use of an experimental model may produce wrong beliefs, from which it is then very hard to escape. Viral- and chemically-induced tumors form highly immunogenic models. Since they are easy to handle, these were used to establish the rules of tumor vaccination. However, it was disputable whether the information from these models was relevant to the situation of patients
with cancer. Using a series of murine spontaneous tumors, Hewitt concluded that it was not possible to immunize against these tumors.\(^\text{15}\) This observation had crucial importance in shaping subsequent studies. The possibility that the experimental work done with high immunogenic transplantable tumors had little relevance to human tumors was a dark shadow that hindered the progress of tumor immunology. Later, more careful use of these spontaneous tumors and more refined immunization techniques showed that Hewitt’s conclusions were wrong.\(^\text{15}\)

Boon led the genetic and molecular identification of a large series of TAA. Initially his studies were performed using conventional transplantable mouse tumors.\(^\text{16}\) Then, tumor antigens were detected on the same spontaneous tumors that had previously been classified as non-immunogenic by Hewitt.\(^\text{15}\) Now many antigens associated with human tumors have been identified.\(^\text{17,18}\)

**The targets**

Boon and others\(^\text{16,19}\) provided an unambiguous definition of TAA, an important finding that definitively laid to rest the doubts on the foundations of tumor immunology in man.\(^\text{20}\) In many cases TAA are peptides presented by class I and class II glycoproteins of the major histocompatibility complex (MHC). Things that may give rise to these tumor-associated peptides are enhanced or diminished expression of some normal proteins and the new expression of altered or normally repressed molecules. Less frequently these antigens are tumor-specific as they derive from mutated proteins. Lastly, various TAA are shared by tumors with distinct histology and origin (Table 1). Telomerase catalytic subunit looks like another widely expressed TAA recognized by T-lymphocytes. It is markedly activated in most human tumors while it is silent in normal tissues.\(^\text{21}\)

**Why?**

The central tenet of antitumor vaccination is that the immune system is able to destroy tumor cells and to retain a long-lasting memory provided that TAA are first efficiently recognized. While the studies aimed at the induction of an immune response.\(^\text{22}\) Finally, technical refinement of genetic engineering is making the development of new cancer vaccines easier. The convergence of these issues is once again placing antitumor vaccination at the cutting edge of biological research. A survey by Science\(^\text{24}\) indicates that antitumor vaccination is expected soon to become an established therapeutic option.

**When?**

Whereas individuals are immunized with microbial vaccines prior to encountering the pathogen, cancer patients have to be immunized when a tumor has been already detected. It is not yet possible to predict which combination of gene mutations will give rise to cancer. Therefore, the common clinical setting is elicitation of an immune response in a tumor-bearing patient, rather than prior to tumor development. The very concept of vaccine is somewhat distorted since it has moved from being preventive to being therapeutic.\(^\text{23}\)

The kind of patients who should be considered eligible for tumor vaccination is not a minor issue. In many trials patients with advanced diseases are enrolled both for compassionate reasons and because of the constraints imposed by ethical considerations. But, do experimental data suggest that vaccination could be effective in advanced stages of neoplastic progression? The experimental data provide an unambiguous picture of the potentials and limits of vaccination. This picture is not, however, generally taken into account. Perhaps unconscious reasons lead to experimental data being assessed with optimistic superficiality.\(^\text{25}\) Many experimental studies have shown that an antitumor response can be elicited by new vaccines. This results in strong resistance to a subsequent tumor challenge and inhibition of residual disease remaining after convention therapy. The pitfall hidden in the evaluation of these vaccine-relay experiments is that successful immunization of healthy mice against a subsequent re-challenge with tumor cells does not demonstrate a true therapeutic effect.\(^\text{26}\) Examination of more realistic studies of the ability of vaccines to cure existing tumors shows that only a minority of tumor-bearing mice could be cured. Furthermore, the limited therapeutic efficacy of vaccines was lost when they were not given in the first few days after the implantation of tumor cells.\(^\text{26}\)

A similar picture is emerging from phase I studies on vaccination of cancer patients. The vaccination is safe, but the results suggest that only a minority of patients (about 10%) display an objective response. The immunologic performance status of these patients is obviously suboptimal for this type of therapy. Even so, one would have expected a greater number of responders to support the promise of new sophisticated vaccines.\(^\text{23}\)

**Therapeutic** vaccination has not had much success in the management of infectious diseases. Its use against the progression of an established tumor is very challenging, since it must secure an effective immune response capable of getting the better of a well-established, proliferating tumor. Of the several objectives that

| Table 1. Cross-expression of some tumor-associated antigens among histologically different human tumors from distinct organs. |
|-------------------------------|-----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| bladder                       | BAGE            | GAGE           |                  |                |                |                |                |
| breast                        | BAGE            | MAGE           | CEA             | p53            | ras            | MUC-1          |
| colon                         | BAGE            | CEA            | p53             | ras            |                |                |
| lung                          | BAGE            | CEA            | p53             | ras            |                |                |
| melanoma                      | BAGE            | GAGE           | MAGE            | CEA            | p53            | ras            | MUC-1          |
| sarcomas                      | GAGE            |                |                  |                |                |                |                |
have been made approachable by antitumor vaccines, the cure of advanced tumors is both the most difficult and at the same time the most common in clinical trials. The strong emotions kindled by cancer suffering provide the main justification for these attempts.27 Perhaps, major improvements in antitumor vaccination will make this goal approachable. However, the data reviewed show that this is far from the present reality.

Nevertheless it should be considered that most tumor lethality depends on a few neoplastic cells remaining after surgical excision of a tumor mass or after having escaped direct killing by chemotherapeutic and radiotherapy. Many experimental findings28-30 suggest that a stage of minimal residual disease is one in which it is possible to foresee a significant cure by immunization. After successful conventional management the tumor burden may be low, and the tumor may reappear after a long dormancy. This is a realistic setting in which vaccination could lead to the induction of anti-tumor immunity capable of extending the survival of patients. As there are grounds for believing that antitumor vaccines could be used as an effective anticancer tool, the purpose of this review is to describe the types of vaccines that are being experimented, emerging clinical results and the new perspectives opened by this scientific endeavor.

Common tools

**Cytokines and cellular signals**

The immunologic attempt of the immune system to prevent the development of a neoplastic disease may be ineffective due to either a lack of immunogenicity of tumor cells, or to a weak reaction unable to contrast the neoplastic proliferation. In both cases, it is likely that most of the physiologic mechanisms of priming of the immunologic effector cells may be impaired or absent. In fact, initiation of immune responses requires that professional antigen-presenting cells (APC) deliver a first signal to T lymphocytes through the binding of the T-cell receptor by the peptide enclosed in the HLA molecule, that is responsible for the specificity of the immune response, and a second or co-stimulatory signal that is not antigen-specific but it is required for T-cell activation31,32 mainly through CD80 (B7-1) and CD86 (B7-2) binding to CD28 receptor, or the CD40:CD40L pathway. Moreover, the capacity of dendritic cells (DC) to activate natural killer (NK) cells by ligation of the CD40 molecule with its counter-receptor has recently been demonstrated.33,34 Immunocompetent cells may also determine the type of immune response by the expression of chemokines and by the release of pro-inflammatory, or anti-inflammatory cytokines which drive T-cells to different activities or even to suppression.35

Therefore, given the complex network of regulatory signals by professional APC and naive and memory lymphocytes occurring in antigen-specific immune responses, it is not surprising that tumor cells may fail to induce efficient humoral and cellular immune reactions even when a well known TAA is present. In this review, several strategies to overcome the immune escape mechanisms of tumor cells will be considered, such as the direct use of TAA to elicit specific reactions, the use of dendritic cells to present TAA in order to enhance the immune response, and the use of tumor cells genetically modified to function as professional APC or to release soluble factors. Animal models have been widely used for many years to demonstrate the effect of different cytokines, added to or secreted by tumor cell-based vaccines, in increasing the in vitro and in vivo cytotoxicity against tumor challenge. The role of the main cytokines involved in activation of humoral and cellular immune responses is represented in Figure 1. On the basis of cytokine functions it has been previously shown in experimental models that: the number of APC in the site of tumor infiltration can be increased by cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4, which should allow also the differentiation of DC precursors; B- and T-cell responses are potently increased by IL-2, IL-12, or GM-CSF; and in particular T-cell cytotoxicity is enhanced by GM-CSF, IL-2, IL-12, interferon (IFN-γ), tumor necrosis factor (TNF-α), while NK activity is enhanced by IL-12 or FLT 3-L.36,37 However, different models and different TAA resulted in controversial findings. Furthermore, since these cytokines are likely to be more effective when released within the tumor area, the transduction of cytokine genes into tumor cells and their use as cellular vaccines after irradiation has been tested in animal models and in humans.38-40

Initial clinical experiences in patients with advanced melanoma or renal cell carcinoma suggest that tumor cell-based vaccines, either engineered to produce GM-CSF or IL-12 or with exogenous GM-CSF, may facilitate marked infiltration of DC and CD4+ and CD8+ T-lymphocytes into tumor lesions, potentially improving the antitumor effect. These data provided evidence of the feasibility of the approach but were unlikely to be able to address the point of efficacy, due to the large tumor burden of these patients. Future immunotherapy attempts should, in fact, focus on the possibility of eradicating minimal residual disease. More recent data demonstrated the role of GM-CSF as a useful adjuvant in peptide-based vaccines in ovarian and breast cancer and in follicular lymphoma, as will be described later in this review.

Figure 1 also shows that cell-to-cell contact via CD40:CD40L plays a pivotal role in activating specific T-cell, B-cell and NK-cell responses. On the other hand, T-cell tolerance can be obtained by blockade of CD40L receptor in non-human primates undergoing solid organ allogeneic transplantation, and in mice receiving either allogeneic bone marrow or solid organ transplantation.41-43

Recent experiments demonstrated that stimulation, via an activating anti-CD40 antibody, resulted in the activation of host APC and could convert lymphocytes of mice treated with a tolerogenic peptide vaccine into cytotoxic T-cells. Moreover, this stimulation induced the
regression of established tumors that had not been affected by previous vaccination alone, thus showing that triggering the CD40 molecule may both overcome T-cell tolerance in a tumor-bearing animal and greatly potentiate a peptide-based vaccine. Moreover, gene transfer by an adenovirus vector of CD40L in human B-cell chronic lymphocytic leukemia (B-CLL) allowed the activation of bystander non-infected B-CLL cells that upregulated co-stimulatory molecules such as CD80 and CD86 and stimulated autologous cytotoxic T-cells.

Another important issue concerns the way T-cells are turned off by CTLA-4 receptor following activation via CD28. In fact, both CD28 and CTLA-4 bind with high affinity to CD80 and CD86 and CTLA-4 physiologically blocks T-cell activation. In the case of antitumor T-cell response it has been demonstrated that blockade of negative regulatory signals by an anti-CTLA-4 monoclonal antibody may retard tumor growth in experimental systems. More recent studies in mice suggested that this molecule was extremely efficient in causing tumor regression when used in combination with subtherapeutic doses of melphalan, or with a GM-CSF-expressing tumor.

Altogether, these studies strongly support the role of cytokines or immunomodulatory molecules in anticancer vaccine strategies. However, they do not clarify whether a strict T-helper (Th1) response is required to achieve tumor killing, or whether a humoral response induced by anti-inflammatory cytokines should also be pursued. Finally, future directions of anticancer vaccine research are likely to deal with monoclonal antibodies enhancing or blocking specific receptors.

**Dendritic cells as initiators of immune response**

Dendritic cells (DC) represent a heterogeneous population of leukocytes defined by morphologic, phenotypic and functional criteria which distinguish them from monocytes and macrophages. From among the professional APC, DC are the most potent stimulators of T-cell responses and play a crucial role in the initiation of primary immune responses. The DC system comprises at least three distinct subsets, including two within the myeloid or non-lymphoid lineage, and a third represented by lymphoid DC. There is also a continuum of differentiation within each of these subsets, from precursors circulating through blood and lymphatics, to immature DC resident in peripheral tissues, to mature or maturing forms in the thymus and secondary lymphoid organs. Recent studies have focused on the different roles of lymphoid and myeloid DC: more resident lymphoid DC induce tolerance to self, whereas migratory myeloid DC, including Langherans cells, are activated by foreign antigens in the periphery and move to lymphoid organs to initiate an immune response.

DC have always been described as having two distinct functional stages: 1) immature, with high antigen uptake and processing ability, and poor T-cell stimulatory function; 2) mature, with high stimulatory function and poor antigen uptake and processing ability. Bacterial products such as lipopolysaccharides, and inflammatory cytokines such as IL-1, TNF-α, type I interferons (IFN-α or β) and prostaglandin E₂ (PGE₂) stimulate DC maturation, whereas IL-10 inhibits it. Interestingly,
human and murine DC upregulate the synthesis of HLA class I and II molecules, and B7-1, B7-2 and CD40 molecules, after ingestion of bacteria or bacterial products, such as lipopolysaccharides, can prime naive T-cells.

An emerging concept is that APC activate T-helper (Th) cells not only with antigen and co-stimulatory signals, but also with a polarizing signal (signal 3). This signal can be mediated by many APC-derived factors, but IL-12 and PGE$_2$ seem to be of major importance. As for Th cells, APC can be functionally polarized. In vitro experiments with monocyte-derived DC showed that the presence of IFN-$\gamma$ during activation of immature DC induces mature DC with the ability to produce large quantities of IL-12 and, consequently, a Th1-driving capacity (APC1 or DC1). In contrast, PGE$_2$ primes for a low IL-12 production ability and Th2-driving capacity (APC2 or DC2).\textsuperscript{32,53} DC-stimulated CD4$^+$ cells upregulate CD40L/CD154 that reciprocally activates DC via CD40. This renders DC more potent stimulators of CD8$^+$ cytotoxic T-cell (CTL).\textsuperscript{34} This novel concept is in contrast to simultaneous stimulation of CD4$^+$ and CD8$^+$ T-cells by DC, whereby the CD4$^+$ T-cells secrete helper lymphokines in support of CD8$^+$ CTL development.\textsuperscript{55} Together with CD40 L and CD40, two different groups have discovered another pair member of the TNF-TNF receptor family. This factor, termed TNF-related activation induced cytokine (TRANCE) or receptor activator of NF-$\kappa$B ligand (RANK-L), is expressed by T cells.\textsuperscript{56} Its corresponding receptor, receptor activator of NF-$\kappa$B (RANK) or TRANCE R, is expressed by mature DCs but not on freshly isolated B cells, macrophages, or T cells.\textsuperscript{57} Ligation to this receptor causes either activation of T-lymphocytes or enhancement of DC survival. In addition, IL-12 is a critical mediator of DC-supported differentiation of naive, but not memory, B-cells,\textsuperscript{58} indicating that direct interactions occur between DC and B cells, apart from those that occur via cognate CD4$^+$ T-cell help.

Lastly, it should be mentioned that the primary and secondary B cell follicles contain another population of DC, the follicular DC (FDC). The origin of these cells is not clear, and most investigators believe that they are not leucocytes. FDC trap and retain intact native antigen as immune complexes for long periods of time, present it to B cells and are likely to be involved in the affinity maturation of antibodies, the generation of immune memory and the maintenance of humoral immune responses.\textsuperscript{59}

In conclusion, there is a general agreement in considering DC as very important players in the game of immune responses against foreign antigens either of infectious agents or of neoplastic cells. Many developing immunotherapeutic strategies against danger antigens are aimed at exploiting the powerful antigen-presenting properties of DC by an in vivo or ex vivo engineering of the DC system. In fact, subcutaneous or intramuscular injection of antigens relies on the local recruitment and activation of DC to capture and present antigens to the immune system. Although the techniques for targeting tumor antigens to DC in situ might eventually obviate the need for ex vivo manipulation of DC, novel methods for ex vivo generation and activation of large amounts of human DC have been developed.

**Antitumor vaccination: types and formulations**

**Killing for priming and killing to destroy**

The way cell vaccines die when injected in vivo influences DC loading. The initial activity of cytokines transduced into the cell vaccine is to select the leucocyte type recruited and stimulated at the site of injection. Tumor cells are killed by effectors of the innate response; NK and polymorphonuclear cells also produce secondary cytokines that set up a local inflammation recruiting DC, whereas T-cell response is activated later. Of note, gene engineering allows the manipulation of the first phase of the process through the choice of cytokine and/or co-factor and by deciding the level of cytokine to be produced. Once the infiltrate leukocytes are activated, their response to the triggering cytokine is physiologic and independent. They produce other cytokines thus amplifying the magnitude and the complexity of response. The initial stage is finalized to T- and B-cell activation, killing of cell vaccine is to provide the antigen(s) and the inflammatory response should provide the right environment for such activation.\textsuperscript{60}

The final stage is aimed at the destruction of existing tumor. Specific immune response is often insufficient to fight solid tumor nodules. Among the possible causes, immunosuppression, low effector-target ratio, MHC downregulation on tumor cells are the most common. Animal studies have shown that these problems can be overcome by general inflammation associated with neotrophil influx. This combination may destroy the tumor-associated blood vessels\textsuperscript{61} in a way that may resemble tissue damage in vasculitis. In this setting a specific immune response is not directly responsible for tumor elimination but should be strong enough to at least begin and direct the inflammatory response to the tumor site. In this perspective, tumor vessels are the main target of a non-specific immune response, their functional impairment increasing the effect of either T- or B-cell-mediated specific immune responses.

**An add to DC-common link?**

Although DC have been indicated as central in alerting and activating the immune system, it is now clear that certain peptides are not and can not be presented by mature DC. This observation, made by Van den Eynde and colleagues,\textsuperscript{62} concerns autoantigens and T lymphocytes that recognize them without being deleted in the thymus and normally without provoking autoimmunity. In fact, APC differ from other cell types by the proteosome that digests protein into immunogenic peptides to fit the MHC groove. APC immunoproteosomes have three catalytic subunits substituted by those induced by IFN-$\gamma$, thus generating slightly different peptides from those generated by non-APC cells.

Several self-antigens identified as tumor-associated because of CTL recognition may not be processed by
immunoproteosome. The implication is that such CTL were not generated through DC presentation or at least not through DC processing unless this happened during transition from immature to mature DC.\(^6\) Perhaps free peptides can be captured on the surface of DC for presentation, or perhaps other as yet unknown mechanisms are involved.

**Genetically modified tumor cell vaccines**

Old and recent discoveries confirm the possibility of a cancer vaccine made of tumor cells.

An empirical approach, such as the use of allogeneic whole-cell vaccine composed of 3 allogeneic melanoma cell lines established in vitro, allowed a 3-fold increase of the five-year survival of patients with stage IV melanoma as reported by Morton et al.\(^3\) The most active component of Morton’s vaccine has been identified by Livingston and colleagues\(^5\) to be a ganglioside (GM2). Patients who develop antibody response to ganglioside showed a significant survival advantage. Whether a CTL response was also activated has not been investigated but does probably exist. However, this finding prompted a phase III study in patients with stage III disease.

In the autologous setting, irradiated melanoma cells were modified with dinitrophenyl and used to treat patients with metastatic disease. Clinical evidence of inflammatory response to superficial metastases was reported. The same treatment administered to phase III patients who remain tumor-free after resection of lymph node metastases has resulted in 50 and 60% 4-year relapse-free and overall survival, respectively.\(^6\) Immuno-staining, TCR repertoire analysis and functional data of node-metastases post-vaccination have shown that treatment with autologous dinitrophenyl-modified melanoma cells can expand certain T-cell clones at the tumor site.\(^7\)

The above results bring up two issues: autologous versus allogeneic tumor cells and chemical or genetic (see below) modifications of tumor cells to be used as cancer vaccines.

Before discussing these issues we should, however, address a more basic question, that is how to use cancer cells as vaccine. Well before identification of tumor antigens, their existence was inferred in melanoma on the basis of expansion and characterization of cytotoxic T-lymphocytes recognizing autologous tumor,\(^8\) whereas antibodies against a variety of tumor types were isolated from patient sera. Antigens recognized by CTL can be tumor-specific and even restricted to the autologous tumor or cross-react among different neoplasms of the same or different tissue origin depending on the mechanism generating such an antigen: point mutation, incorrect splicing, over-expression, translocation and other (see Table 2). The number of tumor antigens is always increasing thus suggesting that our knowledge of the antigene repertoire of tumor cells is partial. In addition, which antigen among those already characterized should be used in a vaccine?\(^9\)

These problems can be solved altogether by using tumor cells that would represent the entire antigenic repertoire with a single drawback, that is immunoselection of certain antigens. Tumor cells from different patients may undergo different processes of immunoselection and therefore a pool of these tumors would be ideal for preparing an allogeneic vaccine. The finding that antigens belonging to allogeneic cells are processed by host antigen-presenting cells,\(^10\) so that they can be recognized by host T-lymphocytes (a phenomenon called cross-priming), removes any conceptual obstacles to the use of allogeneic cells. Allovaccines have several advantages over autologous vaccines: cell lines that are extensively characterized in vitro can be used to treat all the patients included in a clinical study. Genetic modifications of tumor cells have been widely studied as a way to increase immunogenicity. These modifications can be easily made to a cell line, thus avoiding the need to isolate cells from every tumor. The rate of success in culturing tumor cells from a primary tumor varies according to the type of tumor and experience of the operator. Transduced cell lines can be selected for production of a certain amount of transgene thus assuring that the same vaccine will be given to all patients. Which modification is most efficient in inducing tumor immunity has not been unequivocally determined since variability among different tumors has been described. Chemical modification, also called haptenization, aims at adding helper determinant to tumor cells,\(^7\) although the exact mechanism and the downstream pathways activated in this way are not clearly understood. Genetic modification is now preferred since the effect of several cytokines and co-stimulatory molecules are known at molecular level. Their genes can be transduced into tumor cells that acquire new immunoregulatory functions. In this way a desired immune response can be fine-tuned through gene dosage, recruitment of certain cell types, defection of Th1 or Th2 type of response and several other mechanisms.\(^10\)

To summarize the most recent and promising approaches we may consider two strategies aimed at favoring vaccine interaction with DC or directly with T lymphocytes. The two approaches can be viewed for priming or boosting (Figure 2). A combination of cytokine and co-stimulatory factors is likely to be synergistic as generally occurs when soluble and cell–contact signals are given together. Tumor cells transduced with both GM-CSF and CD40L have been shown to be heavily infiltrated by DC. GM-CSF induces proliferation and maturation of hematopoietic cells, and has been shown to stimulate DC accessory properties and enhance the immune response initiated by these cells.\(^10\)

The CD40/CD40L interaction plays a critical role in cell–mediated immunity, and in proliferation and activation of APC, as shown for B cells, monocytes and, more recently, DC.\(^2\) Ligation of CD40 on monocytes and DC results in the secretion of several cytokines, including IL-1, IL-6, IL-12, and TNF-α. The murine tumor transduced with both GM-CSF and CD40L genes showed that tumor infiltrating DC can take up cellular antigen from the tumor and can present it to T lymphocytes in vitro.\(^2\) In this setting, DC bridges cell vaccine–T-lymphocytes
interaction and can be envisaged as the way to prime patients against weaker antigens otherwise ignored by the immune system. A complementary approach would consider the possibility of boosting the primed or the existing immune response. In this case DC that re-encounter activated T-cells can be lysed and may not be appropriate for boosting. Boosting can be done using tumor cells transduced with IL-2 and B7-1 such as to provide both cell expansion and co-stimulation from the tumor cell vaccine directly to T lymphocytes (Figure 2). The way cellular antigen can be captured by DC for priming of T-lymphocytes depends on how tumor cells die after injection. Irradiation of the cell vaccine induces apoptotic cell death and apoptotic bodies can be captured by DC. Others suggest that peptides of cellular protein complexed to heat shock protein (HSP), the natural chaperon of peptide from proteosome to membrane-associated TAP, leave the cells upon necrosis to be taken up by DC. DC loading must be followed by T cells.

The way cytokines and co-signals modulate vaccine-host interactions may determine the extent and the efficacy of treatment. Systemic activation of the immune response (T-cell cytotoxicity, antibodies) is easy to measure but can not be used as a read-out system to predict whether the tumor will be rejected. Tumor nodules might be reached by circulating lymphocytes which, however, may be neutralized because of either immunosuppression or peripheral tolerance. The former is likely dependent on tumor size and is less expected when a small tumor, minimal residual disease or prevention of recurrence is the target to be treated. The latter could be surmounted by appropriate co-signals, for example CD40L.

**Soluble proteins as immunogens**

Soluble proteins derived from autologous cancer cells are not as immunogenic as proteins derived from infectious agents. The degree of foreignness, which depends on the reciprocal distribution between epitopes subject to self-tolerance, is low in TAA. Moreover, TAA do not have the particulate nature of infectious agents that is a key factor in increasing their immunogenicity. Finally, infectious agents are rich in molecules that are able to raise immune responsiveness without being themselves immunogenic. A significant effort has been made over the last few years to translate the immunogenic properties of infectious agents into cancer vaccines for clinical use. The most suitable TAA should be directly involved in the malignant behavior of tumor cells; contain multiple immunodominant B cell and T cell epitopes, including both helper and cytotoxic T-cell epitopes; contain a very high degree of foreignness; be unprotected by self-tolerance mechanisms. Of course, HLA haplotype remains a major constraint in determining whether TAA-derived immunodominant peptides can elicit tumor-specific immune responses in a given individual (see also below).

### Table 2a. Tumor-associated antigens recognized in class I HLA restriction.

<table>
<thead>
<tr>
<th>Antigen defined</th>
<th>Type of antigen and distribution</th>
<th>Type of tumor</th>
<th>HLA-restriction allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp 100</td>
<td>Melanocyte diff.</td>
<td>Melanoma</td>
<td>A2, A3, A24, Cw6</td>
</tr>
<tr>
<td>MART-1/MelanA</td>
<td>Melanocyte diff.</td>
<td>Melanoma</td>
<td>A2, B45</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>Melanocyte diff.</td>
<td>Melanoma</td>
<td>A1, A2, A24, B44</td>
</tr>
<tr>
<td>TRP-1 (gp 75)</td>
<td>Melanocyte diff.</td>
<td>Melanoma</td>
<td>A31</td>
</tr>
<tr>
<td>TRP-2</td>
<td>Melanocyte diff.</td>
<td>Melanoma</td>
<td>A2, A21, Cw8</td>
</tr>
<tr>
<td>p15</td>
<td>Widely expressed</td>
<td>Melanoma</td>
<td>A24</td>
</tr>
<tr>
<td>SART-1</td>
<td>Widely expressed</td>
<td>Lung carcinoma</td>
<td>A2601</td>
</tr>
<tr>
<td>PRAME</td>
<td>Widely expressed</td>
<td>Melanoma, renal</td>
<td>A24</td>
</tr>
<tr>
<td>NAG-P</td>
<td>Melanoma sheared</td>
<td>Melanoma</td>
<td>A2.1</td>
</tr>
<tr>
<td>β catenin</td>
<td>Unique tumor specific</td>
<td>Melanoma</td>
<td>A24</td>
</tr>
<tr>
<td>CDK4-Kinase</td>
<td>Unique tumor specific</td>
<td>Melanoma</td>
<td>A2</td>
</tr>
<tr>
<td>MOM-1</td>
<td>Unique tumor specific</td>
<td>Melanoma</td>
<td>B44</td>
</tr>
<tr>
<td>TRP2/INT2</td>
<td>Unique tumor specific</td>
<td>Melanoma</td>
<td>A6801, Cw6</td>
</tr>
<tr>
<td>CASPASE-8</td>
<td>Unique tumor specific</td>
<td>Head/neck cancer</td>
<td>B35</td>
</tr>
<tr>
<td>HLA A*201 mutated</td>
<td>Unique tumor specific</td>
<td>Renal cancer</td>
<td>A2.1</td>
</tr>
<tr>
<td>MAGE-1, -2 or -6</td>
<td>Cancer/ testis</td>
<td>Melanoma</td>
<td>A1, A2, B44</td>
</tr>
<tr>
<td>MAGE-3</td>
<td>Cancer/ testis</td>
<td>Melanoma</td>
<td>A1, A2, B44</td>
</tr>
<tr>
<td>TYR</td>
<td>Cancer/ testis</td>
<td>Renal cancer</td>
<td>B7</td>
</tr>
<tr>
<td>NY-ESD-1</td>
<td>Cancer/ testis</td>
<td>Melanoma, ovarian, esophagical cancer</td>
<td>A2</td>
</tr>
<tr>
<td>K-RAS-D13 mutated</td>
<td>Cancer/ testis</td>
<td>Colon cancer</td>
<td>A2.1</td>
</tr>
<tr>
<td>HLA-A*201 mutated</td>
<td>Cancer/ testis</td>
<td>Colon and lung</td>
<td>A2.1</td>
</tr>
<tr>
<td>MAGE-3, -2 or -6</td>
<td>Cancer/ testis</td>
<td>Cancer/ testis</td>
<td>CML</td>
</tr>
<tr>
<td>MAGE-4</td>
<td>Cancer/ testis</td>
<td>Colon and lung</td>
<td>A2.1, A3, A11, B8</td>
</tr>
<tr>
<td>MUC-1</td>
<td>Cancer/ testis</td>
<td>Breast, colon, pancreatic cancer</td>
<td>A11</td>
</tr>
<tr>
<td>HPV16E7</td>
<td>Viral related</td>
<td>Cervical cancer</td>
<td>A2.1</td>
</tr>
</tbody>
</table>

### Table 2b. Human tumor antigens recognized by HLA class II-restricted CD4+ T cells.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Tissue distribution</th>
<th>Class II restriction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tnysinase</td>
<td>Melanoma/melanocytes</td>
<td></td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>Melanoma/melanocytes</td>
<td></td>
</tr>
<tr>
<td>Trisphosphate isomerase mutated form</td>
<td>Melanoma, unique</td>
<td></td>
</tr>
<tr>
<td>MAGE-3</td>
<td>Melanoma and other tumors, testis</td>
<td></td>
</tr>
<tr>
<td>MAGE-4</td>
<td>Melanoma and other tumors, testis</td>
<td></td>
</tr>
<tr>
<td>MAGE-5</td>
<td>Melanoma and other tumors, testis</td>
<td></td>
</tr>
<tr>
<td>MAGE-6</td>
<td>Melanoma and other tumors, testis</td>
<td></td>
</tr>
<tr>
<td>MAGE-D12 mutated</td>
<td>Colon and pancreatic cancer</td>
<td></td>
</tr>
<tr>
<td>HER-2/ neu</td>
<td>Breast and ovarian cancer</td>
<td></td>
</tr>
<tr>
<td>PML/RARx</td>
<td>Acute promyelocytic leukemia</td>
<td></td>
</tr>
<tr>
<td>Bcr/abl</td>
<td>Chronic myeloid leukemia</td>
<td></td>
</tr>
<tr>
<td>CD27</td>
<td>Melanoma</td>
<td></td>
</tr>
</tbody>
</table>

*N-acetylgalcosaminyl transferaseV. CML: chronic myeloid leukemia.

### Building up immunogenicity of soluble proteins

Even the most suitable TAA is not immunogenic unless it is processed and presented by professional APC to the host immune system. To this aim, TAA should interact with APC directly. The route of administration and adjuvants are key factors in determining the final outcome.
of immunization. Intravenous injection of soluble antigens induces tolerance, whereas subcutaneous or intramuscular injection of the same antigens results in immunity because of the interaction with epidermal Langerhans cells or dermal dendritic cells. Accordingly, delivery of antigens via mucosal surfaces may result in immunity because antigens may interact with the numerous DC located just beneath the epithelium of mucosal lymphoid organs. The association between soluble antigens and APC is made much more intense by adjuvants. Adjuvants act via different principles and pathways, but the common goals are to prolong the interaction with APC by promoting a slow antigen release, and functionally activate APC themselves by delivering danger signals. Cytokines have also emerged as potent immunoadjuvants since they can influence the immune responses at different levels (see above).

**Particulation of soluble proteins**

Precipitation with aluminium hydroxide or aluminium phosphate has been used to particulate antigens in diphtheria, tetanus, hepatitis B, and other vaccines. These types of vaccines induce antibody formation, but very little delayed cutaneous hypersensitivity (DTH) or cell-mediated cytotoxicity. In a pilot study, five stage I–III patients with multiple myeloma were immunized with autologous idiotype (Id) precipitated in aluminium phosphate suspension. Three patients developed idiotype-specific T- and B-cell responses, but these responses were transient and their amplitude was low.77

The use of immunostaining complexes (ISCOMs) is another strategy that has been used to particulate antigens. ISCOMs are cagelike structures made of cholesterol, saponin, phospholipid, and viral envelope proteins to which other proteins can be associated. Saponin is a plant derivative that is critical to the efficacy of ISCOMs. QS21 is the most effective fraction of saponin and is currently under clinical investigation in several trials.78 ISCOMs may reach the endocytic pathway and induce DTH and CTL responses other than antibody production. Liposomes, virosomes, and proteasomes are alternative strategies to ISCOMs. Experimental data have recently shown in the 38C13 mouse B-cell tumor that liposomal formulation of autologous Id converts this weak self-antigen into a potent tumor rejection antigen.79

**Promoting slow release of soluble antigens**

A slow release of antigen is one of the major goals of adjuvants. Antigen polymerization and emulsifying agents have been put together to achieve this goal. Polymerization can be obtained by association with non-ionic block polymers or by association with carbohydrate polymers. Non-ionic block polymers have been used as components of water-in-oil or oil-in-water emulsions. SAF-1 (Syntex Adjuvant Formulation-1) is an oil-in-water adjuvant formulation containing non-ionic block polymers, squalene, and Tween 80. SAF-1 has been used by Kwak et al. in their pioneering study on idiotype vaccination in follicular lym-

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**Figure 2. Priming of DC and boosting of T-cell responses by transduced tumor cells as vaccine.**
phoma patients.\textsuperscript{80} Chemical immunomodulators such as derivatives of muramyl dipeptide, the smallest subunit of the mycobacterial cell wall that retains immunoadjuvant activity, can be added to oil-in-water adjuvants.\textsuperscript{81} This approach was used in the study by Hsu \textit{et al.} in which idio-type/KLH conjugates were delivered to follicular lymphoma patients after mixing with SAF-165 that contained muramyl dipeptide as immunomodulator.\textsuperscript{82} Lipid components of bacteria have also been used as adjuvants in experimental models. These are portions of the LPS endotoxins of Gram-negative bacteria. These molecules are, however, too toxic and chemically modified derivatives have been developed for clinical use. So far, monophosphoryl lipid A is the least toxic derivative capable of promoting cell-mediated immunity.\textsuperscript{83}

Polysaccharide polymers have also been used as a sustained-release vehicle for TAA. Poly-N-acetyl glucosamine is a highly purified, biocompatible polysaccharide matrix that has recently become available for this purpose.\textsuperscript{84}

**Peptide vaccines**

T lymphocytes recognize small peptides that represent the degradation products of a complex intracellular process and are presented on the cell surface complexed to 1 of 2 types of histocompatibility leukocyte antigen molecules (HLA classes I or II). CTLs (CD8\textsuperscript{+}) mainly recognize peptides of 8 to 10 amino acids derived from intracellular or endogenous proteins and complexed to HLA class I molecules.\textsuperscript{85-89} CD4\textsuperscript{+} T lymphocytes recognize exogenous proteins which are ingested by APC, degraded to peptides of 12-24 amino acids and complexed to HLA class II molecules.\textsuperscript{90,91}

Class I and Class II peptides that are presented on the cell surface, although randomly derived from the original protein, must contain specific amino acids in 1 or 2 critical positions in order to be able to bind the appropriate HLA molecules. Thus, peptide binding is HLA-restricted. The amino acid motifs responsible for the specific peptide-binding to HLA class I and class II molecules have been determined for the common HLA types by analyzing acid-eluted naturally processed peptides and by using cell lines defective in intracellular peptide loading and processing.\textsuperscript{92-94}

Several tumor-specific and some leukemia-specific peptides have so far been identified, and studies aimed at evaluating the potential clinical benefit of peptide vaccines in cancer patients have begun. Among the reasons that make a peptide vaccine strategy interesting are several unique advantages that peptide immunization offers over other vaccine approaches: 1) peptide vaccines permit specific targeting of the immune response against 1 or 2 unique antigens (thus limiting the potential autoimmune cross-reactivity or immunosuppressive activity often observed with more complex immunogens); 2) emerging technology has made it simple, rapid and inexpensive to sequence and prepare larger quantities of tumor antigen peptides for both laboratory and clinical use; 3) use of synthetic peptides greatly reduces the possible risk of bacterial or viral contamination that might derive from autologous or allogeneic tissue for immunization. On the down side, the main disadvantages of peptide immunization are: 1) lack of universal applicability as each peptide is restricted to a single HLA molecule; 2) poor immunogenicity of most native peptides; 3) risk of inducing antigenic tolerance. Successful attempts to enhance HLA binding affinity have been based on synthetically generating peptides with amino acid deletions or substitutions while maintaining antigen specificity.\textsuperscript{95,96} In initial studies, synthetic substituted peptides appeared to enhance immunogenicity and also to overcome the host immune tolerance that exists to native peptides.\textsuperscript{97,98} Conversely it has been reported that changes in the fine specificity of modified peptide-reactive T-cells following vaccination may occur with subsequent loss of tumor cell recognition.\textsuperscript{99}

A peptide vaccine approach involves several steps which are aimed firstly at identifying the appropriate peptide, secondly at checking for its immunogenicity and relevance as a tumor-associated antigen (TAA) \textit{in vitro}, and thirdly at formulating a safe product to be used clinically.

**Identification of the appropriate peptide**

1) \textit{From protein to peptide}. This approach involves the screening of potentially HLA-binding peptides within the sequence of a known tumor-specific protein by using HLA anchor motifs and epitope selection. Peptides derived by mut RAS,\textsuperscript{100} melanoma-associated MAGE protein,\textsuperscript{101} prostate specific antigen\textsuperscript{102} and chronic myelogenous leukemia (CML) specific P210 were identified by this approach.\textsuperscript{103,104} In CML, for example, a possible total of 76 peptides, 8 to 11 amino acids in length, spanning the b2a2 and b3a2 junctional regions of bcr-abl were screened for HLA class I binding motifs and tested for the effective binding property to purified HLA molecules. Four of them, all derived from the b3a2 breakpoint, were found to be able to bind with either intermediate or high affinity to purified HLA A3, A11 and B8.\textsuperscript{103} A similar approach allowed identification of class II b3a2 breakpoint peptides capable of binding HLA DR11,\textsuperscript{105} DR4,\textsuperscript{106} and DR1.\textsuperscript{107} This method for identifying tumor-specific peptides is relatively simple, fast and suitable for any known intracellular protein that may be a potential TAA. Nevertheless, it bears the disadvantage that it cannot, by itself, predict whether the identified peptide is found on HLA molecules of the leukemia or cancer cells that contain the parent protein.

2) \textit{From peptide to protein}. Another strategy used to identify suitable peptides for cancer vaccines involves the structural analysis of naturally processed peptides (NPPs) bound to HLA class I and class II molecules of cancer cells. NPPs were first isolated and sequenced by acid-elution from immunoaffinity purified HLA molecules and subsequently compared with existing protein sequences.\textsuperscript{108} Alternative approaches are to obtain NPPs by mechanically destroying and acid-treating whole tumor cells and/or by exposing living tumor cells to rapid
acid treatment.\textsuperscript{129} These procedures should characterize tumor-, differentiation stage- and tissue-specific self-antigen MHC-bound peptides as well as the naturally processed proteins from which they are derived and use them as tools for immunotherapy. The main disadvantage of this approach is that many tumor cells express low levels of HLA molecules and the yield of NPPs can be scarce. Nevertheless some immunogenic peptides derived from wild-type p53 protein, melanoma associated MART-1 and gp100 proteins were identified by these methods,\textsuperscript{110,111} and naturally processed peptides from acute myeloblastic leukemia cells and CML blasts are now under evaluation.\textsuperscript{112,113} Advantages and disadvantages of synthetic versus natural tumor peptides have been recently reviewed.\textsuperscript{114}

3) From tumor infiltrating lymphocytes to peptide. Probably the most clinically relevant tumor peptides are those identified from the epitope analysis of tumor infiltrating lymphocytes (TIL).\textsuperscript{115-118} In preliminary experiments HLA class I restricted CTL lines were derived by repetitive \textit{in vitro} stimulation of TIL with autologous tumor cells. Subsequently, by transfection of a tumor cDNA library and \textit{in vitro} sensitization assays, the peptide sequences recognized by the tumor-specific CTLs were identified as were the parent proteins (i.e. peptides derived from melanoma associated gp100, tyrosinase and the MAGE family). Most TIL-derived tumor peptides found in the past few years are HLA class I-restricted; however, a novel melanoma antigen resulting from a chromosomal rearrangement and recognized by a HLA-DR1-restricted CD4\textsuperscript{+}-TIL has recently been identified.\textsuperscript{119}

Checking for peptide immunogenicity
Except for TIL-derived peptides, all other tumor-specific, HLA binding, synthetic or naturally expressed peptides still need to be tested for immunogenicity. The ability of inducing CTL or specific CD4\textsuperscript{+} proliferation has been evaluated for all tumor-specific peptides that were subsequently used in clinical trials. P210-derived peptides, for example, were able to elicit peptide-specific T-cell immunity both in normal donors,\textsuperscript{109,120} and CML patients.\textsuperscript{121} Their relevance as TAA was further confirmed by observing peptide-specific HLA restricted CTLs and CD4\textsuperscript{+} cells able to mediated killing of b3a2-CML cells and proliferation in the presence of b3a2 containing cell lysates, respectively.\textsuperscript{106,107} The latter findings were the indirect proof of natural processing of P210 and of HLA presentation of breakpoint-derived peptides. Although strong peptide-specific CTL and CD4\textsuperscript{+} responses have been shown \textit{in vitro} for most tumor peptides so far identified, few data on T-cell induced immunity after peptide vaccination in patients have been generated.\textsuperscript{100,122,123} Thus, strategies to improve peptide immunogenicity, by using different adjuvants and delivery systems, are currently under evaluation.

Peptide vaccine formulation
The goal of experimental clinical protocols using peptide antigens for active vaccination is to induce a strong CTL response against the immunizing antigen and thereby against tumor cells expressing the antigen. The mode of peptide-based cancer vaccination critically affects the clinical outcome. The synthesis of a peptide on a large scale, its purification and testing for common Quality Control/Quality Assurance compliance are simple and fast procedures but the choice of an effective delivery system for the peptide is crucial. Peptide vaccination strategies currently being evaluated include: 1) direct peptide vaccination with immunologic adjuvants and/or cytokines;\textsuperscript{124} 2) lipopeptide conjugates;\textsuperscript{125} 3) peptide loading onto splenocytes or DC;\textsuperscript{126} 4) lysosomal complexes.\textsuperscript{127} Recently, a specific formulation of the polysaccharide poly-N-acetyl glucosamine has been found to be an effective vehicle for sustained peptide delivery in a murine vaccine model able to generate a primary CTL response with a minimal peptide dose.\textsuperscript{128} Finally, triggering CD40 \textit{in vivo} with an activating antibody considerably improved the efficacy of peptide-based anti-tumor vaccines in mice, converting a peptide with minimal immunogenicity into a strong CTL inducer.\textsuperscript{129}

Recombinant viruses
The molecular identification of the antigens on human tumors recognized by T- and B-lymphocytes offers the opportunity to design novel cancer vaccines based on recombinant forms of TAA. Genes coding for TAA can be inserted into the genome of attenuated microorganisms such as bacteria and viruses. Viruses are among the most interesting vectors since they are able to induce antibody, Th, and CTL responses in the absence of co-stimulation.\textsuperscript{128} Their long-lasting cohabitation with human beings has likely favored the evolution of specific patterns recognized by the innate immune system which create an immunostimulatory environment for optimal immune responses. The vector choice, however, is limited by the possible disadvantages of recombinant virus utilization, such as recombination with wild-type viruses, oncogenic potential, or virus-induced immunosuppression.

Vaccinia virus (VV) belongs to the \textit{Poxviridae} family, and its worldwide use in the smallpox eradication campaign demonstrated that it was safe and very effective. To date, no other large-scale vaccination program has had such an impact on human diseases, because smallpox has been virtually eliminated from the world population. Large amounts of foreign DNA can be stably inserted into the VV genome by homologous recombination.\textsuperscript{129} VV employs a built-in transcriptional and post-translational apparatus to produce large amounts of the protein encoded by the inserted gene. VV sojourns within the host cell cytoplasm and does not integrate nor is it oncogenic.\textsuperscript{129} The induction of potent cellular and humoral immune responses with recombinant (r)VV was observed in several tumor systems.\textsuperscript{130-132} Preclinical studies in models of pulmonary metastatization caused by tumors bearing a prototype TAA have revealed some features of rVV that are important in determining successful therapy. In particular, TAA gene must be expressed
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under the control of a strong, early promoter which allows its expression in professional APC such as DC. Moreover, while prevention from tumor challenge requires only the synthesis of the TAA by the recombinant virus, genes encoding immunostimulatory molecules inserted in the recombinant poxvirus, or their combination are required to induce eradication of established tumors.

Encouraging results have also been obtained in mouse models more relevant to the therapy of human cancer. Immunization of mice transgenic for the human HLA-A*0201 allele with an rVV encoding a form of the melanoma antigen gp100, which had been modified to increase epitope binding to the restricting class I molecule, elicited CD8+ T–lymphocytes specific for the epitope that is naturally presented on the surface of an rVV-A*0201-expressing mouse melanoma. Repeated inoculations of an rVV encoding the mouse tyrosinase-related protein-1 (TRP-1/gp75) caused autoimmune attack of normal melanocytes manifested by hair depigmentation (vitiligo) and CD4+–mediated melanoma destruction in mice. In a clinical trial, administration of VV encoding human carinoembryonic antigen (CEA) proved effective in inducing both humoral and cellular immune responses in patients with colorectal cancer.

Poxviruses are not the only choice for tumor immunologists. Adenoviruses in which critical genes that enable viral replication have been deleted and replaced by genes encoding heterologous antigens, have been generally used in gene therapy studies, but have also provided antitumor activity when employed as immunogens. Liver toxicity was described following systemic administration of high titers of first generation E1-deleted Adenovirus vectors optimized for gene therapy. These side effects, which certainly raise some concerns about Adenovirus administration in patients, might not restrain their use in cancer immunotherapy since systemic delivery of high viral titers would certainly not be the favored immunization route.

Initial clinical trials have unveiled some of the intrinsic limitations of recombinant viruses. Many patients, in fact, have high neutralizing antibodies against VV as a consequence of its use as a vaccine for smallpox prevention, and against Adenoviruses, which cause upper respiratory tract infections throughout life. High doses of recombinant adenoviruses expressing the human melanoma antigens MART-1 and gp100 could be safely administered to cancer patients, but the high levels of neutralizing antibodies present in their sera likely impair the ability of these viruses to immunize against the melanoma antigens.

These results, largely expected, have not put an end to the clinical use of recombinant viruses, as various strategies have been exploited to overcome pre-existing immunity. Several groups have engineered non-replicating viruses which normally do not infect human beings. The Avipoxviridae family comprises viruses, such as fowlpox and canarypox, which can productively infect avian but not mammalian cells, and are not cross-reactive with VV. A recombinant fowlpox virus expressing a model TAA was able to cure established tumors in mice; an important aspect of this study was the observation that prior exposure to VV did not abrogate the immune responses induced by the recombinant fowlpox virus. A different non-replicating virus, canarypox virus (ALVAC), has also been employed to elicit immune responses against a variety of antigens.

A highly attenuated strain of VV, known as modified VV Ankara (MVA), has been inoculated as smallpox vaccine into more than 120,000 recipients without causing any significant side effect. Replication of MVA is blocked at the step of virion assembly and for this reason the MVA vectors produce recombinant proteins expressed under the control of both early and late viral promoters, thus mimicking the expression in wild-type virus. An MVA vector, and a fowlpox virus vector expressing a model TAA showed better therapeutic effects on pulmonary metastasis than a VV encoding the same TAA. MVA is a very promising vector for the development of recombinant vaccines for cancer, and can be efficiently used in combination with DNA vaccines.

As an alternative approach, the mucosal route of administration was recently shown to overcome pre-existing immunity to VV. Intrarectal immunization of vaccinia-immune mice with rVV expressing gp160 induced specific serum antibody and strong HIV-specific CTL responses in both mucosal and systemic lymphoid tissue, whereas systemic immunization was ineffective under these circumstances.

Direct immunization of mice with recombinant adenoviruses resulted in the induction of high titers of neutralizing antibodies, which precluded a boost of CTL responses after repeated inoculations. The presence of neutralizing antibodies did not, however, affect the immunogenicity of infected DC, as repeated administration of virus-infected DC boosted the CTL response even in mice previously infected with the recombinant vector. It was also shown that protective immunity against mouse melanoma "self" antigens, gp100 and TRP-2, could be obtained by DC transduced with Adenovirus vector encoding the antigen. Importantly, immunization with Adenovirus-transduced DC was not impaired in mice that had been pre-immunized with Adenovirus. With the help of these novel strategies, recombinant vectors could be used in the general population, including those individuals previously exposed to the viruses. Gene transfer to different human DC subpopulations by vaccinia and adenovirus vectors is a conceivable strategy for TAA loading.

**DNA vaccines**

Following the first and somewhat shocking demonstration that the intramuscular injection of naked DNA (i.e., DNA devoid of a viral coat) encoding the influenza A nucleoprotein could induce nucleoprotein-specific CTL, and protect mice from challenge with heterologous influenza strains, DNA immunization has become a rapidly developing technology. This vaccination method...
provides a stable and long-lasting source of antigen, and elicits both antibody- and cell-mediated immune responses. Compared to recombinant viruses, DNA vaccines offer a number of potential advantages because they are cheap, easy to produce, and do not require special storage or handling. DNA vaccines express virtually only the heterologous gene, therefore, they should induce an immune response selective for the antigen and not the vector, thus supplying a source of antigen suitable for repeated boosting. DNA vaccination has proven to be a generally applicable approach to various preclinical animal models of infectious and non-infectious diseases, and several DNA vaccines have now entered phase I/II human clinical trials. Although the clinical application of DNA vaccines is a very young practice, some trials have already demonstrated that it is possible to elicit a specific CTL response against malaria and HIV proteins in human volunteers.

This novel vaccination approach involves different steps: 1) cloning of a heterologous gene under the control of a viral promoter (ordinarily derived from the CMV immediate early region); 2) purification of the endotoxin-free DNA plasmid from bacteria factories; 3) administration of the expression vectors by direct intramuscular or intradermal injection with a hypodermic needle or using a helium-driven, gene gun to shoot the skin with DNA-coated gold beads. Heterologous DNA can also be introduced into recombinant Salmonella, or Listeria strains that can be thus administered by a mucosal route (Figure 3). In addition to these classic routes of DNA delivery, plasmid-based gene expression vectors have also been admixed with polymers and administered with a needle-free injection device, achieving high and sustained levels of antigen-specific antibodies. The route of DNA delivery can profoundly influence the type of immune response by preferentially activating different Th populations; gene gun bombardment elicits a Th2 response, while intramuscular inoculation induces Th1 activation, even though the antigen form (i.e., membrane-bound vs secreted) can also exert some effect.

The immunostimulatory activity of DNA vaccines has been associated with the prokaryotic-derived portion of the plasmid, which contains a central CpG motif in the sequence PuPuCpGPyPyP. In their unmethylated form, these hexamers stimulate monocytes and macrophages to produce different cytokines with a Th1 promoting activity including IL-12, TNF-α, and IFN-γ. A plasmid that incorporated several CpG islands in the prokaryotic ampicillin-resistance gene induced a stronger immune response when compared to a second plasmid carrying the kanamycin-resistance gene which possesses none. To date, it is not known whether the CpG motifs will have the same immunostimulatory properties when applied to vaccination of human beings. However, it was recently reported that CpG motifs can activate in vitro subsets of freshly isolated human DC to promote Th1 immune responses.

The mechanism of DNA-induced immunization has not yet been fully elucidated. An exclusive role for the direct transfection of normal tissue cells, such as myocytes or keratinocytes, has been debated because surgical ablation of the injected muscle within 1 minute of DNA inoculation did not affect the magnitude and longevity of DNA-induced antibodies. Moreover, studies with bone-marrow chimeras clearly indicated that bone-marrow-derived APC, either transfected by the DNA plasmid or able to capture the antigen expressed by other transfected cells, were necessary to prime T- and B-lymphocyte responses. Indeed, more recent evidence suggests that Th and CTL are activated by DC directly transfected in vivo following DNA immunization.

The first applications of DNA immunization to preclinical models of tumor growth revealed some interesting aspects. In general, the potency of naked DNA does not equal that of recombinant viruses, probably because DNA does not undergo a replicative amplification in the transfected cells, which in turn limits the amount of heterologous antigen produced. Inflammatory responses caused by DNA inoculation are more contained than those occurring during infection with viruses; for this reason, repeated inoculations of plasmid DNA, or the use of adjuvants such as cardiototoxic are generally required for the induction of an optimal response. Another emerging issue is that the efficacy of the vaccination approach depends more on the type of antigen than on the route of administration (Table 3). Vaccines based on shared viral antigens, or model TAA artificially introduced in the experimental tumors can be used to induce a strong, and often therapeutic immune response. Using a gene gun for DNA immunization, Irvine et al. observed effective treatment of established pulmonary metastases, but recombinant cytokines were necessary to enhance the therapeutic effects. Unlike viral and model TAA, self/TAA fail to induce sterilizing immunity since therapy of established tumors has been rarely reported, and prevention from challenge is often partial. Central and peripheral tolerance to self antigen has thus emerged as the main limitation to the successful application of DNA vaccines to the therapy of cancer. This conclusion seems to apply to several mouse melanocyte differentiation antigens, a class of molecules that is expressed in both melanomas and melanocytes and includes tyrosinase, TRP-1/gp75, TRP-2, and gp100/pmel 17. However, tolerance can be broken by the use of a xenogenic source of TAA. While immunization with mouse TRP-1/gp75 or TRP-2 antigens failed to induce a detectable immune response, vaccination with a plasmid DNA encoding the human homologous antigens elicited autoantibodies and CTL in C56BL/6 mice; immunized mice rejected metastatic melanoma and developed patchy depigmentation of their coats (vitiligo). This “obligatory” association of vitiligo and anti-tumor response was recently questioned by a study showing that immunization with mouse TRP-2-encoding plasmid could protect CB6 F1 mice in the absence of overt vitiligo, suggesting a role for the genetic background in controlling both the extent and the consequences of the immune activation against self TAA.
A novel vaccine that combines the properties of viruses and DNA is based on antigen production in the context of an alphavirus replicon. These new vectors rely on the ability of the alphavirus RNA replicase to drive the replication of its own gene, as well as of subgenomic RNA encoding the heterologous antigen. This loop of self-replication results in a several-fold amplification of protein production in infected cells. Compared with traditional DNA vaccine strategies, in which vectors are persistent and the expression constitutive, the expression mediated by the alphaviral vector is transient and lytic, resulting in a decrease of biosafety risks as well as the risk of inducing immunologic tolerance due to long-lasting antigen expression. A single intramuscular injection of a self-replicating RNA immunogen at doses lower than those required for standard DNA-based vaccines elicited antibodies, CD8+ T-cell responses, and prolonged the survival of mice with established tumors. Interestingly, the enhanced immunogenicity of these vectors correlated with the apoptotic death of transfected cells, which facilitated their uptake and presentation by DC.

**Methodology for ex vivo generation of DC**

Investigators working in human and murine systems have discovered culture conditions that use hematopoietic cytokines to support the growth, differentiation, and maturation of large amounts of DC. Therefore, DC can also be purified from peripheral blood after removal of other defined T, B, NK and monocyte populations by using antibodies and magnetic beads or a cell-sorter. However, the very low frequencies of DC in accessible body samples, especially blood, limits the use of these DC for vaccination protocol. The ex vivo differentiation of DC progenitors can be traced easily by monitoring changes in some key surface molecules such as CD1a (acquired by DC) and CD14 (expressed by monocytes and lost by DC). Furthermore expression of co-stimulatory molecules such as CD40, CD80, CD86, as well as HLA antigens, can be used to evaluate the stage of differentiation and the degree of maturation of DC during *in vitro* culture. In addition, two new markers, CD83 and p55, have been shown to be selectively expressed by a small subset of mature DC differentiated in *in vitro* culture. According to the knowledge of DC ontogeny, two major strategies are used. The first is based on the ability of CD34+ progenitors isolated from bone marrow, peripheral blood, or neonatal cord blood to differentiate *ex vivo* within 12–14 days into mature CD1a+/CD83+ HLA-DR+ DC in the presence GM-CSF and TNF-α. Both stem cell factor (SCF) and FLT3 ligand are able to augment the DC yield if these key factors are present in the culture.

The maturation of DC from progenitors is influenced not only by cytokines, but also by extracellular matrix (ECM) proteins, such as fibronectin which has been reported to enhance DC maturation by mediating a specific adhesion through the α5β1 integrin receptor. The choice of culture conditions, especially the cytokine combination, will influence DC purity, maturation and function, and this is a consideration of prime importance before starting a DC-based immunotherapy strategy. A more practical approach is the production of DC from CD14+ monocytes, in the presence of GM-CSF and IL-4.

A future strategy for easy achievement of large amounts of DC is the *in vivo* injection of the same cytokines utilized for *ex vivo* DC generation. In fact, the administration of FLT3 ligand either in animals or in humans results in a reversible accumulation of functionally active DC in both lymphoid and non-lymphoid tissues. Therefore, in murine models it has been demonstrated that FLT3 ligand caused the regression of various tumors supporting the suggestion that DC may be directly involved in the antitumor effect of FLT3 ligand.

**Strategies for delivery of TAA into DC**

Several approaches for delivery of TAA into DC have been utilized. To date, in the clinical protocol of vaccination by DC both synthetic peptides corresponding to known tumor antigens and tumor-eluted peptides have been used for DC-mediated antigen presentation. While synthetic peptides represent only the limited antigenic repertoire of the presently known tumor antigens, tumor-eluted peptides, though originating from unknown proteins, may reflect a wider antigenic spectrum. Another potential disadvantage of using defined synthetic peptides to activate tumor-reactive T-cells is that the generated peptide-specific T-cells may not recognize autologous tumor cells expressing the antigen of interest. Loading DC with cocktails of different synthetic peptides, corresponding to different tumor antigens expressed by the same tumor, has been demonstrated to be a clinically effective procedure. Nevertheless it is possible that the synthetic peptide-approach will limit patient selection, on the basis of the HLA phenotype, and will prevent the possibility of activating both CD4 and CD8 T-cells directed to different epitopes of the same antigen. To bypass these disadvantages, several alternative methodologies using a mix of TAA have been developed. DC are able to internalize complete tumor lysates or apoptotic cells and to present derived antigen in an HLA I-restricted manner. In addition, DC secrete antigen-presenting vesicles, called exosomes, which express functional HLA class I and class II, and T-cell co-stimulatory molecules. Tumor peptide-pulsed DC-derived exosomes prime specific cytotoxic T-lymphocytes *in vivo* and eradicate or suppress growth of established murine tumors in a T-cell-dependent manner. However, a possible limitation of these approaches is the need for large numbers of primary samples or tumor cell lines and the complete lack of control of the nature of the antigens that are being presented by the DC. The use of RNA instead of protein could constitute a good alternative since it could be amplified *in vitro*. In addition, subtractive hybridization could allow the enrichment of tumor-specific RNA, thus limiting immune response against self antigen. A further possibility is the engineering of DC with expression vectors carrying TAA genes. Among the viral vectors, retroviral, adenoviral, and vaccinia vectors have been widely utilized to
transduce either monocyte or CD34+ cell-derived DC. Many authors have chosen retroviral vectors because retroviral transduced-DC should be able to constitutively express and process TAA to produce long-term antigen presentation in vivo. Specific CTLs against transduced-TAA are elicited by retrovirus-engineered DC. However, their low efficiency of transduction limits the clinical use of retroviruses.

In contrast, adenoviral vectors infect replicating and non-replicating cells, are easy to handle, and supernatant with clinical grade high titer is readily achievable. Both monocyte and CD34+ cell-derived DC can be transduced with high efficiency by adenovirus combined with poly-cations. Moreover, DC transduced by adenovirus maintain their APC functions. However, the use of adenovirus vectors is hampered by their immunogenicity which causes the rapid development of a CTL response that eliminates virus-infected cells and generation of neutralizing antibodies in recipients. Moreover, vaccinia virus which is a member of the poxvirus family, is not oncogenic, does not integrate into the host genome, is easy to manipulate genetically and is capable of accepting large fragments of heterologous DNA. The transduction of CD34+ cell-derived DC is feasible but their use is limited by the narrow therapeutic index between optimal transduction and target cell viability.

Antitumor vaccination: emerging clinical results

Clinical trials in solid tumors

Despite the fact that the large number of ongoing clinical trials which can be derived from the Physician Data Query (PDQ) of NCI (Figure 4) suggests a diffuse interest in immunotherapy, there is still a strong need to define the clinical impact of immunotherapy in the treatment of solid tumors. Table 4 summarizes the already published vaccination trials carried out using a) autologous or allogeneic neoplastic cells, b) synthetic peptides corresponding to defined TAA, alone or pulsed on autologous monocytes or DC.

Melanoma

Melanoma is the most striking example of a non-virus-induced immunogenic tumor in man that is able to elic-
it T-cell-mediated antitumor immunity. The majority of tumor antigens defined by T-cells have been identified utilizing patients’ T-lymphocytes as effector cells and tumor cells obtained from autologous (metastatic) tumor deposits as the target. Several investigators have isolated cross-reactive tumor-specific CTLs from peripheral blood, lymphocytes, or tumor-infiltrating lymphocytes of melanoma patients, and these CTLs are able to recognize common tumor antigen expressed in melanomas that share the restricting HLA class I allele. Thus, large numbers of ongoing or published clinical trials have been carried out on patients with metastatic melanoma. Experimental strategies are encouraged since with current standard therapies the prognosis of patients with metastatic melanoma is poor with a median survival of about 6 months. Several approaches to induce antitumor immune response have been reported.

**Irradiated tumor cells.** The initial anti-melanoma vaccines were similar in their preparation to the vaccines against infectious diseases. Crude preparations of homogenized tumor cells were mixed with immune-stimulating adjuvants such as viral or bacterial particles. Some of these early vaccines were tested in clinical trials and induced objective clinical responses in about 25% of the cancer patients. The most impressive trial was reported by Morton et al. who administered, to 136 stage IIIA and IV (American Joint Committee on Cancer, AJCC) melanoma patients, a polyvalent melanoma cell vaccine (MVC) comprising 3 allogeneic melanoma cell lines. Of 40 patients with evaluable disease, 9 (23%) had regressions (3 complete). Induction of cell-mediated and humoral immune responses to common melanoma-associated antigens present on autologous melanoma cells was observed in patients receiving the vaccine. Survival correlated significantly with DTH and antibody response to MVC and there was a 3-fold increase in 5-year survival of patients with stage IV melanoma. Livingston et al. randomized 122 stage III melanoma patients free of disease after surgery to receive treatment with the ganglioside GM2/BCG vaccine or with BCG alone. All patients were pretreated with low-dose cyclophosphamide. In most patients vaccinated with GM2/BCG, an antibody production against ganglioside was demonstrated and this was associated with a prolonged disease-free interval and survival, although the improvement did not reach statistical significance.

**Gene-modified tumor cell vaccine.** Autologous or allogeneic tumor or fibroblast cells have been modified to express cytokines and/or co-stimulatory molecules and/or a suicide compound. This genetic engineering is mainly performed ex vivo using retroviral vectors. In the published human trials, tumor cells have been transduced to express several cytokines. Arienti et al. described 12 stage IV melanoma patients who underwent vaccination with HLA-A2-compatible allogeneic human melanoma cells (5x10⁷ or 15x10⁷ cells) engineered to release IL-2. Little toxicity with three mixed clinical responses was recorded. Among the nine patients immunologically evaluated, peripheral blood lymphocytes from three patients displayed enhanced non-HLA-restricted cytotoxicity, and two of those individuals had an increased reactivity against tyrosinase peptide or gp-100 peptide after immu-

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Table 3. DNA vaccines in active immunotherapy of experimental mouse tumors.

<table>
<thead>
<tr>
<th>TAA</th>
<th>Experimental tumor</th>
<th>Route of inoculation</th>
<th>Adjuvant</th>
<th>Vaccine effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta-galactosidase</td>
<td>adenocarcinoma</td>
<td>gene gun bombardment</td>
<td>cytokines (IL-2, IL-12)</td>
<td>treatment of 2-day-old pulmonary metastases</td>
<td>(47)</td>
</tr>
<tr>
<td>(model TAA)</td>
<td></td>
<td>followed by cytokine i.p.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gag from M-MuLV</td>
<td>leukemia</td>
<td>i.m., 3 inoculations every 10 days</td>
<td>none</td>
<td>complete protection from challenge</td>
<td>(56)</td>
</tr>
<tr>
<td>HPV-E7</td>
<td>sarcoma</td>
<td>3 gene gun bombardments, every 2 weeks</td>
<td>none</td>
<td>complete protection from challenge</td>
<td>(48)</td>
</tr>
<tr>
<td>Neu</td>
<td>spontaneous mammalian tumor</td>
<td>i.m., 4 weekly inoculations</td>
<td>IL-2-encoding plasmid</td>
<td>partial protection from challenge</td>
<td>(50)</td>
</tr>
<tr>
<td>P1A</td>
<td>mastocytoma</td>
<td>i.m., 3 inoculations every 10 days</td>
<td>none</td>
<td>partial protection from challenge</td>
<td>(49)</td>
</tr>
<tr>
<td>Idiotyp/GM-CSF</td>
<td>B-cell lymphoma</td>
<td>i.m., 3 inoculation every 3 weeks</td>
<td>none</td>
<td>partial protection from challenge</td>
<td>(57)</td>
</tr>
<tr>
<td>fusion protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mouse TRP-2</td>
<td>melanoma</td>
<td>3 gene gun bombardments, every 2 weeks</td>
<td>IL-12-encoding plasmid</td>
<td>partial protection from challenge</td>
<td>(48)</td>
</tr>
<tr>
<td>human TRP-1</td>
<td>melanoma</td>
<td>5 gene gun bombardments, weekly</td>
<td>none</td>
<td>reduction of pulmonary metastases upon challenge; vitiligo</td>
<td>(52)</td>
</tr>
<tr>
<td>human TRP-2</td>
<td>melanoma</td>
<td>1-4 gene gun bombardments</td>
<td>GM-CSF (in therapy setting)</td>
<td>reduction of pulmonary metastases (prevention and therapy); vitiligo</td>
<td>(51)</td>
</tr>
<tr>
<td>mouse TRP-2</td>
<td>melanoma</td>
<td>i.m.</td>
<td>cardio-toxin</td>
<td>protection from challenge; no vitiligo</td>
<td>(53)</td>
</tr>
</tbody>
</table>
nization. In the only patient for whom the autologous melanoma line was available, and following in vitro stimulation of the PBLs after vaccination, the frequency of CTL precursor (CTLp) was significantly enhanced. Other groups utilized a similar approach transducing different cytokines, i.e. IL-12, IL-7, and GM-CSF. Despite documented specific antitumor reactivity with an increased frequency of anti-melanoma cytolytic precursor cells, negligible clinical results were demonstrated. To summarize, the advantage of a genetically modified autologous cell vaccine is that it contains the whole collection of tumor proteins and therefore has the greatest chance of inducing an immune response against relevant tumor antigens. However, growing autologous tumor cells in vitro to establish tumor cell lines is time-consuming and often unsuccessful. These studies are relevant since they show that injection of gene-modified cells into a patient a) is safe; b) is followed by efficient and variable transduction rate of host tissues; c) is associated with transgene expression in the patient; d) is associated with biological activity of the transgene product in most instances.

**Synthetic and natural peptides.** Phase I clinical trials have been carried out using synthetic peptides corresponding to defined TAA. The clinical trial using melanoma differentiation antigen (MAGE-3.A1) by Marchand et al.,\(^{123}\) enrolling 39 chemoresistant stage IV melanoma patients, was encouraging because monthly injection of 100-300 μg peptide alone was associated with tumor regression in 7 out of 26 patients who received the complete treatment. All but one of these regressions involved cutaneous metastases. No evidence for CTL response was found in the blood of the 4 patients who were analyzed, including 2 who displayed complete tumor regression. In contrast, Jager et al.\(^{211}\) immunized similar patients with gp100 peptide along with GM-CSF and, in some patients, were able to document an increase in the specific CTL activity against the immunizing peptide. Rosenberg et al.\(^{212}\) reported that 31 metastatic melanoma patients were immunized with the modified g209–2M peptide in incomplete Freund's adjuvant (IFA) along with IL-2 obtaining tumor regression in 42% of patients. Peripheral blood mononuclear cells harvested from these patients after, but not before, immunization exhibited a high degree of reactivity against the native g209–217 peptide, as well as against HLA-A2+ melanoma cells.

These studies indicate that vaccination with synthetic peptides is well tolerated, with occasional occurrence of mild fever and inflammation at the site of injection. Nonetheless, it should be pointed out that there is usually a poor correlation between induction of specific T-cells and the clinical response. The reasons for this discrepancy might be the selection of the patients enrolled in the trials since the majority of patients were in stage IV with large amounts of disease.

**Dendritic cells.** Autologous DC generated from peripheral blood monocytes have been utilized as antigen-presenting cells after their loading with specific melanoma antigens. Chakraborty et al.\(^{214}\) found that intradermal administration of DC pulsed with a MAGE-1 HLA class I-restricted peptide could elicit peptide and autologous melanoma reactive-CTLs in patients with advanced melanoma. However, despite the presence of these CTLp in the vaccination site, peripheral blood, and distant tumor sites, no significant therapeutic responses were seen.

Nestle et al.\(^{195}\) recently described the immunization of 16 melanoma patients using DC loaded with melanoma peptides or tumor lysates. DC were pulsed with a cocktail of gp100, MART-1, tyrosinase, MAGE-1, or MAGE-3 peptides chosen to suit the individual patient class I HLA molecules. Four patients whose HLA haplotype was inappropriate for peptide pulsing received DC pulsed with autologous tumor lysate. Keyhole limpet hemocyanin (KLH) was included during antigen pulsing. DC were administered by direct injection into uninvolved lymph nodes via ultrasound guidance to facilitate entry into the lymphatics and to minimize DC loss. Patients received 6-10 injections of 1x10⁶ cells every 1-4 weeks. Toxicity was limited to mild local reactions at the injection sites. Immunologic monitoring revealed DTH skin reactions to peptides in 11 cases, and peptide-specific CTLs could be recovered from the skin biopsies of some patients. Regression of tumor was seen in 5 out 16 patients, including 2 complete responses lasting over 15 months. Responding tumor sites included skin, lung, soft tissue, bone, and pancreas. Importantly, two of the responding patients received only tumor lysate-pulsed DC, suggesting an approach applicable to cancers lacking defined tumor antigens.

**Colon cancer**

Colon cancer is potentially curable by surgery; the cure rate is, however, moderate to poor depending on the extent of disease. Adjuvant chemotherapy with 5-fluorouracil plus levamisole or folinic acid is the standard treatment for stage III colon cancer based on the results of numerous co-operative and intergroup clinical studies. In contrast, adjuvant chemotherapy for stage II disease has no benefit.\(^{216}\) Despite several immunotherapeutic approaches having been tested for colon cancer patients, only one study has reported clinical results. In a prospective randomized study,\(^{217}\) 254 patients with stage II or III post-surgery colon cancer were randomly assigned to receive active specific immunotherapy, namely autologous tumor cell–bacille Calmette-Guérin (BCG) or no adjuvant treatment. The immunotherapy program comprised three weekly intradermal injections starting 4 weeks after surgery, with a booster vaccination at 6 months with 10⁷ irradiated autologous tumor cells. The first vaccination contained 10⁷ BCG organ-
isms. The 5-year median follow-up showed a 44% reduction of risk of recurrence in all patients receiving the vaccinations. The major impact of immunotherapy was evident in patients with stage II disease, who had a significantly longer disease-free period and 61% risk reduction. In addition, no patient discontinued treatment early because of side effects.

Recently, Foon et al. generated anti-idiotype antibody, designated CeaVac, that is an internal image of CEA. Thirty-two patients with resected Dukes’ B, C, and D, and incompletely resected Dukes’ D disease were treated with 2 mg of CeaVac every other week for four injections and then monthly until tumor recurrence or progression. Fourteen patients were treated concurrently with a 5-FU chemotherapy regimen. All 32 patients entered into this trial generated a potent anti-CEA humoral and cellular immune response. Interestingly, the 5-FU regimen did not affect the immune response. A phase III trial for patients with resected colon cancer is ongoing.

Prostate cancer

Several prostate-tissue-associated antigens, including prostatic alkaline phosphatase (PAP), prostate-specific membrane antigen (PSMA), and prostate-specific antigen (PSA), are now being explored as targets for prostate cancer immunotherapy. Valone et al. have carried out a dose-escalation trial of peripheral blood DC pulsed with recombinant PAP protein in 12 patients with advanced prostate cancer. Intravenous administration of 0.3, 0.6, and 1.2x10^9 pulsed cells/m^2 monthly for three months resulted in T-cell proliferative responses against PAP in all patients, the magnitude of which was related to cell dosage. Toxicity was limited to myalgias in three patients. Clinical outcomes have not been reported.

Renal cell carcinoma

Renal cell carcinoma accounts for 2% of all malignancies and many patients have metastatic disease at diagnosis and the prognosis is unfavorable. At present, neither chemotherapy nor radiation therapy has any significant influence on the course of disease or the survival time. Immunotherapy using recombinant IL-2 alone or combined with interferon-α is currently the standard therapy for metastatic renal cell carcinoma. Cellular immunotherapy includes the adoptive transfer of in vitro expanded tumor infiltrating lymphocytes as well as active immunotherapy with an autologous tumor cell vaccine engineered to secrete GM-CSF. Although each of these attempts generated promising results neither attempt met the expectations. Recently, Holtl et al. have administered, to 4 metastatic renal cell carcinoma patients, autologous monocyte-derived DC pulsed with autologous tumor cell lysate. Each patient received 3 monthly intravenous infusions with the immunogeneic KLH. Initial results have shown
Table 4. Phase I/II trials of vaccination in patients with solid tumor.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Stage</th>
<th>Pts</th>
<th>Vaccine</th>
<th>Route of Admin.</th>
<th>TA Ads</th>
<th>Side Effects</th>
<th>Clinical Results</th>
<th>Immunol Results</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanoma</td>
<td>III/IV</td>
<td>136</td>
<td>Melanoma Cell Vaccine (MCV)</td>
<td>ID</td>
<td>Human Melanoma Cell Lines</td>
<td>Yes/No</td>
<td>Mild (Erithema, Fever)</td>
<td>9/40 evaluable pts (3 CR, 8 PR)</td>
<td>Cell mediated and humoral responses to MCV; 1 Activation of TIL</td>
</tr>
<tr>
<td>N</td>
<td>12</td>
<td></td>
<td>IL-2 Transduced-Melanoma Cell Line</td>
<td>SC</td>
<td>Melanoma Cell Line</td>
<td>No</td>
<td>Mild (Erithema, Fever)</td>
<td>3 MR; 1 SD</td>
<td>1 Melanoma-Specific CTLp</td>
</tr>
<tr>
<td>N</td>
<td>10</td>
<td></td>
<td>IL-7 Transduced-Autologous Melanoma Cells</td>
<td>SC</td>
<td>Autologous Melanoma Cells</td>
<td>No</td>
<td>Mild (Fever)</td>
<td>2 MR 5 SD</td>
<td>1 Melanoma-Specific CTLp</td>
</tr>
<tr>
<td>N</td>
<td>21</td>
<td></td>
<td>GM-CSF Transduced-Autologous Melanoma Cells</td>
<td>ID and SC</td>
<td>Autologous Melanoma Cells</td>
<td>No</td>
<td>Mild (Erithema, Induration, Itching)</td>
<td>1 PR; 1 MR; 3 Minor Resp</td>
<td>1 Melanoma-Specific CTLs; 80% Tumor Destruction into Metastases</td>
</tr>
<tr>
<td>N</td>
<td>31</td>
<td></td>
<td>Peptide+IL-2</td>
<td>SC</td>
<td>modified g209-2M</td>
<td>IL-2 and IFA</td>
<td>Mild (Erithema)</td>
<td>6 PR; 3 MR; 3 SD</td>
<td>1 Melanoma-Specific CTLp</td>
</tr>
<tr>
<td>N</td>
<td>39</td>
<td></td>
<td>Peptide</td>
<td>SC and ID</td>
<td>MAGE-3 (HLA-A1)</td>
<td>No</td>
<td>Mild</td>
<td>3 CR; 4 PR</td>
<td>No Increase of antMAGE-3 CTLs even in Responders</td>
</tr>
<tr>
<td>N</td>
<td>16</td>
<td></td>
<td>Ag Pulsed Autologous Monocyte-derived DCs</td>
<td>Lymph nodes</td>
<td>Pulsed Peptide Cocktail</td>
<td>MAGE-1</td>
<td>Mild</td>
<td>2 CR; 3 PR; 1 MR</td>
<td>DTH to KLH in 16; DTH to peptide-pulsed DCs in 11</td>
</tr>
<tr>
<td>N</td>
<td>17</td>
<td></td>
<td>Ag Pulsed Autologous Monocyte-derived DCs</td>
<td>ID</td>
<td>Autologous Tumor Lysate</td>
<td>No</td>
<td>No</td>
<td>1 PR</td>
<td>DTH to Vaccine in SH7; CD8 Cells in expanded-VL</td>
</tr>
<tr>
<td>Colon Cancer</td>
<td>II and III</td>
<td>254</td>
<td>Irradiated Autologous Tumor Cells</td>
<td>ID</td>
<td>Autologous Tumor Cells</td>
<td>BCG</td>
<td>Mild</td>
<td>Stage II: 61% Risk Reduction for Recurrence; Stage III: no Significant Benefits</td>
<td>DTHs+ ≥30% Pts</td>
</tr>
<tr>
<td>Prostate Cancer</td>
<td>Locally Advanced</td>
<td>82</td>
<td>Ag Pulsed Autologous Tumor Cells</td>
<td>Lymph nodes</td>
<td>Peptide Cocktail</td>
<td>Autologous Tumor Lysate</td>
<td>KLH</td>
<td>Mild</td>
<td>2 CR; 3 PR; 1 MR</td>
</tr>
<tr>
<td>Renal Cell Cancer</td>
<td>N</td>
<td>12</td>
<td>Ag Pulsed Monocyte-derived DCs</td>
<td>IV</td>
<td>Autologous Tumor Lysate</td>
<td>KLH</td>
<td>Mild</td>
<td>1 PR</td>
<td>DTH to KLH after 1+ and 2+ Vaccination</td>
</tr>
</tbody>
</table>

| Pts: Patients; TA: Tumor Antigens; Ads: Adjuvants; SC: Subcutaneous; MR: Mixed Response; SD: Stable Disease; CTLp: Cytotoxic T Lymphocyte precursor; ID: Intradermic; PR: Partial Response; TIL: Tumor Infiltrating Lymphocytes; IFA: Incomplete Freund's Adjuvant; KLH: Keyhole Lymocianin; DTH: Delayed Type Hypersensitivity; IV: Intravenous

that a potent immunologic response to KLH and, most importantly, against cell lystate could be measured in vitro after the vaccinations. In addition, the treatment was well tolerated with moderate fever as the only side effect. In contrast, only one partial response after 2 vaccinations was observed.

Recently, Kugler et al.228 vaccinated 17 patients with metastatic renal cell carcinoma using hybrids of autologous tumor and allogeneic DC generated by an electrofusin technique. After vaccination, and with a mean follow-up time of 13 months, four patients completely rejected all metastatic tumor lesions, one presented a mixed response, and two had a tumor mass reduction of greater 50%. These promising data indicate that hybrid cell vaccination is a safe and effective therapy for renal cell carcinoma and may provide a broadly applicable strategy for other malignancies with unknown antigens.

Hematologic malignancies

Tumor vaccines in B-cell lymphoproliferative disorders

Multiple myeloma (MM) and low-grade non-Hodgkin’s lymphomas (NHL) are clonal expansions of lymphoid cells that have rearranged immunoglobulin (Ig) genes. Early during development, pre-B-cells become committed to the expression of a heavy and light chain Ig variable region. The heavy chain derives from the recombination of variable (V) with diversity (D) and joining (J) region genes with a constant region (C). The V-D-J joins occur with a variable number of nucleotide insertions or deletions resulting in a unique sequence which creates the third hypervariable region (CDR III) and contributes to the antigen-binding site. These antigenic regions (idiotype; Id) are characteristic for any given Ig-producing tumor (e.g. MM and NHL) and can be recognized by an immune response consisting of anti-Id antibodies and/or by Id reactive T-cells.229-235 The tumor-derived Id is a self protein which, in most circumstances, is poorly immunogenic. However, hapten and adjuvants, including cytokines, have been used in several animal models to increase Id immunogenicity and establish protective anti-Id-immunity.236 Lately, Id vaccines have come into medical use in patients with lymphoma and MM.

Idiotype vaccination in human lymphoma

A pioneering study was carried out in 9 lymphoma patients in CR or partial remission. They were immunized with subcutaneous injections of autologous Id, conjugated to KLH and emulsified in an oil-in-water emulsion containing non-ionic block polymers.80 Specific anti-Id humoral and/or cellular responses were observed in 7/9 patients. Two patients with measurable disease showed a clinical improvement. These results have been con-
confirmed in a larger series of patients. Following standard chemotherapy, 41 patients with B-cell lymphoma received subcutaneous injections of autologous Id-KLH conjugates mixed with an oil-in-water emulsion containing non-ionic block polymers and threonyl-muramyl dipeptide. Approximately 50% of the patients generated specific anti-Id responses and isolated tumor regressions were observed. In particular, 11/16 patients had a significant increase in the frequency of tumor-specific cytotoxic T-lymphocytes precursor (CTLp). The median duration of freedom from cancer-progression was significantly prolonged and this resulted in a survival advantage, especially in patients who generated cell-mediated anti-Id immunity.

These pioneering studies did not prospectively investigate the effect of Id vaccines on tumor burden, since most patients were already in clinical remission, and standard tumor regression criteria could not be used. A recent study has directly evaluated the ability of Id vaccines to eradicate residual t(14;18)- lymphoma cells in 20 patients in first remission after ProMACE-based chemotherapy. These patients received multiple injections of Id /KLH conjugates in the presence of GM-CSF. Eight of eleven patients with detectable translocations in the peripheral blood converted to a PCR negative status after vaccination. Tumor-specific cytotoxic CD8+ and CD4+ T-cells were uniformly seen in most patients. Antibodies to autologous Id were also detected, but they were apparently not required for molecular remission since the latter was achieved in some patients without a detectable antibody response. Clinical monitoring indicates a 90% disease-free survival after a median follow-up of 3 years. This is encouraging compared with the 44% disease-free survival (after a median follow-up of 3 years) in another series of patients treated at the same Institution with anti-B4-blocked ricin in first remission.

In a pilot study, five MM patients were repeatedly immunized with autologous Id precipitated in alumini-um phosphate suspension. Four patients were previously untreated and one patient was in stable-partial remission following chemotherapy. Three patients developed specific anti-Id T- and B-cell responses, but these responses were transient and their magnitude was low. A more effective immunization schedule was developed with the goal of achieving long-lasting T-cell anti-Id immunity. This effort was focused on early stage MM, based on the assumption that Id-specific T-cells are present at higher frequency mainly in patients with early stage MM or MGUS. Most of these Id-reactive T-cells are Th1-type cells in early stage MM, whereas they are predominantly Th2-type in patients with advanced disease. Thus, a more effective antitumor T-cell immune response may be expected if vaccines are delivered when the frequency of T-cells with the potential to develop cytotoxic activity is higher. In this series, patients received subcutaneous injections of autologous Id precipitated in aluminium phosphate suspension, together with free GM-CSF. Long-lasting Id-specific T-cell responses were induced in all five immunized patients. Moreover, one patient showed a decrease of circulating Id upon immunization.

A vaccination trial in which Id/KLH conjugates and GM-CSF were administered subcutaneously as a maintenance treatment after high-dose chemotherapy and PBPC infusion has recently been published. Most patients generated Id-specific DTH reactions. DTH specificity was confirmed in one patient by investigating the reactivity to synthetic peptides derived from the VDJ sequence of the tumor-specific Ig heavy chain. In 3 patients with minimal residual disease, the DTH skin tests remained positive up to two years after the last immunization, but residual tumor cells were not eliminated by these long-lasting immune responses. Nevertheless, these patients remained in clinical remission without any further maintenance treatment. Thus, it is possible to generate anti-Id immune responses that are not potent enough to eliminate residual tumor cells, but are sufficient to hold the disease in check for extended periods.

These results have been confirmed in a preliminary report of 18 patients with MM receiving Id/KLH conjugates and GM-CSF in first remission after high-dose chemotherapy and tandem transplantation followed by PBPC infusions. In particular, 50% of the patients generated positive DTH reactions and 2 patients, in partial remission at the time of vaccination, achieved a complete remission following vaccination. A retrospective pair-mate analysis has shown a trend for a better clinical outcome in MM patients receiving Id vaccines compared to those receiving IFN-α alone. This tendency is particularly evident in patients who generated positive Id-specific T-cell responses. Although preliminary and retrospective, this is the first study providing clinical evidence that the generation of T-cell immune response against tumor cells may positively influence the clinical outcome of MM patients in the remission phase (N. Munshi and L. Kwak, personal communications).

**DC-based anti-Id vaccination**

As previously discussed, a proportion of lymphoma and MM patients enrolled in clinical trials mounted an Id-restricted antibody and T-cell response and some of them showed tumor regression. However, there are important differences between lymphoma cells and MM
plasma cells. In the case of lymphomas, the cells are characterized by high surface expression and little antibody secretion whereas myeloma cells have very low levels of cell surface Ig with high levels of antibody secretion. Thus, it is unlikely that the sole generation of an antibody-based anti-Id immune response will be beneficial for MM patients. In fact, anti-Id antibodies may be blocked from reaching the tumor cells by the high levels of circulating Id. Moreover, despite the existence of a pre-plasma cell stem cell compartment in MM with a higher expression of surface Ig, it is possible that tumor cells would not express enough target protein for the antibodies to be effective. Conversely, an Id specific T-cell response would not need to bind to cell surface Ig to be active. T-cells do not recognize intact protein, but are specific for processed peptide fragments of the Id expressed on class I or II molecules. The advantage of a cytotoxic T-cell response is that it would not be blocked by free circulating paraprotein and would not depend on the expression of the native protein on the surface of tumor cells. Moreover, B-cells, including putative myeloma stem cells, are known to process and present peptides of the Ig on their membrane associated with class I and II molecules. Therefore, optimal strategies for Id vaccination may require the induction of a T-cell-mediated immune response which is best achieved by the use of APC. In this view, the rapid generation of a T-cell immunity in healthy volunteers after a single injection of mature DC has recently been described. 243 Nine normal subjects were given subcutaneous injections of monocyte-derived immature DC pulsed or pulsed with KLH, tetanus toxoid (TT) or HLA-A*0201-positive restricted influenza matrix peptide. Four other individuals received these antigens without DC. Of note, administration of unpulsed DC or antigens alone failed to induce any T-cell response. Conversely, a CD4+ T-cell response was observed in 9/9 and 5/6 subjects injected with KLH or TT-pulsed DC, respectively. Moreover, a significant stimulation of effector and memory CD8+ CTLs was also reported. This feasibility trial provides the first controlled evidence of the capacity of DC to stimulate T-cell immunity.

DC-based anti-Id vaccination has been reported in B-cell malignancies in a few papers. Hsu et al. have described 4 low-grade non-Hodgkin’s lymphoma (NHL) patients resistant to conventional chemotherapy or relapsed who were injected intravenously with Id-pulsed DC freshly isolated from PB by subsequent enrichment steps. A tumor-specific T-cell response was observed in all cases associated, in one case, with tumor regression. Sixteen patients have been treated so far and an anti-Id restricted cellular response has been observed in 8 subjects (R. Levy, personal communication). The same strategy of targeting the Id has been applied by the same group to induce a T-cell immune response in MM patients. 249 Twelve patients were injected, 3 to 7 months after autologous stem cell transplantation, with Id-pulsed DC followed by 5 subcutaneous boosts of Id/KLH administered with adjuvant. Whereas 11/12 patients developed a strong KLH-specific cellular proliferative response, thus suggesting immunocompetence after high-dose chemotherapy, only 2 individuals generated an anti-Id restricted T-cell proliferation and only 1/3 patients showed a transient Id-specific CTLs response. This approach raises concerns about the efficacy of uncultured blood DC of stimulating efficiently T-cells, the capacity of Id-loaded DC to reach secondary lymphoid tissues to prime T-cells escaping the entrapment of the lungs and the role of Id-KLH boosts after DC administration.

Wen et al. 176 reported immunization of one MM patient injected with Id-pulsed DC derived from adherent mononuclear cells in the presence of appropriate cytokines. In this paper, Id-specific T-cell proliferation and secretion of IFN-γ were reported, as were the production of anti-Id antibodies. Similar results have recently been reported by Cull et al. who treated two patients with advanced refractory myeloma with a series of four vaccinations using autologous Id-protein pulsed DC combined with adjuvant GM-CSF. 247 DC were derived from adherent mononuclear cells. Both patients generated a specific T-cell proliferative response that was associated with the production of IFN-γ, indicating a Th1-like response. However, no Id-specific cytotoxic T-cell response could be demonstrated. Lim and Bailey-Wood have also treated 6 MM with DC generated from adherent mononuclear cells. 248 DC were pulsed with the autologous Id and KLH as a control vaccine. All patients developed both B- and T-cell responses to KLH, suggesting the integrity of the host immune system. Id-specific responses were also observed. In one patient, a modest but consistent drop in the serum Id level was observed. Lastly, a vaccine formulation based on CD34 stem cell-derived DC pulsed with Id-derived peptides has recently been used in 11 MM patients with advanced disease. 249 Five patients generated Id-specific immune responses and one patient showed a decreased plasma cell infiltration in the bone marrow.

New strategies in Id vaccination

The generation of an effective antitumor response greatly depends on the final activation of tumor-specific cytolytic DC8 cells. This is the final event resulting from a series of cognate and non-cognate interactions occurring among tumor cells, professional APC, CD4, and CD8 cells. Each of these cell populations plays a unique role, and may represent a possible target of immune intervention to improve the efficacy of vaccination. Malignant B-cells can be modified to become efficient APCs themselves and present peptides from their own tumor-specific antigens to autologous T cells. To this end, a number of strategies are currently under preclinical and clinical evaluation. One possibility is to fuse tumor cells with dendritic cells. The fusion product will combine the functional properties of DC with the full antigenic repertoire of tumor cells. 250 As an alternative, malignant B-cells can be turned into effective APC by stimulating cell surface CD40 with its specific counter-receptor CD40 ligand. 251 Genetic engineering
is another approach that may turn malignant B-cells into effective APC. Transfection with immunologically relevant DNA sequences coding for cytokines or co-stimulatory molecules greatly enhances the ability of malignant B-cells to activate antitumor immune responses. This approach has recently been used in MM taking advantage of the selective expression of functional adenoviral receptors on the cell surface of myeloma cells. Interestingly, there has been a description of the immunization of a matched related donor of allogeneic bone marrow with the myeloma derived Id (conjugated with KLH) isolated pretransplant from patients. After transplantation, a CD4+ T-cell line was established from the peripheral blood of the recipient and found to be of donor origin and proliferate specifically in response to the myeloma Id. Thus, this experience demonstrates the principle of transfer of donor immunity with the advantage of immunizing a tumor naive donor who may be more likely to be able to generate an immune response against the tumor Ig without interference or suppression by the malignant cells. However, the lack of an anti-Id CTL response and ethical concerns on the immunization of healthy donors with tumor-derived products, suggest that in the future an alternative strategy based upon the generation, ex vivo, of Id-reactive CTL clones by means of APC, will be needed.

The prerequisite for any vaccine-based strategy is the possibility of differentiating tumor cells from normal cells. Id is absolutely tumor-specific, but is not directly related to the malignant phenotype of myeloma cells, and is self-Ag. As such, it is protected by self-tolerance mechanisms. There is a growing list of alternative tumor-specific antigens that can be exploited as targets for active specific immunotherapy. Among others, the core protein of Muc-1, antigens encoded by MAGE-type genes, overexpressed or fusion proteins resulting from chromosomal abnormalities may all represent alternative immunogens. Compared to Id, some of these antigens may be more intrinsically related to the malignant phenotype of tumor cells.

Polymorphism of the HLA molecules is a major obstacle in the outcome of vaccines aimed at triggering cytolytic T-cell responses. By combining amino acid sequencing of tumor-specific antigens and HLA typing, it is now possible to predict whether the HLA alleles of a given individual can bind tumor-derived peptides. Several groups are planning to use Id vaccination only in those patients for whom preliminary sequencing demonstrates a compatible restriction between HLA and peptides.

Finally, a new generation of immunogens has been developed using DNA-based technologies. The whole tumor-specific immunoglobulin or the variable region sequences of both heavy- and light-chains have been used as immunogens or to produce recombinant proteins in bacteria. However, it has soon become clear that naked DNA is not immunogenic per se and additional sequences are required to elicit protective immune responses. Sequences coding for cytokines, chemokines or xeno-}

**Antitumor vaccination**

Chronic myelogenous leukemia (CML) is a biphasic neoplastic disorder with a prolonged indolent phase lasting an average of 4 years followed by an acute phase of blastic transformation which inevitably leads rapidly to death. There is no curative therapy for CML other than allogeneic bone marrow transplantation, an option that is available only to a small fraction of patients who have both a matched donor and are young enough to tolerate the procedure.

Recently, IFN-α has been shown to induce hematologic remissions in most CML patients, with a relevant portion of them also experiencing several degrees of cytogenetic response and ultimately a statistically significant prolongation of their chronic phase. However, still too few CML patients are long survivors if not cured regardless of the treatment option they received. Because of the unique features of this disease, the hallmark translocation that characterizes all neoplastic cells, a therapeutic targeting approach only the Ph+ clone could be a powerful tool in the treatment of CML.

The first direct evidence of the immune system’s crucial role in recognizing and eliminating Ph+ CML cells came from the demonstration that infusion of large doses of peripheral blood leukocytes from the marrow donor induced durable remission in patients with CML who had relapsed following a T cell depleted marrow allograft. This latter finding proved that in CML the graft-versus-tumor effect is mediated by the cellular arm of the immune system. While the nature of the response is likely to be largely allogeneic a possible role for specific anti-CML responses is suggested by the lack of this observation in patients with other myeloid leukemias undergoing the same treatment.

The hypothesis that CML cells could be recognized by the immune system through the presentation of P210, the tumor-specific product of the bcr/abl hybrid gene, was first tested. The evidence that P210 b3a2-breakpoint peptides were able to bind HLA class I and HLA class II molecules and to elicit specific T cell responses in normal donors provided the rationale for a peptide vaccine in CML patients. Pinilla-Ibarz et al. have recently completed a phase I dose escalation trial (5 doses over 10 weeks) of a multivalent peptide vaccine (5 peptides) plus QS21 in patients with CML and b3a2 breakpoint. Patient characteristics included hematologic remission, IFN-α therapy and no HLA restriction. In a preliminary report the peptide vaccine appeared safe with patients experiencing only minimal discomfort at the site of injection.

With regards to the immune response, peptide-specific delayed hypersensitivity (DTH) in vivo and peptide-specific proliferation in vitro were shown but no peptide-specific CTL response was induced. Bocchia et al. are currently conducting a multicenter phase I/II trial of a pen-
tavalent peptide vaccine plus QS21 and GM-CSF in b3a2-
CML patients expressing any of HLA A3, A11, B8 or DR11. 
Patient characteristics also include major or complete 
cytogenetic response with or without IFN-α maintaining 
therapy. The protocol comprises 6 s.c. vaccinations with 
peptides + QS21 at 2 weekly intervals, with GM-CSF 
 injected at the vaccine site for 4 consecutive days start-
ing the day before each vaccination. Goals of the study 
are the evaluation of the induction of peptide-specific T-
cell response, of the role of GM-CSF as immunologic 
adjuvant in CML patients and the impact of the peptide 
vaccine on minimal residual disease.

As in other cancers, the use of DC as powerful induc-
ers of an active specific immune response in CML is now 
under in vitro evaluation. Interestingly, most CML-
derived DC carry the t(9;22) translocation and therefore 
could naturally present P210 derived peptides. In fact, 
CML derived Ph+ DC were able to strongly stimulate 
autologous T-cells that displayed vigorous cytotoxicity 
activity against autologous CML cells but low reactivity 
to HLA-matched normal bone marrow cells or autolo-
gous remission state bone marrow mononuclear cells. P210-
derived peptides could have a role in inducing this 
leukemia-specific response and a vaccine strategy which 
combines Ph+ DC and breakpoint peptides will be inves-
tigated.

**Conclusions**

This review shows that there are many prospects of 
curing cancer through the active induction of a specific 
immune response to TAA. The terms of the matter are 
now defined with molecular and genetic details for 
melanomas. Ongoing research is aimed at defining TAA 
on other forms of tumors. Indeed, experimental data and 
very recent clinical evidence suggest that antitumor vac-
cines will soon be a new form of tumor treatment that 
will be able to be adopted for the management of 
defined stages of neoplastic disease, in sequential asso-
ciation with conventional treatments.

Prediction of when the efficacy of antitumor vaccina-
tion will be assessed and will become a routine proce-
dure is beyond a simple scientific evaluation. While pre-
clinical research has identified several possible targets 
and strategies for tumor vaccination, the clinical sce-
nario is far more complex and as yet no specific clue has 
emerged to clearly envisage a clinical development strat-
agy which could make biotechnology investments in this 
area attractive enough to pharmaceutical companies. 
Patent issue complexity further contributes to slowing 
down the development of expensive clinical trial pro-
grams. A cautious, yet attentive attitude seems, at the 
moment, to be the general behavior of the pharmaceu-
tical industry.

At present peptide vaccination may appear of more 
immediate application. Several phase I clinical trials have 
already been carried out using synthetic peptides from 
defined TAA. These peptides have been administered 
alone or combined with adjuvants, or presented by 
monocytes or DC. Nearly all studies indicate that this 
form of vaccination is well tolerated, mild fever and 
inflammation at the site of injection being the only occa-
sional side effects observed. Nonetheless it should be 
pointed out that there is usually a poor correlation 
between peptide ability to induce a T-cell response and 
clinical response. Among the several reasons that may 
account for this discrepancy, the choice of the peptides 
may have a critical importance. Most peptide-based vac-
cines have considered HLA class I restricted peptides 
only, whereas there is increasing evidence that tumor-
specific CD4+ T-cells may be important in inducing an 
effective antitumor immunity. The addition of peptides 
that bind class II HLA glycoproteins to peptide vaccines 
could lead to an amplification of the immune response 
as well as to better clinical effect.

A survey of the outcomes of vaccination trials shows 
that the poor correlation between induction of immuno-
logic responses and the clinical results is a consistent 
finding, independently of the immunizing strategy 
adopted. Many factors may contribute to this poor cor-
relation, e.g.:

a) the selection of the patients enrolled in the trial: 
tumor burden, stage of disease, and others, as discussed 
above;

b) the techniques used for the immune monitoring in 
*vitro*: most of the current studies evaluate T-cell induc-
tion through *in vitro* peptide stimulation of PBMC, while 
the use of tetrameric soluble class I-peptide complex-
es or reverse solid phase ELISPOT analysis may pro-
vide complementary information;

c) the assays for immune monitoring *in vivo*: often a 
positive DTH test does not correlate with evident tumor 
regression.

Perhaps, fine needle biopsies at the site of regressing 
and non-regressing tumors could provide a more direct 
insight into the events associated with the clinical out-
come. The immune pattern within the tumor should be 
compared to the one found in the skin. This should allow 
evaluation of the exact role of vaccine-induced T-cells. 
Which of the existing *in vitro* and *in vivo* assays corre-
lates most accurately with clinical responses remains to 
be established.

Several recent studies showing that the immune sys-
tem recognizes TAA during tumor growth did not clari-
fy whether such recognition was indeed associated with 
subsequent tumor cell destruction. The development of 
reliable assays for efficient monitoring of the state of 
immunization of cancer patients against TAA is as an 
important goal that will markedly affect the progress of 
antitumor vaccines. A major problem in testing the effi-
cacy of antitumor vaccination in adjuvant settings 
depends on both the long period of time and large num-
ber of patients required. The possibility of effectively 
monitoring the immune response induced acquires crit-
ical importance since it may provide a much earlier sur-
rrogate end-point, predictive of the clinical outcome.

The downregulation of the expression of TAA repres-
ents another crucial issue for vaccination therapies, 
since it may lead to the immunoselection of tumor cell
clones that hide the target TAA. An ideal TAA is a protein that is essential for sustaining the malignant phenotype, and that is not stripped or downregulated by the immune reaction. Mutations that give rise to TAA of this kind have been described.\textsuperscript{272} However, they will be an appropriate target only for the tumors expressing these particular mutations and will not be suitable for more general cancer vaccines. Improvements in the identification of tumor-associated mutations that may be potentially recognized by the immune system may also open up the possibility of tailoring individual cancer vaccines. The recent report of the construction of fusion cells composed of autologous tumor cells and DC or TAA pulsed–DC represents a step forward towards the quick manufacture of tumor-specific and individualized vaccines. In contrast, the characterization of the telomerase catalytic subunit (hTERT) expressed in more than 85% of human cancers appears to open the way to a novel strategy for a general anticancer vaccination targeting a widely distributed TAA.\textsuperscript{273}

The \textit{in vivo} or \textit{ex vivo} introduction of TAA genes into DC through recombinant viral vectors is still hampered by the lack of an ideal viral vector and by the induction of an immune response against the viral proteins. Nonetheless, many viral vectors successfully used in animal models and currently tested in clinical trials appear to be safe vehicles for gene transfer, without any major toxicity. To control transgene expression levels better, investigators are exploiting tissue- or cell-specific regulatory elements such as cytomegalovirus promoters and enhancers that are preferentially expressed in tumor cells.

Certainly the new prospects opened by antitumor vaccines are fascinating. When compared with conventional cancer management, vaccination is a soft, non-invasive treatment free from particular distress and iatrogenic side effects. Antitumor vaccines can be expected to have a considerable social impact, but a few large clinical trials enrolling the appropriate patients are now necessary to assess their efficacy.

This review will end considering their use not in the treatment of cancer patients but to prevent cancer in healthy persons, a so far neglected prospect. Current studies are leading to the detection of gene mutations that predispose to cancer.\textsuperscript{274} Identification of the gene at risk and its mutated or amplified products would provide the opportunity to vaccinate susceptible subjects against their foreseeable cancer. Molecular characterization of altered gene products predictably destined to become a tumor antigen will be the first step towards the engineering of effective vaccines to be used for this purpose.\textsuperscript{26}

An unrestrained imagination may picture an even broader application of antitumor vaccines, i.e. their use to prevent tumors in the general population. Molecular and genetic data suggest that the number of TAA is not endless. Several of the TAA detected so far are shared by histologically distinct tumors arising in different organs (Table 1). The possibility of vaccinating against most common human cancers by using not many more than twenty TAA may perhaps be conceivable. Experimental data suggest that the immunity elicited by specific vaccination is much more effective in the inhibition of incipient tumors than in the cure of those that have already progressed.\textsuperscript{275} The risk of inducing an autoimmune disease would be a major worry since not rarely antigens acting as TAA are expressed by normal tissues.\textsuperscript{276} This risk would be much harder to accept when treating healthy individuals than in the vaccination of cancer patients, where the scales of risk-benefit are biased by a short life-expectancy. On the other hand failure to intervene when a disease so diffuse and dramatic as cancer can be prevented could also be viewed as harmful.\textsuperscript{277} Lastly, it should be considered that the same or even a higher risk of inducing autoimmune reactions is associated with many antimicrobial vaccines. Fortunately, they started to be used when this risk was not yet perceived.

In conclusion, even if cancer vaccines are an old dream,\textsuperscript{278} only recently has their design become a rational enterprise. There are now many ways of constructing vaccines able to elicit a strong protective immunity. This progress is offering ground for optimism.

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Conflict on interest: Dompé Biotec Spa sells G-CSF and rHuEpo in Italy, and Amgen Italia Spa has a stake in Dompé Biotec Spa.

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\textbf{References}

19. Robbins PF, Kawakami Y. Human tumor antigens recog-
18. Coulie PG. Human tumour antigens recognized by T
15. Boon T. Antigenic tumor cell variants obtained with
14. Hewitt HB, Blake ER, Walder AS. A critique of the evi-
13. Jaffe EM, Pardoll DM. Murine tumor antigens: is it
12. Klein G, Sjogren HO, Klein E. Demonstration of resis-
11. Gross L. Intradermal immunization of C3H mice
23. Colombo MP, Forni G. Immunotherapy I: Cytokine
20. Nossal GJV. The case history of Mr. T.I. - terminal
35.
12. Klein G, Stjernholm HO, Klein E. Demonstration of resist-
20. Robbins PF, Kawakami Y. Human tumor antigens recog-
21. Nossal GJV. The case history of Mr. T.I. - terminal
22. Vanderheide RH, Hahn WC, Schulze JL, Nadler LM. The telomerase catalytic subunit is a widely expressed tumor-associated antigen recognized by cytotoxic T lymphocytes. Immunology 1999; 100:673-9.
26. Lollini PL, Forni G. Specific and nonspecific immune
27. Allione A, Consalvo M, Nanni P, et al. Immunizing and curative potential of replicating and nonreplicat-
ing murine mammary adenocarcinoma cells engineered with interleukin (IL)-2, IL-4, IL6, IL-7, IL-10, tumor necrosis factor α, granulocyte-macrophage colony-stimulating factor, and γ-interferon gene or admixed with conventional adjuvants. Cancer Res 1994; 54:6022-6.
31. Janeway CA Jr. Bottomly K. Signals and signs for lymph-


74. Albert ML, Bhardwaj N. Resurrecting the dead: DCs cross-present antigen derived from apoptotic cells on MHC class I. The Immunologist 1998; 5:194-8.


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