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ALLOGENEIC HEMATOPOIETIC STEM CELLS FROM SOURCES OTHER THAN BONE MARROW: BIOLOGICAL AND TECHNICAL ASPECTS

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ABSTRACT

Background and Objective. 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 alloreactivity, offers an exciting perspective for widening the number of potential stem cell donors and greater leeway for graft manipulation than is possible with BM.

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.[1] 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

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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.[2] 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 characterization. An *ad hoc* working group reviewed the biology and clinical relevance of CD34-positive cells in this journal in 1995.[3] In particular, techniques for CD34-positive cell separation and procedures for their collection from peripheral blood were analyzed. A brief chapter was also devoted to CD34-positive cells in cord blood.[3]

The working group on CD34-positive hematopoietic cells subsequently reviewed the use of PBSC in acute myeloid leukemia[4] and multiple myeloma.[5] The growing interest in the use of PBSC and cord blood stem cells in the setting of allogeneic transplantation has now prompted the working group to review this subject by analyzing its biological and technical aspects.

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] 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.[8] The advantage is quicker hematopoietic recovery[9,10] 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]

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.[3,21] 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

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

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

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.[13,24-29]

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.[30] 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) $\times 10^6$/kg $CD34^+$ cells with two aphereses. All transplants were syngeneic, and recovery of 0.5×10^9/L granulocytes and 20×10^9/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) 147×10^6/L $CD34^+$ cells on day 4, a 65-fold increase over the baseline level. The median collection was 754×10^6 $CD34^+$ cells and 270×10^8 $CD3^+$ cells with 2 aphereses. With 12 μg/kg for 6 days, Körbling *et al*.[16] collected a mean of 13.1×10^6/kg $CD34^+$ cells with 3 aphereses, and their patients recovered $>0.5\times10^9$/L granulocytes on day 10 and $>20\times10^9$/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).[32] Dührsen[33] 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.[2]

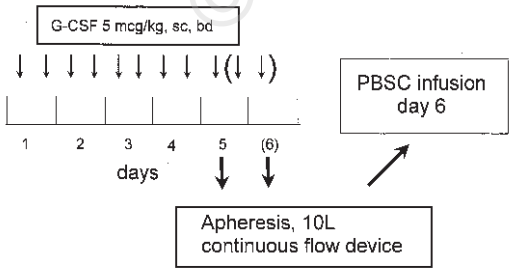

Figure 1. Schematic representation of timing in PBSC mobilization in healthy donors. G-CSF is administered at a daily dose of 10 μg/kg, apheretic harvest is performed on day 5 (and 6). Day 5 collection cells are stored at 4°C till the following day, when they are infused together with day 6 cells. Parentheses indicate that G-CSF is given and aphereses performed only if the target number of $CD34^+$ cells is not reached with the day 5 apheretic run.

The number of apheretic 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 apheretic procedures. With this dose, a median of approximately 7×10^6/kg $CD34^+$ cells are obtained with a single apheresis performed on day 5. At the M.D. Anderson Cancer Center in Houston[29] 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 $> 4\times10^6$/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 2.5×10^6/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*.[35] were able to collect 13×10^6/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, 3.2×10^3/mL and 1.75×10^3/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 $>1\times10^6$/kg required 3 to 4 aphereses. With this method, median time to ANC $> 0.5\times10^9$/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.[2,20,27,28,32,39] Since the $CD34^+$ cell level rapidly declines after growth factor withdrawal, it is highly recommended that its administration be continued till the end of apheretic 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,[39] 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 >4×10⁶/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.[28,39] 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 >2×10⁶/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[28] 4 out of the 53 normal donors yielded only 0.6, 1.49, 1.55 and 1.74×10⁶ CD34⁺ cells/kg. Despite the low cell numbers, successful engraftment was achieved

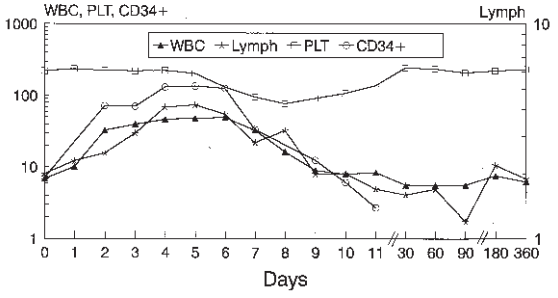

Figure 2. Variations of blood cell counts in normal donors during G-CSF treatment and apheretic collection of mononuclear cells. The curves represent mean values. Data are those of 76 normal donors.[39]

Table 2. Incidence and grading of side effects reported during G-CSF administration in 76 healthy donors from the GITMO.[39]

	% donors			
	Absent	*Mild*	*Moderate*	*Severe*
Bone pain	27.4	59.6	9.6	3.2
Arthralgias	54.8	32.2	11.2	1.6
Headache	70.9	20.9	8	0
Fatigue	74.5	22	3.3	0
Fever	91.5	6.7	1.6	0

in all cases. We suggest that collection of >4×10⁶/kg CD34⁺ cells is the target for a safe allogeneic transplantation. Lower doses, however, may be sufficient. A lower limit of >2×10⁶/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).

Twenty-three percent of donors also said the apheretic procedures were *demanding*. Comparable side effects are reported in other studies.[34,28] Additional problems could include pneumothorax due to jugular vein cannulation and paresthesia.[34] Nonetheless, donors who had previously given marrow mostly agreed that they preferred blood cell mobilization and collection to marrow harvest.[34-39] 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 (<70×10⁹/L) to moderate (<50×10⁹/L) followed PBSC harvests in 40% and 10% of cases, respectively. WBC counts exceeded 50×10⁹/L in 40% of cases, and 70×10⁹/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 $<100\times10^9$/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 apheretic 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.[39] 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;[44] 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 mandatory to follow the PBSC donors regularly, and to register carefully any variation in their blood counts.

The studies on leukemia development after G-CSF treatment must be considered with caution. A Japanese group[46] 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[47] 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.[48] 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.[2] 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*.[49] 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.[2]

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[50] has shown a high expression of myeloid antigens on PB CD34$^+$ cells (i.e. CD 33, CD 13) 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[51] 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[52] 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.[53] 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.[54]

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 compared 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.[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/G2M 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.[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.[57]

Nevertheless, even though in a cohort of heavily treated cancer patients[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 chemo-

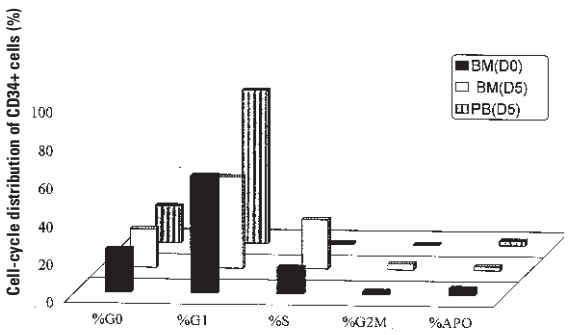

Figure 3. Analysis of cell-cycle distribution of PB and BM $CD34^+$ cells.

therapy + 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.[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 G_1-phase but are easily recruitable into S-phase.[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}H-Tdr) in a 16-24-hr suicide assay.[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}H-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.[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% SEM of BM), whereas the proportion of LTC-IC cycling was superimposable on that of BM. The frequency was not different in the two compartments (48.2±35 SEM and 62.5±54 SEM for 10^4 $CD34^+$ cells in PB and BM, respectively; p = ns). Thus these data, coupled with previous observations from nonhuman primates on cell-cycle status and response to CSF of cytokine-mobilized $CD34^+$ cells,[61] suggest that many circulating progenitors are not deeply quiescent in G0-phase. Rather, they are actively cycling and their high clonogenic efficiency and prompt proliferative response to CSFs may indicate a faster progression through cell-cycle mediated, perhaps, by G-CSF priming. A kinetic and functional pattern of $CD34^+$ cells similar to that observed in normal PBSC donors has been found in acute leukemia and multiple myeloma patients mobilized with chemotherapy and G-CSF, and in lymphoma individuals receiving G-CSF alone (Lemoli, unpublished observations). Thus, regardless of the mobilization protocol, the administration of G-CSF and/or the change of compartment (i.e. egress into peripheral blood) induces a profound effect on the characteristics of hematopoietic progenitor cells. Further studies are presently directed toward investigating the modulation of the expression of integrin adhesion molecules critical for mobilization and related to cytokine-induced cell-cycle transit.[62] Moreover, pharmacological doses of steel factor determine a redistribution of stem cells in mice[63] 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.[65]

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.[66] 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*.[67] 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[68] 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

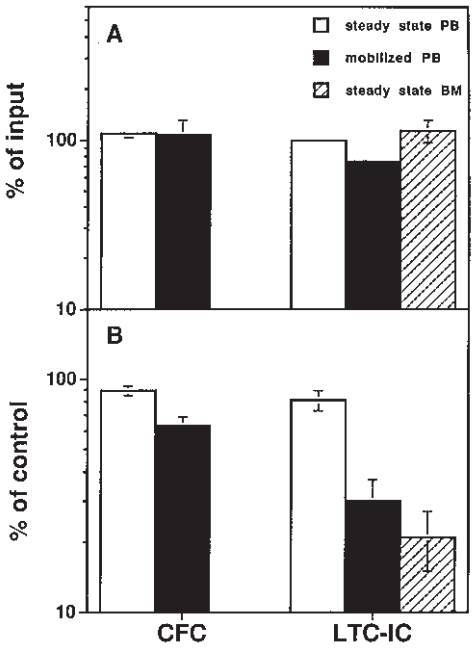

Figure 4. Recovery and proliferative status of CFC and LTC-IC in steady-state normal blood and bone marrow and in the leukapheresis products of cancer patients obtained after chemotherapy + G-CSF. The cells were cultured for 16 hrs in a medium containing serum substitutes, SF (100 ng/mL), IL-3 (20 ng/mL) and G-CSF (20 ng/mL) in the presence or absence of ^{3}H-Tdr (panel B and A, respectively).

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 1-8×10^{6} CD34^{+} cells and 10-120×10^{3} 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 0.05-0.5×10^{6} CD34^{+} cells/kg b.w., while it has been suggested that recipients of allogeneic PBSC must receive at least 2.5-5×10^{6} 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.[72] 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*.[73] sorted CB-derived, CD34^{+}CD45RA^{lo}CD71^{lo} 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^{+} CD45RA^{lo} CD71^{lo} cells obtained from adult donors. This advantage was clearly ontogeny-related, since the proliferative potential of purified CD34^{+} CD45RA^{lo} CD71^{lo} cells collected from fetal liver was superior to that of CB-derived cells. In this context, Hows *et al*.[74] demonstrated 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*.[75] 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*.[76] demonstrated that LTC-IC and cells presumably capable of *in vivo* engraftment reside in the CD34^{+}HLA-DR^{+}Rh123^{dull} fraction. The cycling status of CB progenitors is still a matter of debate. In fact, some investigators using the tymidine suicide technique reported a higher frequency of cells in S phase,[77] whereas others did not find any difference between CB and adult BM in the frequency of actively cycling progenitors.[78] 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,[79] and since entering S phase is required for gene transfer through safety modified retroviruses. Interestingly, a higher efficiency of retrovirus-mediated gene trasfer has been reported in CB than in BM progenitors.[78,80,81] 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*.[82] 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.[83] As described in the CB processing section, *ex vivo* expansion of CB progenitors prior to trasplantation might be useful to hasten hematopoietic engraftment; however, since the long-term engraftment capability of *ex vivo* cultured cells might be lost or impaired,[84] 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 registeries, 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.[85]

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*.[67] included CB collection in heparinized tubes by gravity. Further studies[69,86] 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*.[87] Another approach, proposed by Turner *et al*.,[88] 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.[89] It has been demonstrated, moreover, that CPD/CPD-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.[90,91] Interestingly, when newborns are delivered following the Leboyer method there is no *transfusion effect* since the newborn is kept above the level of uterus.[92] 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,[93-97] and recent data on the immediate follow-up of newborns who underwent early clamping of the the umbilical cord and CB collection support the safety of this procedure.[98] 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 banking, 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 Ficoll[99] or red cell sedimentation by means of animal gelatin,[69,100] i.e. reagents that are currently not (and probably will never be) licensed for use in humans by regulatory agencies like the FDA. Consequently, procedures involving the use of licensed products like HES[101,102] 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.[101,103] Conversely, data on the engraftment potential of purified, CB-derived $CD34^+$ cells are still poorly reproducible in the SCID-hu mouse model[104] and totally lacking in humans, so for the time being the storage of purified $CD34^+$ CB progenitors for clincal 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 myeloid[107] or megakaryocytic lineage.[108]

Rubinstein *et al*.[101] 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

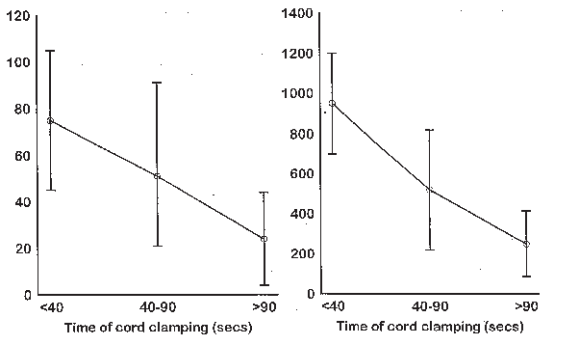

Figure 5. Effect of the time of umbilical cord clamping on the mean (±1 SD) volume and nucleated cell count of cord blood collections (n=67). As in most European OB/GYN units, soon after birth the newborn is kept below the level of the uterus, and delayed clamping of the umbilical cord is associated with a reduction of cord blood volume and cellular content.

futher 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 differentiatation 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 interaction.[110,111] 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, anti-microbial 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 vivo*[114] 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 CBL 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.[121] Moreover, recent data demonstrate that the innate immunity directed towards Epstein-Barr virus-infected cells is remarkably high in CBL collected from the majority of neonates.[122] 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, anatomo-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).[111,123] Several lines of clinical and experimental evidence support this hypothesis, in particular: i) CBL preferentially display suppressor rather than helper immunological functions[111,113] and ii) both B and T lymphocytes maintain a state of hyporesponsivity towards noninherited maternal HLA molecules for a long time after birth.[124,125] A further confirmation of this peculiar state of tolerance derives from a recently reported observation[126] 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.[127] The concept of neonatal tolerance was recently re-examined in mice[128-130] and it was demonstrated that induction of this phenomenon may depend on sev-

eral elements, including the nature of the antigen-presenting cells,[128] the dose of antigen administered,[129] and the mode of immunization.[130]

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.[117,131-133] However, normal CBL alloreactivity has been documented in other studies.[134,135] 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 alloreactive lymphocytes and a state of unresponsiveness in CBL.[136] 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),[126,135] 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.[119,137] 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.[138,139] Indeed when T-cells are removed from the graft before transplantation, the incidence of GvHD decreases sharply.[71,140-143] On the other hand, the possible beneficial roles of T-lymphocytes include sustaining engraftment[144] and preventing relapses through the graft-versus-leukemia effect.[145,146] 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 anti-viral or anti-neoplastic effect.

Standard T-cell depletion techniques

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

Method of T-Cell Depletion	*Cells Removed*	*T-Cell Depletion (x log_{10})*
SBA lectin and E-rosette depletion	T and B lymphocytes, monocytes, neutrophils	2.5 - 3.0
Multiple E-rosette depletions	T lymphocytes	2.0
Mouse MoAb (anti-CD2, CD8) + rabbit C'	T lymphocytes	2.0
Mouse MoAb (anti CD6) + human C'	T lymphocytes	1.5-2.0
Rat MoAb(CAMPATH-1) + human C'	T and B lymphocytes, monocytes,	2.5
Anti-CD5 immunotoxin-Ricin A Immunomagnetic separation (anti-CD3, CD8)	T lymphocytes	2.0
SBA lectin + immunomagnetic separation	T and B lymphocytes, monocytes, neutrophils	3.1
AIS CD%/8T-CELLector	T lymphocytes	2.5
Autologous Immunorosettes (anti-CD2 and CD3 tetrametric complexes)	T lymphocytes	2-3

Over the past 15 years, several techniques have been developed for depleting T-cells from human marrow allografts.[147] 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 leukaphereses from the peripheral blood of donors previously stimulated by hemopoietic growth factors.

Kessinger *et al*.[148] first reported allogeneic transplantation utilizing T-cell depleted peripheral blood mononuclear cells and a sheep erythrocyte rosetting technique. Engraftment was rapid and grade II acute GvHD was observed.

In a preliminary study after monocyte lysis with L-phenylalanine methyl ester, Suzue *et al*.[149] depleted T-lymphocytes from apheresis products harvested after stimulation of healthy donors with G-CSF using both E-rosettes with sheep red blood cells and panning with flasks coated with anti CD5/CD8 monoclonal antibodies. They reported an unsatisfactory depletion of T-cells (99.5%) and a stem cell recovery of only 7.5%.

Aversa *et al*.[71] employed soybean lectin agglutination followed by 2 to 4 rounds of E-rosetting with sheep red blood cells on the leukapheresis product from donors stimulated with G-CSF. This approach achieves approximately $3\times\log_{10}$ 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 $CD34^+$ hemopoietic progenitors appears to be a valid technical alternative. Table 4 shows the basis of the main techniques for positive selection of hemopoietic 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.[150]

The $CD34^+$ stem cell selection systems adopted in clinical use are based on immunoadsorption 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 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.[151] recovered a mean of 30% $CD34^+$ cells, with a purity of 70%. Peripheral blood $CD3^+$ cells were reduced by 3 logs. Other investigators have reported similar results.[152-155] 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.[140] 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 1×10^5/kg b.w.,[156] which is difficult to achieve with one-step positive selection of hemopoietic stem cells. For a successful mismatched bone marrow transplant the T-lymphocyte threshold must be < $3\text{-}5\times10^4$/kg b.w.[157] in the inoculum because of the greater likelihood and increased severity of GvHD in these patients. On

Company	*Method*	*Antibody*	*Detachment*
CellPro	Immunoadsorption	12.8	Mechanical
Baxter	Magnetic beads indirect	9C5	Chymopapain
Baxter	Magnetic beads indirect	9C5	$PR34^{+™}$ oligopeptide
Dynal	Magnetic beads direct	BI3C5	Anti-Antibody (anti Fab of mouse MoAb)
AIS	Panning	ICH3	Mechanical
Immunotech	Magnetic latex beads direct	QBEND10	Not required
Milteny	Magnetic colloid indirect	QBEND10	Not required
Terry Fox Laboratory	Magnetic Colloid Indirect	8G12	Not required
System x	FACS (high speed)	Various	Not required

Table 4. Methods available for stem cell positive selection.

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*.[157] employed an E-rosetting procedure before positively selecting hemopoietic 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%.[158]

In small-scale experiments, Fernandez *et al*.[159] applied the E-rosetting procedure after positive selection of $CD34^+$ cells with this same ®Ceprate system to obtain a mean $\log_{10}$ T-cell depletion of 4. Slaper-Cortenbach *et al*.[160] 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 immunorosettes 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.[40,161] 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.[161] 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%.[162]

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.[163-165]

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.[166-171] 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.[172-177] Since it has been demonstrated that T-cell receptor (TCR): antigen interaction, in the absence of appropriate costimulation, may induce T-cell unresponsiveness or even apoptotic deletion,[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 receipient.[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. Cytofluorimetric 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,[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 hemopoietic 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.[184] Development of mobilization regimens capable of increasing the number of peripheral blood hemopoietic 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.[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)± methotrexate (MTX).[187] Another study reported 2 cases out of 5 who died from aGVHD.[188] By contrast, other groups reported a very low incidence of GVHD.[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 $0.5\text{-}1\times10^6$ $CD3^+$ T cells/kg could be potentially capable of initiating GVHD, which would be prevented by further steps in T-cell depletion.[158] 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 hemopoietic growth factors used to mobilize PBSC on peripheral blood lymphocytes.

Weaver *et al*.[191] 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 ug/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.[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 sug-

Authors	*TNC* *(x 10^8/kg)*	*$CD34^+$* *(x 10^6/kg)*	*$CD3^+$* *(x 10^6/kg)*	*NK* *(x 10^6/kg)*
Dreger et al. (26)	13.52	8.16	404	N.R.
Weaver et al. (191)*	20.53	9.6	450	N.R.
Körbling et al. (16)	16.5	10.7	300	64.3
Schmitz et al. (17)	8.6	13.1	340	94.0
Bensinger et al. (18)	10.6	13.1	385	N.R.
Majolino et al. (27)	9	6.84	250	27
Rambaldi et al. (203)	8	6.9	279	N.R.

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.*

gest 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 Bekkum[200] 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 anergy 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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