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(54) Title: ENRICHMENT OF HEMATOPOIETIC STEM CELLS FROM BLOOD OR BONE MARROW

(57) Abstract

Process for enrichment of hematopoietic stem cells in a stem cell preparation, e.g. bone marrow, umbilical cord blood and peripheral blood stem cell preparation. Non-stem cells, in particular monocytes and myeloid cells, are depleted from the preparation by adherence to an opsonized material, e.g. a scrubbed nylon fiber coated with human IgG and blocked with human serum albumin. Platelets are depleted from the stem cell preparation before contacting the opsonized material, e.g. by counterflow centrifugal elutriation. Alternatively, adherence of platelets to stem cells is prevented by the addition of a chelating substance to the medium in which the enrichment process is performed.

Title: Enrichment of hematopoietic stem cells from blood or bone marrow

Field of the invention

The invention is in the area of hematopoietic stem cell transplantation and is concerned with a novel process for the preparation of enriched suspensions of hematopoietic stem cells. The invention relates to a method to deplete non-stem cells from hematopoietic stem cell suspensions used for transplantation, without the use of xenogeneic antibodies directed against cell surface molecules.

The process will be suitable for the processing of autologous transplants, as it will result in a reduction of the volumes of transplants to be cryopreserved, a reduction of the amount of (toxic) cryoprotectant to be infused into the patient, a diminished risk of transfusion problems related to lysis of granulocytes and erythrocytes upon freezing and thawing, and probably a reduction of the number of contaminating malignant cells.

Its use will also be advantageous in both autologous and allogeneic transplantations when further purification of the stem cells is desirable. A smaller scale immunoselection technique, requiring less monoclonal antibody can be used for final purification after stem cells have been enriched by the invented procedure.

Background of the invention

Hematopoietic stem cell transplantation.

Although results in the treatment of cancer patients have improved, the prognosis with current treatment protocols in some patient groups is still poor. One approach to improve cure rate is intensification of chemo- and/or radiotherapy. However, this is hampered by irreversible damage to hematopoiesis in the bone marrow, even with the use of hematopoietic growth factors. This problem can be corrected by the administration of bone marrow cells.

This can be done in an allogeneic setting, with bone marrow from an HLA-matched related donor. Two problems restrict the application of allogeneic bone marrow transplantations: firstly, the fact that only 30% of the patients have an HLA-identical sibling, and secondly, the frequent occurrence of Graft-versus-Host Disease (GvHD). GvHD is caused by T cells from the graft, which react to the patient's alloantigens, thereby damaging patient tissues. This is in some cases a severe, sometimes fatal, disease.

Alternatively, autologous bone marrow transplants are being used. For this purpose bone marrow is harvested in the remission phase of the disease, cryopreserved, and reinfused into the patient after high-dose chemotherapy. However, in some tumors, bone marrow infiltration with tumor cells is a common feature. Furthermore, intensive pretreatment (such as pelvic irradiation) may lead to damage to bone marrow, thereby hampering the harvest of sufficient bone marrow stem cells.

A few years ago it has been found that hematopoietic progenitor cells can be mobilized to the blood by treatment with certain chemotherapeutic drugs. Moreover, it was observed that the mobilizing effect of chemotherapy can be enhanced by in vivo administration of hematopoietic growth factors, such as G-CSF. Peripheral blood stem cells (PBSC) are now increasingly used as an alternative for bone marrow in autologous transplantations.

PBSC are collected by leukocytapheresis. This is a centrifugation technique in which low density cells (i.e. mononuclear cells, including the progenitor cells) are collected. The other blood components are reinfused (continuously or intermittently) into the donor.

PBSC transplantation has several advantages over bone marrow transplantation:

1. The chemotherapy applied before harvesting largely eradicates circulating tumor cells, thereby reducing the tumor load in peripheral blood stem cell transplants. However, with recently developed more sensitive detection techniques, (low numbers of) malignant cells in the PBSC transplants can still be detected.

2. Treatment protocols of patients in which mobilized PBSC were infused, resulted in more rapid hematopoietic recovery as

compared to bone marrow transplantation, thus reducing the dangerous period of aplasia.

3. Less morbidity of the harvesting procedure (no general anesthesia).

Recently, it has been found that considerable numbers of stem cells are present in umbilical cord blood. Worldwide about 50 (allogeneic) umbilical cord blood transplantations in children have been performed so far. The majority of these patients showed hematopoietic recovery. Several centers have started umbilical cord blood banking. Major advantages of the use of umbilical cord blood stem cells will be:

1. The ready availability for transplantation of fully typed material with known progenitor cell content.

2. A harvest procedure without any discomfort to the mother/child.

Umbilical cord blood stem cell transplantations have mainly been performed in children, in view of the limited numbers of stem cells harvested. Expansion in vitro may however facilitate transplantation of umbilical cord blood stem cells in adults.

Processing of stem cell transplants.

It is generally assumed that purification of stem cells from the transplants would be desirable. Purification will provide major advantages:

a. In autologous transplantations, purification will lead to a reduction in the number of malignant cells.

b. In autologous and umbilical cord blood transplantations, purification will lead to a reduction in the volume of cells to be cryopreserved, to a reduction of the amount of (toxic) cryoprotectant to be infused into the patient, and to diminished risks of infusion which are related to the lysis of granulocytes and erythrocytes upon freezing and thawing (such as formation of aggregates, hemoglobinuria).

c. In allogeneic transplantations, the resulting depletion of T cells will lead to diminished GvHD.

d. Growth-factor induced expansion and differentiation of stem cells in vitro is more effective when purified stem cells are cultured.

e. For effective transfer of foreign genes into stem cells (for gene therapy) purified preparations of stem cells may be necessary.

Several immunological techniques have been developed for the isolation of stem cells. In all the available methods, CD34 monoclonal antibodies (mAbs), which react specifically with hematopoietic stem cells, are being used for the isolation of progenitor cells. One of the first reports was by Berenson et al (J. Clin. Invest. 81 (1988): 951) who isolated $CD34^+$ cells from bone marrow, transplanted these cells into irradiated baboons and achieved hematological reconstitution. Because leukemic blasts from patients with acute leukemia also carry the CD34 antigen, this approach of stem cell purification cannot be used in acute leukemia. However, in solid tumors like breast carcinoma, neuroblastoma etc. and in other hematological disorders, the malignant cells are CD34-negative.

The following companies have developed systems for the enrichment of $CD34^+$ cells:

* Applied Immune Sciences introduced the microCELLector. In this system, Soybean agglutinin (SBA) or CD34 mAb is covalently bound to polystyrene flasks. In the first step of the procedure, mature differentiated cells are (partly) depleted by binding to SBA. $CD34^+$ cells are then selected from the SBA-negative population by binding to CD34 mAb. Finally, bound cells are released by physical agitation.

* CellPro introduced the CEPRATE SC Stem Cell Concentration System, which is based on the binding of biotinylated CD34 mAb to progenitor cells, and subsequent separation over a column with avidin-coated polyacrylamide beads. Progenitor cells are liberated by mechanical stirring of the gel bed. The procedure results in a > 50-fold enrichment of $CD34^+$ cells and CFU-C, with approximately 50% yield.

* Baxter introduced the Isolex system, comprising immuno-magnetic beads coated with CD34 mAb for the selection of progenitor cells. In this system the positively selected stem cells are treated with chymopapain to strip the beads from the surface of the cells.

* Dynal introduced a procedure similar to that of Baxter, but which differs in the method used to detach the beads from the cell surface. Cell release is performed using a polyclonal antibody directed against murine Fab antibody fragments.

Some of the techniques reported so far have actually been applied for isolation of progenitor cells in a clinical setting and transplantations with selected stem cells have been performed successfully.

One major disadvantage of the available techniques is the large amount of CD34 monoclonal antibody needed, which makes these techniques very expensive. A second disadvantage is the need of some techniques to release the cells, which may result in damage to the progenitor cells. Moreover, the purity and yield achieved in isolations of stem cells from PBSC transplants is in general lower than purity and yield of stem cells isolated from bone marrow. Therefore, a process leading to substantial enrichment of stem cells by depletion of non-stem cells without the use of mAb would be a considerable advantage.

The following non-immunological techniques are used for processing of stem cell grafts:

* Density separation for depletion of erythrocytes and granulocytes from bone marrow.

* Counterflow centrifugal elutriation for depletion of lymphocytes from bone marrow grafts.

However, no non-immunological procedure is available by which a substantial depletion of all non-stem cell types from grafts can be obtained. PBSC transplants are in general infused into the patients without any separation step. PBSC leukocytapheresis products obtained after treatment of the patients with chemotherapy and G-CSF cannot be enriched by density separation because all cells collected by an optimal leukocytapheresis procedure have a low specific density.

Summary of the invention

According to a first embodiment of the invention there is provided a process for the enrichment of hematopoietic stem cells in a stem cell preparation, comprising depleting non-stem cells from the preparation by adherence to an opsonised fibre material and recovering a hematopoietic stem cell-enriched preparation, wherein adherence of stem cells to the opsonised fibre material is prevented either by pretreating the stem cell preparation to remove platelets therefrom, or by including a chelating substance in the medium in which the non-stem cell depletion process is performed to prevent adherence of platelets to the stem cells.

The invention comprises a procedure for enrichment of hematopoietic progentior cells based on a selective adherence of certain non-progenitor cells to a fiber material coated with an opsonizing substance. In a preferred form, the adherence step is preceded by counterflow centrifugal elutriation. The invented procedure is based on two discoveries:

1. Selective removal of monocytes and myeloid cells from stem cell preparations can be achieved by passage of the cell suspensions over scrubbed nylon fibers, provided that first platelets are rigorously depleted from the graft. Alternatively a chelating substance (e.g. EDTA or trisodium citrate) may be included in the filtration medium, thereby preventing the adherence of platelets to leukocytes. Without platelet depletion or prevention of the adherence of platelets to leukocytes by capture of divalent cations, stem cells adhere to nylon fiber. The optimal way of platelet removal is counterflow centrifugal elutriation.

2. The selective adherence of monocytes and immature myeloid cells to nylon fiber is strongly promoted by coating the fiber with opsonizing human plasma proteins.

When the enrichment step with coated nylon fiber is preceded by elutriation, substantial depletion of all non-stem cell types from the transplant can be achieved. By each of these two techniques different cell types are removed: the elutriation technique separates platelets, erythrocytes and part of the lymphocytes from the progenitor cells. The passage over coated nylon fiber results in depletion of monocytes and myeloid cells from the transplant. With the combination of these techniques substantial enrichment of stem cells is achieved without the use of monoclonal antibodies. In peripheral blood transplants approximately 10-fold reduction of the total number of non-stem cells is achieved.

The invention provides a process for the enrichment of hematopoietic stem cells in a stem cell preparation (usually umbilical cord blood, a peripheral blood stem cell suspension, or bone marrow), the process comprising depleting non-stem cells (more particularly comprising monocytes and myeloid cells) from the preparation by adherence to an opsonized material and recovering a hematopoietic stem cell-enriched preparation.

Preferably, said opsonized material comprises a fiber material, in particular a polyamide fiber material, treated with an opsonizing substance, wherein the opsonizing substance is preferably selected from the group consisting of IgG, IgA, C3b, C3bi, C3dg, C1q, iC3, conglutinin, mannan-binding protein (MBP), CL-43, SP-A and SP-D. Most preferably, the opsonized material comprises a scrubbed nylon fiber material coated with human IgG. Furthermore, the opsonized material is preferably treated with a blocking substance which reduces aspecific cell binding, wherein the blocking substance is preferably selected from the group consisting of human serum albumin, animal serum albumin, gelatin, Ficoll 70 and Ficoll 400.

Preferably, the stem cell preparation is pretreated to remove platelets (thrombocytes) therefrom. The pretreatment of the stem cell preparation preferably comprises a counterflow centrifugal elutriation in which smaller cells including platelets, erythrocytes and small lymphocytes are removed and larger cells including hematopoietic stem cells, monocytes and myeloid cells are recovered. Alternatively, the platelets can be removed by sequential low-speed centrifugation steps.

In a second preferred embodiment a chelating substance (e.g. EDTA or trisodium citrate) is included in the medium in which the process of depletion of monocytes and myeloid cells is performed, thereby preventing the adherence of platelets to the stem cells. In this embodiment of the invention, removal of platelets is not necessary.

A preferred process comprises providing a stem cell preparation from bone marrow or, preferably, from peripheral blood or umbilical cord blood, depleting platelets, erythrocytes and small lymphocytes therefrom by a counterflow centrifugal elutriation in which smaller cells including platelets, erythrocytes and small lymphocytes are removed and larger cells including hematopoietic stem cells, monocytes and myeloid cells are recovered, depleting monocytes and myeloid cells from said recovered larger cells by adherence to a material coated with an opsonizing substance and blocked against aspecific cell binding, and recovering a preparation enriched in hematopoietic stem cells.

In another preferred embodiment a stem cell preparation from bone marrow, cord blood or peripheral blood is passaged directly (without prior removal of platelets) over a material coated with an opsonizing substance and blocked against aspecific cell binding, in a medium to which a chelating substance has been added. Thereby, monocytes and myeloid cells (and B cells) are depleted and a preparation enriched in hematopoietic stem cells will be recovered. In this embodiment, erythrocytes, lymphocytes and platelets are not depleted, and the process will result in a more limited enrichment.

Of course, the invention covers the combination of both methods, i.e. prior depletion, e.g. by counterflow centrifugal elutriation, of platelets, erythrocytes and small lymphocytes, and passage of the resulting composition, which is enriched in the larger cells, over the non-stem cell depletion material in a medium containing a chelating substance.

The process may also comprise a step of immunoselection using an antibody which binds hematopoietic stem cells, or an antibody which binds non-stem cells or malignant cells, to effect a further enrichment of hematopoietic stem cells.

The invention also provides an assembly of means and instructions for use in a process for the enrichment of hematopoietic stem cells in a stem cell preparation, said means comprising (the constituents of) an opsonized material capable of binding non-stem cells including monocytes and myeloid cells present in a stem cell preparation.

The assembly may further comprise at least one member of the group consisting of elutriation media, cell suspending media, opsonization media, blocking media, washing media, and components of said media.

<u>Brief description of the drawings</u>

Figure 1 shows the principle of counterflow centrifugal elutriation.

Figure 2 shows the result of separation of PBSC leukocytapheresis product by counterflow centrifugal elutriation. The figure depicts the recovery of cells (as percentage of input) in the elutriation fraction containing the large cells (n=14). CFU-GM = a measure of the outgrowth of monocytic and myeloid precursors in semi-solid medium during a 12 day culture.

Figure 3 shows that yield of PBSC $CD34^+$ cells upon passage over nylon wool in the presence of fetal bovine serum is enhanced after depletion of thrombocytes, erythrocytes and part of the lymphocytes by counterflow centrifugal elutriation. The figure depicts recovery of cells in the filtrate as percentage of the cells applied to the nylon wool.

Figure 4 shows the effect of an addition of paraformaldehyde-fixed resting or thrombin-activated (non-autologous) platelets on the recovery of various cell types in elutriated PBSC upon nylon fiber filtration in the presence of fetal bovine serum. The figure depicts the recovery of cells in the filtrate as percentage of the cells applied to the nylon wool.

Figure 5 shows the effect of elutriation on platelet binding to $CD34^+$ cells in PBSC leukocytapheresis products. Adherence of platelets to $CD34^+$ cells was measured by determination of CD41 mAb binding to $CD34^+$ cells by FACS-analysis. The figure depicts the percentage of $CD34^+$ cells binding CD41 mAb (minus the percentage of $CD34^+$ cells binding non-specific mAb) in unseparated leukocytapheresis product and in elutriated stem cell fraction.

Figure 6 shows that the yield of $CD34^+$ cells after filtration of PBSC samples is enhanced when instead of removing the platelets by elutriation, a chelating agent is added to the medium prior to filtration. On the x-axis the composition of the filtration medium is given. The figure depicts the recovery of cells in the filtrate as percentage of the cells applied to the nylon wool. As can be seen, another consequence of the addition of EDTA to the filtration medium is that myeloid cells are less well depleted from the cell preparation.

Figure 7 shows recovery of cells upon passage of elutriated PBSC cells over nylon fiber preincubated with:
a. IMDM supplemented with 10% fetal calf serum and 9 mM $MgCl_2$; b. IMDM supplemented with human serum albumin (10 mg/ml);
c. IMDM supplemented with human immunoglobulin (1 mg/ml) followed by incubation with IMDM supplemented with HSA (10 mg/ml). The figure depicts the percentages of cells applied to the nylon wool which were recovered in the filtrate.

Figure 8 shows recovery of cells upon passage of elutriated PBSC cells over nylon wool preincubated with either IMDM supplemented with 10 % fetal calf serum or IMDM supplemented with immunoglobulin (1 mg/ml), without subsequent saturation with HSA. The figure depicts the recovery of cells in the filtrate as percentage of cells applied to the nylon wool.

Figure 9 shows recovery of cells upon filtration of elutriated PBSC over nylon wool preincubated with either IMDM supplemented with human serum albumin (10 mg/ml) or IMDM supplemented with human complement factor iC3 (0.1 mg/ml) followed by saturation of the fiber with HSA. The iC3 was prepared by chemical cleavage of the intrachain thioester bond which is present in the α-chain of the molecule. The figure depicts the recovery of cells in the filtrate as percentage of cells applied to the nylon wool.

Figure 10 shows the effect of addition of trisodium citrate to the filtration medium, instead of removal of platelets, on filtration of PBSC samples over IgG-coated nylon wool. The figure depicts recovery of cells in the filtrate as percentage of the cells applied to the nylon wool. On the x-axis the composition of the filtration medium is given.

Figure 11 shows the yields of various cell types present in PBSC leukocytapheresis products after counterflow centrifugal elutriation and passage over nylon wool coated with human immunoglobulin and saturated with HSA (three experiments with PBSC from different patients). The figure depicts recovery of cells after the whole procedure as percentage of the cells started with.

<u>Detailed disclosure of the invention</u>

The invented procedure was developed in the first instance for enrichment of progenitor cells from peripheral blood stem cell leukocytapheresis preparations obtained after treatment with chemotherapy and G-CSF. These preparations contain leukocytes, erythrocytes (10 to 20 fold excess to leukocytes) and platelets (5 to 50 fold excess to leukocytes). From about 1% to about 4% of the leukocyte fraction consists of progenitor cells ($CD34^+$ cells), the remainder mainly consisting of lymphocytes, monocytes and myeloid cells. We looked for a non-immunological technique or a combination of techniques which would result in depletion of all these different non-stem cell types, with high recovery of progenitor cells.

In blood transfusion technology, leukocyte-depleted red cell concentrates are routinely prepared by passage over fibers ("filtration"). Such a procedure for removal of leukocytes is simple, fast, and does not require expensive equipment. A selective depletion of leukocytes occurs, while erythrocytes are recovered with high efficiency in these so-called "filtrations". This is due to differences in size and adherence properties of the cells (cell sieving and cell adherence). Several types of leukocyte removal filters are being used, which are composed of either polyester, cellulose acetate, cotton or nylon fibers.

With nylon fiber not all leukocytes are depleted, but a selective removal of certain leukocyte types from blood can be achieved. Passage of blood cells over nylon fiber in the presence of human plasma (Greenwalt et al, Transfusion 2, 221-229 (1962); Roos and Loos, Biochim. Biophys. Acta 222, 565-582 (1970)) or fetal calf serum (Litvin and Rosenstreich, Methods in Enzymology 108, 298-302 (1984); de Boer et al, J. Immunol. Methods 43 (1981): 225) is known to result in adherence of virtually all monocytes, granulocytes and B lymphocytes to the fibers, while T lymphocytes do not stick to them. Platelets are retained partially and erythrocytes do not adhere. For optimal adhesive properties, the finish has to be removed from the fiber (Greenwalt, 1962). Nylon fiber from which the finish has been removed is called: scrubbed nylon fiber.

We have investigated whether nylon fiber can be used for selective depletion of monocytes and myeloid cells from peripheral blood stem cell leukapheresis suspensions. The most important question was, whether progenitor cells would adhere to the nylon fiber or not. We observed that upon passage of PBSC leukocytapheresis products over nylon fiber in the presence of fetal calf serum, recovery of stem cells (enumerated as $CD34^+$ cells) was strongly variable, ranging from 12 to 112% (Table 1). The procedure resulted in considerable depletion of monocytes, but depletion of myeloid cells was only partial and variable.

The incomplete removal of myeloid cells is in strong contrast with the nearly complete removal of mature granulocytes achieved by passage of peripheral blood from normal donors over nylon fiber. Morphological examination of the cells showed that in PBSC leukocytapheresis products obtained from patients pretreated with chemotherapy and G-CSF, in contrast to blood from normal donors, nearly exclusively immature myeloid cells (band forms, promyelocytes, myelocytes and metamyelocytes) are present. This is caused by the pretreatment of the patients with chemotherapy and hematopoietic growth factors. In particular the more immature myeloid cells did not adhere to the fiber. Because of their lower density, as compared to mature granulocytes, these immature myeloid cells are enriched in leukocytapheresis products compared to whole blood.

To make passage over nylon fiber a suitable technique for enrichment of stem cells, the recovery of progenitor cells had to be optimized. We discovered that adherence of $CD34^+$ cells to the nylon fiber could be prevented by prior depletion of platelets from the graft or by addition of a chelating substance to the filtration medium.

Platelets can be separated from leukocytes by sequential centrifugation washing steps, or by counterflow centrifugal elutriation. Counterflow centrifugal elutriation is a technique in which particles are exposed to two opposite forces in a specially designed flow chamber within a centrifuge: the centrifugal force and a centripetal hydrodynamic force, caused by a fluid flow in the opposite direction of the centrifugal force. By increasing the fluid flow or decreasing the rotor speed, particles are separated (Figure 1 and Lutz, M.P. et al, Analytical Biochemistry 200 (1992): 376). Separation occurs mainly according to size and to a lesser extent to density.

In counterflow centrifugal elutriation stem cells co-separate with the larger blood cells (monocytes and myeloid cells), while the smaller cells (i.e. platelets, erythrocytes and small lymphocytes) are washed away. Figure 2 shows that a nearly complete removal of thrombocytes, erythrocytes and a partial removal of lymphocytes from PBSC leukocytapheresis products was achieved by elutriation with a >85% yield of $CD34^+$

cells and of myeloid progenitor cells (colony-forming units granulocyte-monocyte (CFU-GM)). When the resulting stem cell preparations were subsequently subjected to nylon fiber filtration in the presence of fetal calf serum, we observed that recovery of $CD34^+$ cells in the filtrate was strongly increased in comparison with filtration of the original unseparated PBSC leukocytapheresis products (Figure 3).

The adherence of $CD34^+$ cells to nylon fiber appeared to be mediated specifically by platelets. Particularly, activated, but not resting platelets were found to be effective in promoting retention of stem cells on nylon wool. When thrombin-activated platelets were added to the elutriated stem cells, recovery of $CD34^+$ cells after passage over nylon wool was drastically lowered. Conversely, resting platelets had no effect (Figure 4).

The effect of platelets on adhesion of stem cells to nylon wool may be caused by two mechanisms:

1. Adherence of platelets to the nylon fiber may secondarily lead to trapping of stem cells. We found that during passage of PBSC leukocytapheresis products over nylon fiber 44 $\pm$ 22% (n=5) of platelets adhered to the nylon wool.

2. Platelets or platelet fragments may adhere to the stem cells after which the stem cells adhere to the nylon wool. In our institute, Dercksen et al (Blood, in press) have observed that in PBSC leukocytapheresis products platelets and platelet fragments are bound to part of the $CD34^+$ cells. This was reflected in binding of mAb directed against CD41 to the $CD34^+$ cells. CD41 is an antigen which is expressed nearly exclusively on platelets. Upon extensive washing of the cells in the presence of EDTA, CD41 mAb binding to $CD34^+$ cells was strongly diminished. It was proposed that the residual CD41 mAb binding after washing reflected cellular CD41 expression on megakaryocyte progenitors. Figure 5 shows that elutriation not only results in removal of free platelets but also in removal of bound platelets or platelet fragments from the $CD34^+$ cells, as indicated by the lowered percentage $CD34^+$ cells which bind CD41 mAb.

In a preferred embodiment of the invention, platelets are removed from the stem cell graft by a counterflow centrifugal elutriation. Alternatively, the same objective may be achieved by sequential differential (low-speed) centrifugation steps. However, elutriation has in the invented procedure the following advantages:

1. Non-bound platelets are more effectively removed by elutriation than by differential centrifugation.

2. Platelets which are bound to cells are more efficiently removed by elutriation than by low-speed centrifugation.

3. Additional cell types (erythrocytes and a larger part of lymphocytes) are removed from the graft by counterflow centrifugal elutriation.

Since the adherence of platelets to $CD34^+$ cells (Dercksen et al, Blood, in press) is dependent on the presence of divalent cations, it was investigated whether the mere addition of chelators like EDTA or trisodium citrate to the filtration medium could prevent the adherence of $CD34^+$ cells to nylon fiber. As is shown in Figure 6, inclusion of EDTA in the filtration medium resulted in enhanced yield of $CD34^+$ cells in the filtrate without prior removal of platelets from the PBSC preparations. Unfortunately, EDTA also resulted in strongly diminished adherence of the myeloid cells to the nylon fiber. Similar results were obtained with addition of trisodium citrate to the filtration medium. However, as is shown in Figure 10, lowered adherence of myeloid cells in the presence of a chelating substance does not occur when the fiber is coated with an opsonizing substance. Thus, in a second preferred embodiment, when only removal of monocytes and myeloid cells is desired, a chelating substance is included in the filtration medium to prevent the adherence of $CD34^+$ cells to the nylon fiber, thereby circumventing the need of removal of platelets from the stem cell transplants.

To obtain reasonable enrichment of progenitor cells upon passage of PBSC transplant over nylon fiber, it was necessary to enhance the adhesive properties of the fiber for the (immature) myeloid cells. We discovered that this can be achieved by coating the fiber with an opsonin, in its most preferred form the opsonin being human IgG.

Myeloid cells are phagocytotic cells. Binding of phagocytes to foreign particles, such as bacteria, is strongly enhanced by opsonization, i.e. coating of the particle with specific proteins. Proteins with the ability to enhance the binding of phagocytotic cells to foreign particles are called opsonins. In vivo, the main opsonins are immunoglobulins (especially IgG and to a lesser extent IgA) and complement factors C3b, C3bi and C3dg. These complement factors are enzymatic cleavage products of complement factor C3, which are generated upon activation of the complement cascade. Moreover, complement factor Clq is thought to have opsonizing properties. We hypothesized that coating of the fiber with opsonins would result in enhanced adherence of the (immature) myeloid cells.

To investigate this possibility we used a human immunoglobulin product prepared from plasma, containing at least 90% IgG (CLB Immunoglobulin I.M.). C3b, C3bi and C3dg cannot be purified from plasma. However, the opsonizing capacity of C3 cleavage products can be mimicked by chemical cleavage of the thioester bond between a cysteine and glutamine residue in the α-chain of C3. The resulting molecule is called iC3. Due to a conformational change of the molecule phagocytotic cells can bind to it via their complement receptors with high affinity.

When we replaced the fetal calf serum (FCS) by a non-opsonizing protein (1% (w/v) human serum albumin (HSA)), both in the medium used for preincubation of the fiber and in the filtration medium, no influence on the adherence pattern of cells was found (Figure 7). However, when the nylon fiber was preincubated with immunoglobulin I.M. and subsequently saturated with human serum albumin (HSA), strongly enhanced depletion of myeloid cells (and also further enhancement of the adherence of monocytes) from elutriated PBSC was observed (Figure 7). We found that after coating with immunoglobulin, saturation of the fiber with a non-opsonizing protein was necessary to prevent aspecific adherence of all cell types. Omission of such a protein addition (from the preincubation and from the filtration medium) led to binding of almost all cells, including the $CD34^+$ cells, to the immunoglobulin-coated nylon fiber (Figure 8).

Coating of fiber with iC3, followed by saturation with HSA, resulted in moderately enhanced binding of monocytes and myeloid cells to the nylon fiber (Figure 9).

After coating of the nylon fiber with human IgG, myeloid cells also adhered to the fiber in the presence of a chelating substance. Figure 10 shows the effect of addition of trisodium citrate to the filtration medium on the yield of $CD34^+$ cells from PBSC leukocytapheresis preparations after filtration over IgG-coated nylon wool, without prior removal of platelets. Yield of $CD34^+$ cells was enhanced in the presence of trisodium citrate, while depletion of monocytes or myeloid cells was similar to that in filtrations in which no chelating substance was included in the filtration medium. Similar results were obtained upon addition of EDTA to the filtration medium.

Thus, the invention shows that phagocytic cells can be removed from stem cell products by passage over a fiber coated with an opsonin. It can be used for the enrichment of stem cells from bone marrow, PBSC grafts, and umbilical cord blood grafts. In a preferred embodiment, the passage over the opsonized fiber material is preceded by counterflow centrifugal elutriation, by which not only platelets, but also erythrocytes and a larger part of the lymphocytes can be removed from the grafts. By counterflow centrifugal elutriation similar depletions of non-stem cells can be achieved from bone marrow, from umbilical cord blood and from PBSC leukocytapheresis product. Therefore, the combination of these techniques may result in a significant enrichment of progenitor cells from all these types of grafts.

This is illustrated for PBSC leukocytapheresis products in Figure 11. PBSC grafts were subjected to counterflow centrifugal elutriation and subsequently subjected to passage over human immunoglobulin-coated nylon fiber. This resulted in nearly complete removal of erythrocytes and thrombocytes, in more than 95% removal of myeloid cells, monocytes, and B lymphocytes from PBSC, and in at least 80% removal of T and NK cells. The mean enrichment of stem cells obtained by this procedure was 9-fold. No procedure is known which results in similar enrichment of stem cells from peripheral blood stem cell leukocytapheresis products, without the use of specific monoclonal antibodies.

In another preferred embodiment, stem cell containing preparations are directly passaged, without prior removal of platelets, over a fiber material coated with an opsonin in a medium containing a chelating substance. This will result in removal of monocytes, myeloid cells and B lymphocytes, and therefore in a lower enrichment of stem cells than after processing stem cell grafts by consecutive elutriation and filtration. However, the removal of these cell types may be of great advantage for further (immuno)selection steps, since especially monocytes are sticky cells, which have a tendency to adhere to many types of material, thereby interfering with purification. Furthermore, myeloid cells are very susceptible to freezing/thawing damage, and removal of them will probably lead to better quality of stem cell grafts after thawing. These reasons, together with the fact that a filtration technique is in principle a simple and cost-effective technique, may make it very attractive to introduce it into current stem cell processing protocols as such.

It has been reported that in separations of human bone marrow by counterflow centrifugal elutriation, a primitive, pluripotent stem cell type, which does not grow out in colony assays but may be responsible for long-term engraftment, co-elutriates with the smaller lymphocytes. A fraction enriched for such primitive stem cells, and depleted of colony-forming cells, has been isolated from human bone marrow by separating by counterflow centrifugal elutriation and collecting the smallest cells, followed by isolation of $CD34^+$ cells from this fraction (International patent application WO 94/11493; Wagner, J.E. et al (1995) Blood 86, 512-523). This raises the question whether pluripotent stem cells from PBSC products might be lost together with the platelets, erythrocytes and lymphocytes in elutriation separations. To answer this question, the frequency of primitive hematopoietic stem cells in elutriation fractions was assayed in a limiting dilution type long-term stem cell culture on FBMD-1 stromal feeder cells by determination of cobble-stone-area-forming cells (CAFC) at week 4 and week 6 (according to Breems et al (1994) Leukemia 8, 1095-1104). In mouse studies, CAFC week 4 to 6 frequencies have been found to correlate with the ability of bone marrow cells to induce long-term hematopoietic repopulation. PBSC leukocytapheresis samples were separated into a fraction containing the small cells and a fraction containing the large cells, which include the majority of $CD34^+$ cells and CFU-GM. It was shown that the majority of week 4 and week 6 CAFC were recovered in the elutriation fraction containing the large cells. Moreover, as a control on the quality of the primitive stem cells in these fractions, the production of CFU-GM was assessed in parallel long-term cultures in flasks. For this purpose, cells from these long-term cultures were replated in appropriate semisolid medium at week 4 and week 6 and colony formation was determined after 14 days. These measurements demonstrated that all CFU-GM producing long-term-culture-initiating cells resided in the fraction containing the larger cells. No CFU-GM formation was observed in the cultures inoculated with cells from the elutriation fraction containing the small cells. Together, these results indicate that in peripheral blood stem cell grafts at least the majority of the stem cells capable of initiating long-term hematopoietic reconstitution is recovered in the elutriation fraction containing the large cells (together with the majority of $CD34^+$ cells and CFU-GM).

Instead of nylon, also other fiber materials may be used to remove phagocytotic cells from stem cell preparations. Appropriate fibers should not bind progenitor cells, or, alternatively, should not bind progenitor cells after being coated with a non-opsonizing protein (i.e. blocked with a blocking substance).

Enhancement of the adherence of phagocytotic cells is achieved by coating with an opsonin. In a preferred embodiment of the invention the opsonizing substance is human IgG. We observed that upon coating with human immunoglobulin the removal of (immature) myeloid cells and monocytes from PBSC transplants was more efficient than upon coating with human complement factor iC3. However, the enhanced adherence of phagocytotic cells to fiber may also be achieved by other opsonizing proteins. In principle, also IgA may be used, or the complement proteins C3b, C3bi, C3dg or C1q. Recently, a new class of opsonizing proteins has been discovered, called collectins (Holmskov, U. et al., Immunology Today 76 (1994): 67). These are the plasma lectins conglutinin, mannan-binding protein (MBP), CL-43 and the pulmonary surfactant proteins SP-A and SP-D. These proteins are ligands for the collectin receptor on phagocytes and can bind via their lectin domains to carbohydrates on microorganisms. These proteins may also be able to enhance binding of phagocytes to fibers.

For most fiber materials, it may be necessary to cover the fiber with some non-opsonizing substance to prevent aspecific cell loss (i.e. as a blocking substance). This can be done with biocompatible substances, which do not have adherent properties for cells and bind easily to the fiber. Several kinds of human or animal proteins (e.g. human serum albumin, bovine serum albumin) or colloids (e.g. gelatin) may be used. However, it is preferred to cover the fiber with human serum albumin. This is a plasma protein of human origin which is non-immunogenic and has very good coating properties for many types of material.

The invented procedure as such, or in combination with elutriation, may result in removal of malignant cells from the graft in several types of malignancies. Malignant cells are different in adherence properties and/or size from normal blood cells. For example, malignant B cells may be depleted by passage over opsonized fiber, but also by counterflow centrifugal elutriation. Carcinoma cells are generally more adhesive and larger than blood cells, and may also be separated from the progenitor cells during elutriation and/or passage over opsonized fiber.

Several procedures have been described for depletion of Fcγ-receptor bearing cells from stem cell containing cell preparations, e.g. by using magnetic beads coated with immunoglobulin (international patent application WO 94/02016) or sheep red blood cells coated with rabbit anti-sheep red blood cell IgG (Sawada, K. et al (1990) J. Cell. Physiol. 142, 219-230). It may be possible to deplete monocytes and myeloid cells from stem cell grafts by such techniques, since a large proportion of these cells expresses Fcγ-receptors. However, there are many obvious advantages of a filtration technique, as described here. No foreign particles are introduced into the stem cell preparation, the technique is simple and can be performed rapidly, is relatively inexpensive, and can be easily brought to a clinical scale on the basis of existing blood-filtration technology. Moreover, depletion of phagocytotic cells by the filtration technology described here, may be more efficient than by using immunoglobulin-coated beads or cells. The adherence in the filtration technique is not only based on affinity of the cells for immunoglobulin, but also on the natural affinity of phagocytotic cells for polyamide fiber.

When further purification of the progenitor cells is necessary, or further removal of malignant cells is necessary, the invented procedure may be followed by an immunoselection step. The immunoselection may be performed by a positive selection of progenitor cells (e.g. by using CD34 mAb) using any of the available immunoselection techniques described above, or by new immunoselection techniques. Alternatively, residual non-progenitor cells may be removed by negative selection using mAb directed against the various types of non-progenitor cells and/or the malignant cells.

The immunoselection can be performed after applying the invented procedure. Alternatively, an immunoselection may be integrated in the "filtration" procedure. For example, beads suited for capture of cells which are labeled with a specific antibody, may be integrated in a column of opsonized fiber material. Alternatively, beads to which cell-specific antibodies have been attached, can be applied.

The invention will be illustrated by the following examples of experimental work. It is noted, however, that these examples merely serve to a better understanding of the invention and do not intend, nor should be construed so as to limit the scope of the invention.

Examples

1. *Counterflow centrifugal elutriation of stem cell grafts.*

In case of PBSC leukocytapheresis products no preceding processing of cells before elutriation is necessary. Cells (a preparation containing $3x10^8$ - $7x10^8$ leukocytes) were suspended in 10-20 ml phosphate-buffered saline (PBS) supplemented with

20 mg/ml human serum albumin (HSA) and 5 mM EDTA. The human serum albumin preparation used was from the CLB and prepared by ethanol fractionation of human plasma and contained more than 95% albumin. Counterflow centrifugal elutriation was performed in a Curamé 3000 elutriation system (Dijkstra Vereenigde BV, Lelystad, The Netherlands) equipped with disposable polycarbonate separation chambers (International Medical BV, Zutphen, The Netherlands). A fluid counterflow within the elutriation chamber was achieved by a roller pump. In the inlet tubing, a pulse flattening air chamber was placed. The cell suspension was introduced into the system at a fluid flow of 12.5 ml/min and a rotor speed of 3000 rpm. The elutriation medium consisted of PBS supplemented with 4 mg/ml HSA and 5 mM EDTA. Alternatively, instead of EDTA, 0.38% (w/v) trisodium citrate was added. After introduction of the cells, the liquid flow was increased to 15 ml/min. The separation was carried out at 6°C. Rotation speed was decreased by steps of 50 to 100 rpm until a rotor speed of 1800 rpm was reached. At each rotation speed, fractions of 50 to 100 ml were collected from the outlet. After that the speed was slowed down to 1000 rpm and an additional fraction of 50-100 ml was collected. Subsequently, the size of the cells in each fraction was determined with a Coulter Counter equipped with a Channelyzer. Fractions containing the cells with larger size (= monocytes, myeloid cells and progenitor cells) were pooled, sedimented by centrifugation and suspended in a medium appropriate for filtration over nylon fiber.

2. *Passage over nylon fiber.*

Filtration over IgG-coated nylon wool: Elutriated stem cell preparations were suspended in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10 mg/ml HSA. 1.7 Gram nylon wool (type 200 combed, scrubbed nylon wool; Robbins Scientific Corporation, CA) was tightly packed in a 20 ml syringe. A plastic tubing was connected to the outlet of the syringe via a luer connection. A roller pump was used to obtain a fluid flow. The syringe was placed in a 37°C water bath and approximately 20 ml of IMDM containing 1 mg/ml human immunoglobulin (Immunoglobuline I.M., CLB, Amsterdam; obtained from normal human plasma, and containing at least 90% IgG) was pumped into the syringe from below until the nylon wool was completely wetted. The nylon fiber was incubated at 37°C during 30 min with the IgG, and subsequently 30 ml IMDM supplemented with 10 mg/ml HSA was pumped through it in the opposite direction (from above). The nylon wool was incubated at 37°C during 20 min. Then the cells ($45x10^6$ - $150x10^6$ leukocytes), suspended in IMDM with 10 mg/ml HSA, were applied to the nylon wool and pumped through with a flow rate of 1.0 to 2.5 ml/min. The non-adherent cells were washed from the fiber at the same flow rate with at least 30 ml IMDM supplemented with 10 mg/ml HSA. Cells present in the filtrate were sedimented by centrifugation and suspended in PBS supplemented with 0.38% trisodium citrate and 2 mg/ml HSA.

It was found that IMDM could be replaced by phosphate buffered saline (PBS), without any effect on the outcome of the filtration process. For enrichment of non-elutriated stem cell preparations by filtration, cells were suspended in PBS supplemented with HSA plus EDTA (5 mM) or trisodium citrate (13 mM). Likewise, as filtration medium, PBS supplemented with HSA plus EDTA or trisodium citrate was used.

Protocol using fetal bovine serum: In the first series of experiments, the nylon fiber was not coated with IgG, but IMDM containing 10% fetal bovine serum and 9 mM $MgCl_2$ was used as filtration medium. The nylon wool was also preincubated with this medium for 30 min at 37°C. Cells were suspended in IMDM supplemented with 10% heat-inactivated fetal bovine serum and 9 mM $MgCl_2$ (de Boer et al, J. Immunol. Methods 43 (1981): 225). The nylon wool was preincubated during 30 min at 37°C with this medium. No subsequent saturation of the fiber with IMDM supplemented with HSA was done. After the cells were applied, the non-adherent cells were removed by washing with at least 30 ml IMDM supplemented with 10% heat-inactivated fetal bovine serum and 9 mM $MgCl_2$.

To investigate the effect of chelating substances on the yield of stem cells in filtrations of non-elutriated stem cell preparations, the filtration medium was either or not supplemented with 13 mM trisodium citrate or 5 mM EDTA.

Protocol using iC3: Similar to the protocol with human immunoglobulin, except that the nylon wool was preincubated with 0.1 mg/ml iC3 in IMDM instead of immunoglobulin; the iC3 was prepared from complement factor C3 purified from human plasma. C3 was incubated with 0.2 M methylamine in PBS (37°C, 60 min). Non-reacted methylamine was removed by dialysis.

Protocol using HSA: Similar to the protocol using human immunoglobulin, except that the nylon wool was preincubated with IMDM supplemented with 10 mg/ml HSA instead of immunoglobulin.

3. Analysis of samples.

The PBSC leukocytapheresis samples, the fractions obtained after elutriation and the filtrate obtained after passage of cells over coated nylon wool were analyzed as follows:

* Leukocytes and erythrocytes were counted using a Coulter Counter (model ZF).

* Thrombocytes were counted using a Cell-Dyn 100 thrombocytometer (Sequoia-Turner).

* Leukocytes were differentiated by direct immunofluorescence and FACS analysis with a FACSscan flow cytometer (Becton and Dickinson, Mountain View, CA). The following mAb were used, conjugated either with fluorescein isothiocyanate (FITC) or phycoerythrin (PE): Leu-4 (CD3) for T cells, Leu-M3 (CD14) for monocytes, Leu-16 (CD20) for B cells, HPCA-2 (CD34) for progenitor cells, Leu-19 (CD56) for NK cells (all obtained from Becton and Dickinson), and CLB-gran/2 (CD15) for myeloid cells. Platelet adhesion to progenitor cells was studied using non-conjugated CLB-thromb/7 (CD41) followed by incubation with FITC-conjugated goat anti-mouse immunoglobulin (CLB).

experiment	lymphocytes	monocytes	myeloid cells	$CD34^+$ cells
1	89	27	50	103
2	78	2	51	69
3	112	3	83	64
4	36	5	8	29
5	63	0	10	12
6	71	22	48	112
7	40	3	18	42
8	69	10	42	36
9	80	3	11	29
10	83	1	5	21
11	71	8	41	37
12	83	1	73	114
13	65	1	15	34

Table 1: Recovery of cells in the filtrate after nylon wool filtration of PBSC leukocytapheresis products. Nylon wool (1.7 g in 20 ml syringe) was pre-incubated with IMDM supplemented with 10 % FCS and 9 mM $MgCl_2$. Cells were suspended in this medium and allowed to pass through the column at a controlled flow rate (1-2.5 ml/min.). The non-adherent cells were removed from the nylon wool by washing with at least two column volumes of medium. The recovery of cells in the filtrate is expressed as the percentage of cells which were applied to the nylon wool.

The claims defining the invention are as follows:

1. A process for the enrichment of hematopoietic stem cells in a stem cell preparation, comprising depleting non-stem cells from the preparation by adherence to an opsonised fibre material and recovering a hematopoietic stem cell-enriched preparation, wherein adherence of stem cells to the opsonised fibre material is prevented either by pretreating the stem cell preparation to remove platelets therefrom, or by including a chelating substance in the medium in which the non-stem cell depletion process is performed to prevent adherence of platelets to the stem cells.

2. The process of claim 1, wherein prior to opsonisation treatment said fibre material is a polyamide fibre material.

3. The process of claim 2, wherein said fibre material is treated with an opsonising substance selected from the group consisting of IgG, IgA, C3b, C3bi, C3dg, Clq, iC3, conglutinin, mannan-binding protein, CL-43, Sp-A and SP-D.

4. The process of claim 1, wherein said opsonised material comprises a scrubbed nylon fibre material coated with human IgG.

5. The process of any one of claims 1-4, wherein said opsonised material is treated with a blocking substance which reduces aspecific cell binding.

6. The process of claim 5, wherein said blocking substance is selected from the group consisting of human serum albumin, animal serum albumin, gelatin, Ficoll 70 and Ficoll 400.

7. The process of any one of claims 1-6, wherein said non-stem cells comprise monocytes and myeloid cells.

8. The process of any one of claims 1-7, wherein the stem cell preparation is pretreated to remove platelets therefrom.

9. The process of claim 8, wherein said pretreatment of the stem cell preparation comprises a counterflow centrifugal elutraition in which smaller cells including platelets, erythrocytes and small lymphocytes are removed and larger cells including hematopoietic stem cells, monocytes and myeloid cells are recovered.

10. The process of any one of claims 1-9, wherein a chelating substance is included in the medium in which the process of depletion of non-stem cells is performed, to prevent adherence of platelets to the stem cells.

11. The process of claim 10, wherein said chelating substance is EDTA or trisodium citrate.

12. The process of any one of claims 1-11, wherein the stem cell preparation is derived from blood or from bone marrow.

13. The process of any one of claims 1-11, wherein the stem cell preparation is an umbilical cord blood preparation.

14. The process of any one of claims 1-11, wherein the stem cell preparation is a peripheral blood stem cell preparation.

15. The process of any one of claims 1-14, comprising providing a stem cell preparation from bone marrow or, preferably, from peripheral blood or umbilical cord blood, depleting platelets, erythrocytes and small lymphocytes therefrom by a counterflow centrifugal elutriation in which smaller cells including platelets, erythrocytes and small lymphocytes are removed and larger cells including hematopoietic stem cells, monocytes and myeloid cells are recovered, depleting monocytes and myeloid cells from said recovered larger cells by adherence to a material coated with opsonising substance and blocked against aspecific cell bin and recovering a preparation enriched in hematopoietic stem cells.

16. The process of any one of claims 1-15, comprising providing a stem cell preparation from bone marrow or, preferably, from peripheral blood or umbilical cord blood, adding a chelating substance, such as EDTA or trisodium citrate, to the preparation to prevent adherence of platelets to leucocytes, depleting monocytes and myeloid cells from the preparation by adherence to a material coated with an opsonising substance and blocked against aspecific cell binding, and recovering a preparation enriched hematopoietic stem cells.

17. The process of any one of claims 1-16, further comprising a step of immunoselection using an antibody which binds hematopoietic cells, or an antibody which binds non-stem cells or malignant cells, to effect a further enrichment of hematopoietic stem cells.

18. A process for the enrichment of hematopoietic stem cells in a stem cell preparation, substantially as hereinbefore described with reference to any one of the Examples.

Dated 19 February, 1999
Stichting Centraal Laboratorium van de Bloedtransfusiedienst van het Nederlandse Rode Kruis

Patent Attorneys for the Applicant/Nominated Person
SPRUSON & FERGUSON

Figure 1: Principle of counterflow centrifugal elutriation.

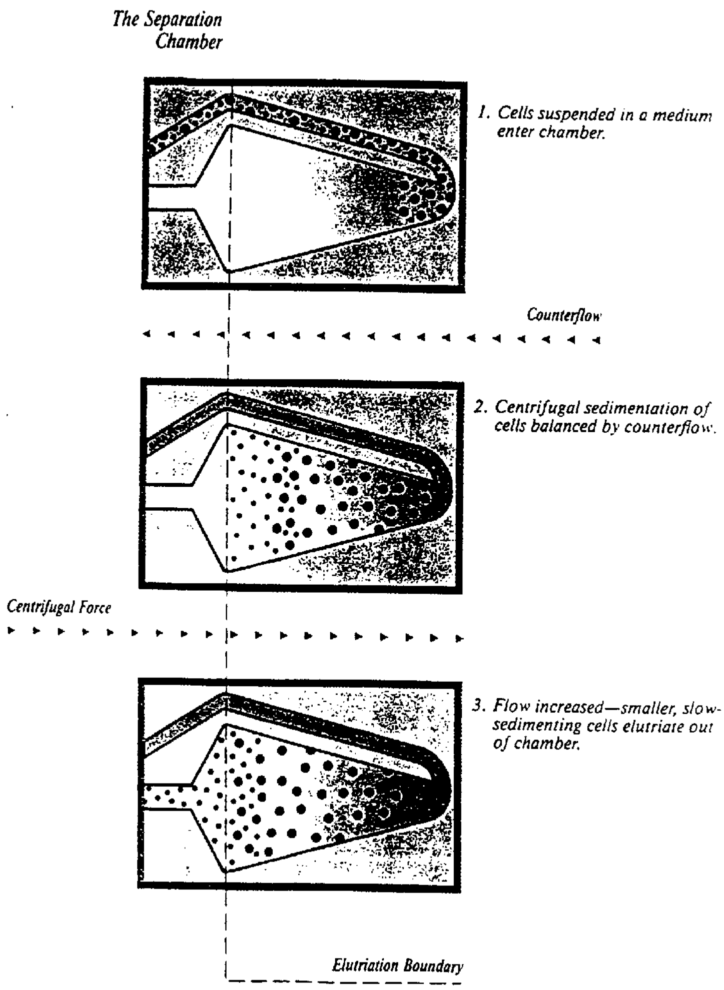
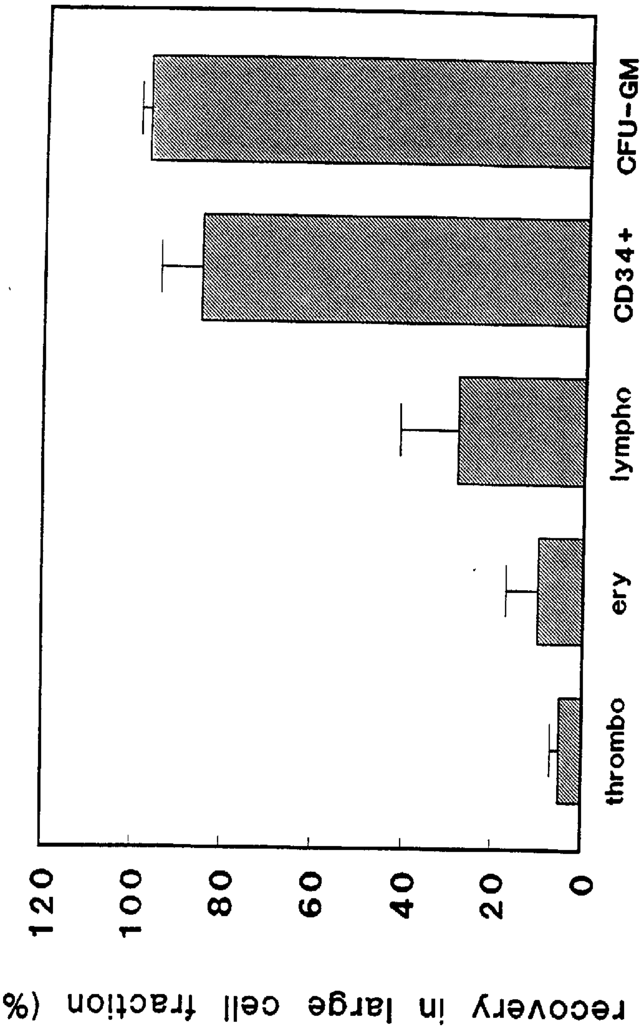


Figure 2: Effect of elutriation of PBSC leukocytapheresis product (n=14)



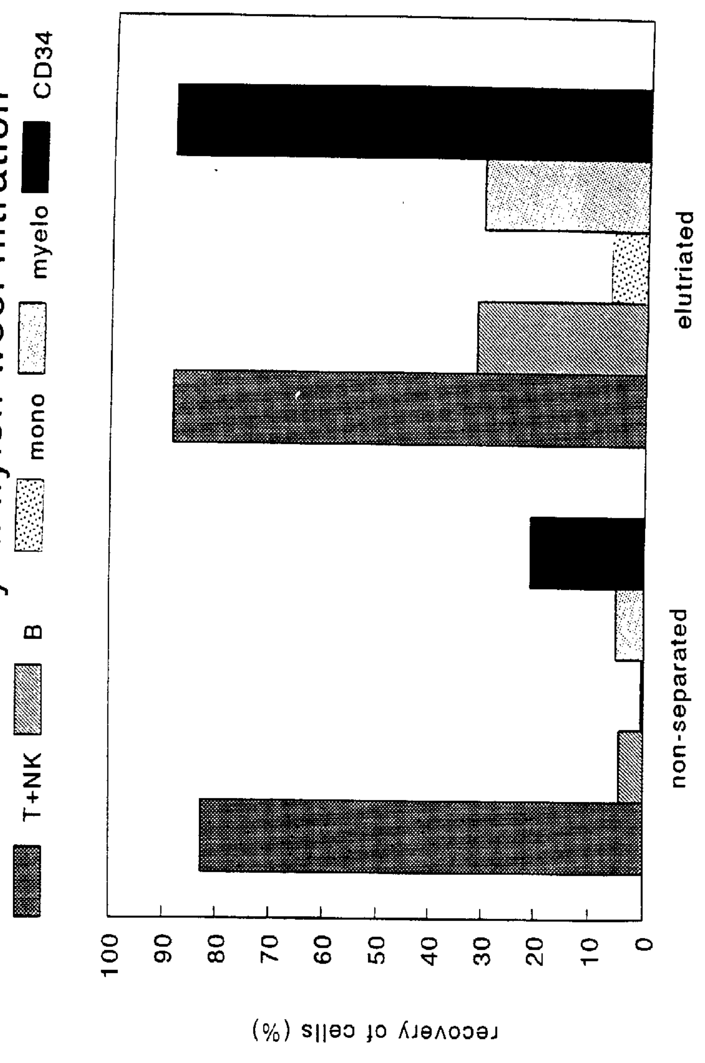
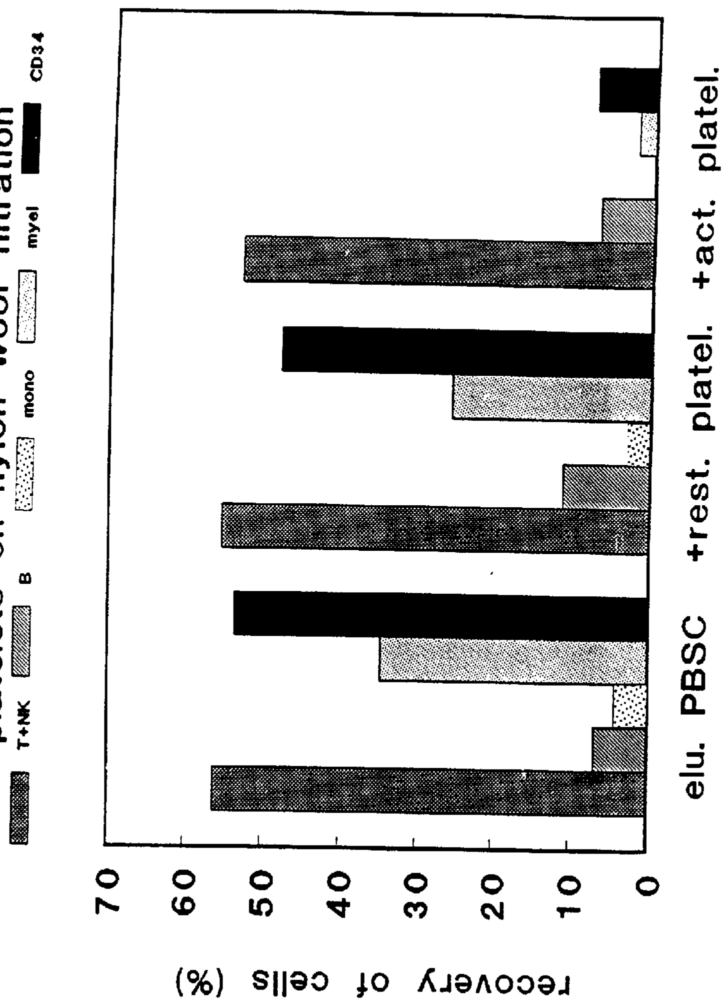


Figure 3: Effect of elutriation on cell recovery in nylon wool filtration

Figure 4: Effect of resting or activated platelets on nylon wool filtration



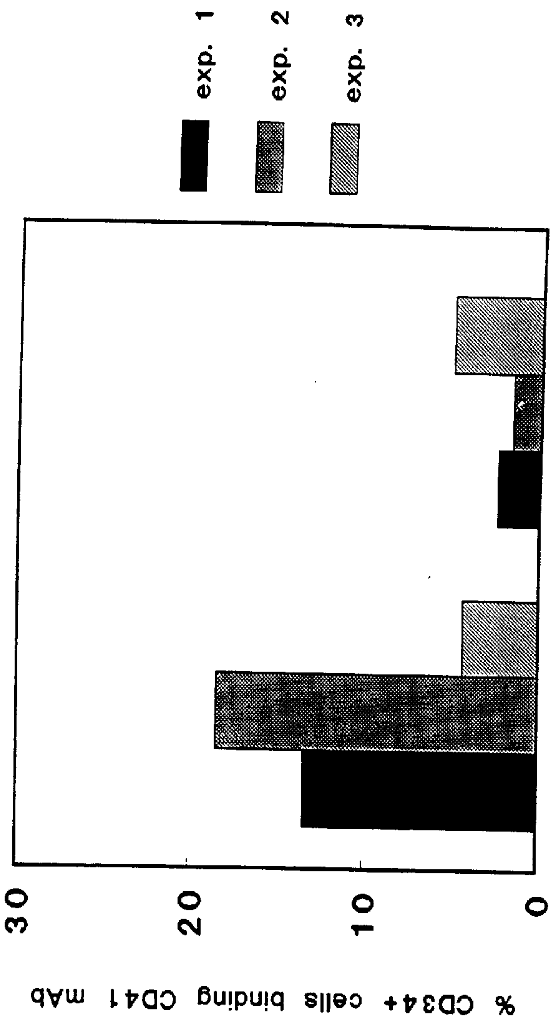


Figure 5: Effect of elutriation on platelet adherence to CD34+ cells

Figure 6: Effect chelators on filtration of PBSC over FCS-coated nylon wool

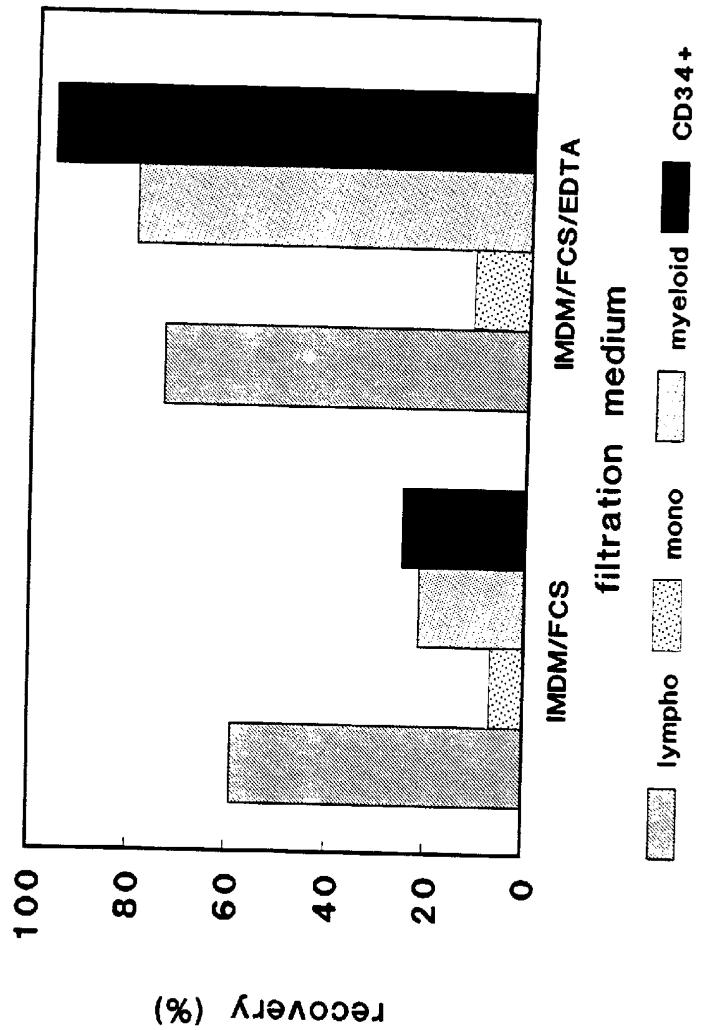


Figure 7: Effect of coating the fiber with HSA or IgG

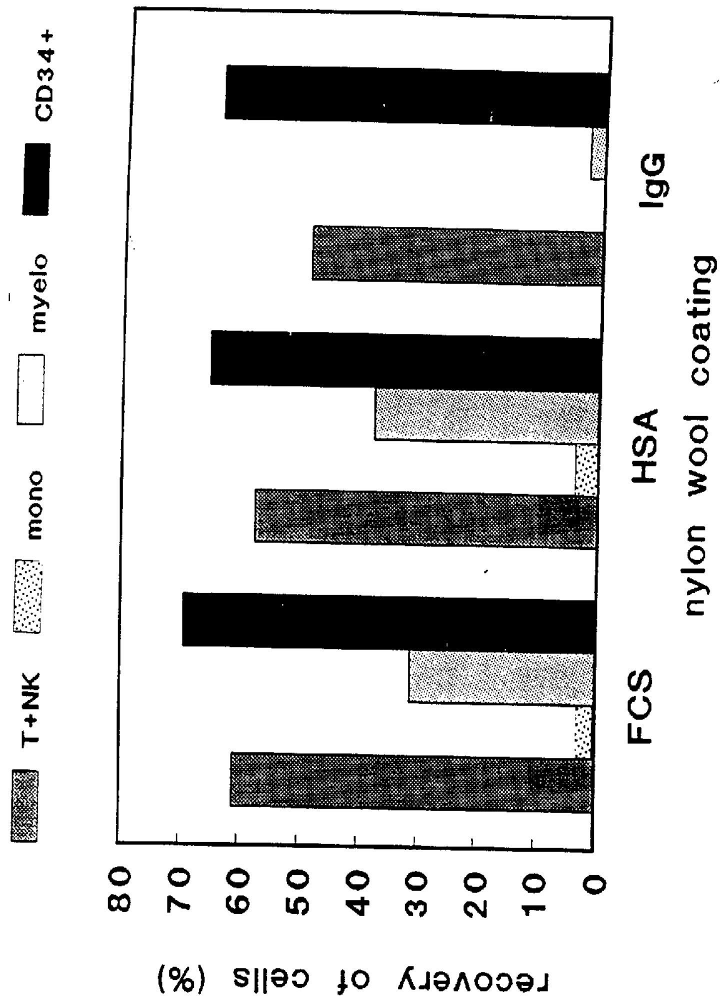


Figure 8: Effect of coating the fiber with IgG without subsequent saturation with HSA.

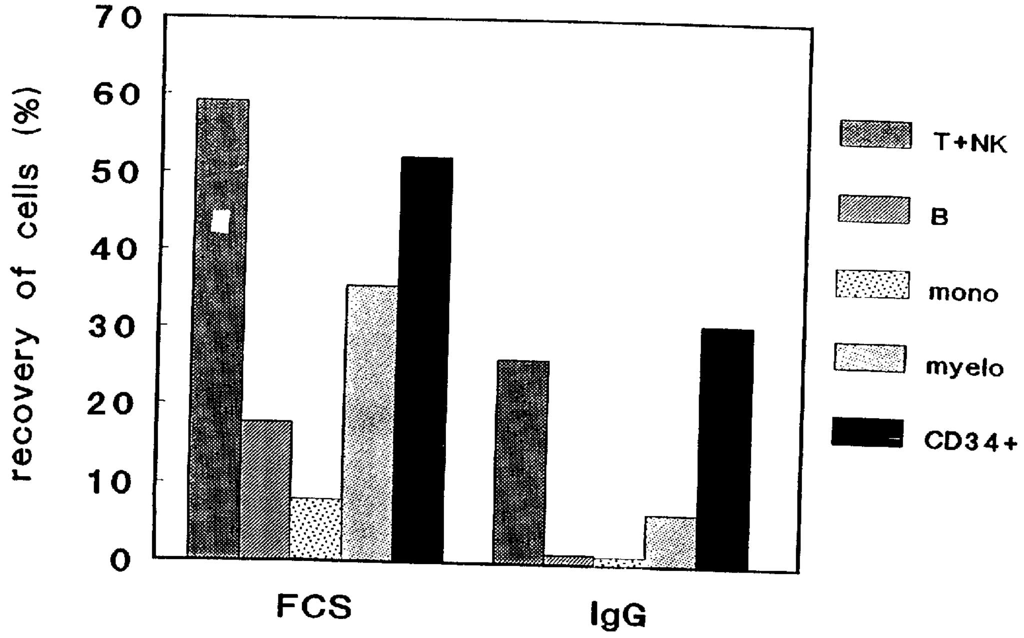


Figure 9: Effect of coating the fiber with iC3.

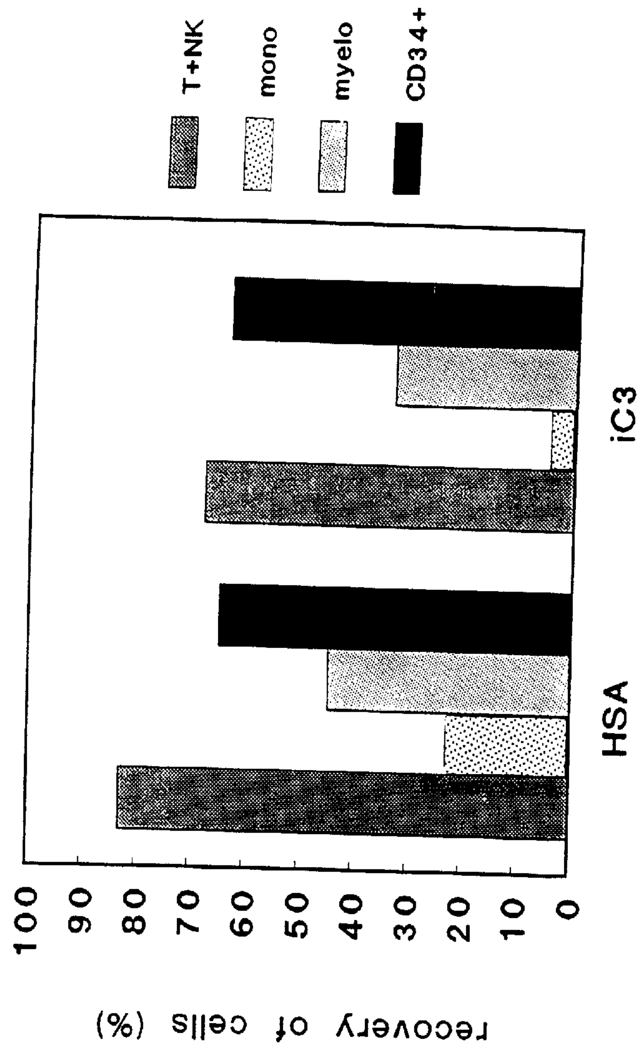


Figure 10:Effect chelators on filtration of PBSC over IgG-coated nylon wool

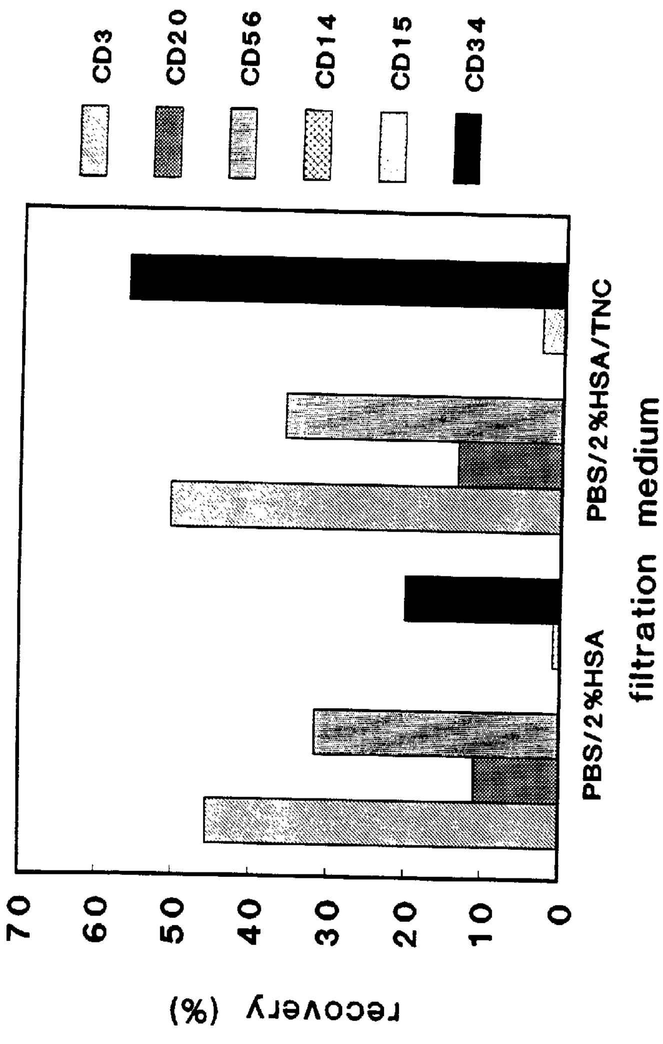


Figure 11: Removal of cells from PBSC by elitriation and nylon wool filtration.