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(54) Title: METHODS FOR POSITIVE IMMUNOSELECTION OF STEM CELLS

(57) Abstract

The invention relates to improved methods and kits for the positive immunoselection of target cells wherein the cells are immobilized to a solid matrix via an immune complex containing a soluble hapten and an anti-hapten antibody and are dissociable by hapten competition. The invention in particular relates to an improved positive immunoselection for purifying lymphohematopoietic stem and progenitor cells from heterogeneous cell compositions, and a novel immune reagent for effecting this purification.

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"Methods for Positive Immunoselection of Stem Cells"

The invention relates to improved methods for positive immunoselection of target cells from a heterogeneous cell composition. The invention also relates to immunological kits for effecting said immunoselection of cells. The invention also relates to improved methods for assaying the purity of the immunoselected cells using indicator cells, which may be autologous or heterologous, as well as methods for subjecting the recovered cells to further immunoselection procedures.

The invention in particular relates to improved methods for the positive immunoselection of stem and progenitor cells, and a specific immunological reagent (monoclonal antibody) for effecting said positive immunoselection of stem and progenitor cells. The invention also relates to improved methods for assaying the purity of said immunopurified stem and progenitor cells using indicator cells (e.g., autologous) as well as methods for subjecting the immunoselected cells to further immunoselection. Finally, the invention relates to purified stem and progenitor cells resulting from these methods, and methods of using these purified stem cells diagnostically and therapeutically.

BACKGROUND OF THE INVENTION

The use of immunoselection for the specific recovery of desired moieties from heterogeneous compositions is conventional in the art. Essentially immunoselection techniques broadly embrace the isolation of a desired moiety from a heterogeneous composition on the basis of its specific reactivity with its immunological binding partner. Essentially, these techniques exploit the specific reactivity of the desired moiety contained in a heterogeneous system, e.g., a particular antigen, bacteria, virus, cell, hapten, antibody, etc. with its immunological counterpart, an immunological reagent which is generally an antibody, antibody-like molecule, or an antigen so as to effect the desired separation.

Immunoselection techniques have in particular been utilized for the separation of desired cells from heterogeneous compositions comprising these cells. This application of immunoselection for the purification of specific cell types has been the focus of much recent research given the significant potential applications of purified cell compositions as diagnostics and therapeutics, as well as for biomedical research.

For example, the selective recovery of specific highly purified cell types will better enable biological researchers to study the differentiation of these specific cell types, will facilitate the identification of the specific lineage or function-specific antigens comprised on these cell types and will enable researchers to identify the specific discrete functions and bioproducts produced by these cells.

A particular relevant example of the applications of cellular immunoselection is in the area of bone marrow transplantation (BMT). BMT essentially involves the infusion of healthy bone marrow cells into a recipient in need thereof. The recipients for BMT generally have been patients exhibiting blood and immune system defects or diseases. In particular, bone marrow transplants have been performed in patients whose immune and hematopoietic systems are devastated or severely immuno-compromised by conditions including leukemia, cancer, chemotherapy, radiation therapy, lympho-hematopoietic malignancies, solid tumors, or

congenital metabolic defects. BMT has further been contemplated as an adjunct to gene therapy.

As noted, the general objective of BMT is the infusion of healthy bone marrow cells into a recipient in need thereof. However, a more specific objective of BMT is the infusion of healthy stem cells and progenitor cells into a recipient in need thereof. This is essentially because these cells are the "master cells" from which all the cells of the immune and hematopoietic system develop. Accordingly, an effective method for the infusion of sufficient quantities of healthy stem and progenitor cells into a recipient in need thereof could result in the entire reconstitution of the immune and hematopoietic system.

Thus, the more specific objective of BMT is the infusion of healthy stem and progenitor cells into a recipient in need thereof. BMT is classifiable into two general types. Autologous BMT comprises the reinfusion of healthy bone marrow or stem cells into the same recipient from which these cells have been derived. Conversely, heterologous or allogeneic BMT involves the infusion of healthy bone marrow or stem cells into a recipient other than from whom the cells were derived. These two distinct forms of BMT have different applications and comprise different constraints.

Autologous BMT is utilized in particular in cancer patients being treated by chemotherapy or radiation. While these treatment protocols are designed to eradicate tumor cells, unfortunately, these methods may also damage normal cells, in particular cells of the hematopoietic and immune system. Therefore, as a means for alleviating these adverse side effects of cancer therapy, it has been known to remove a portion of the subject's bone marrow prior to initiation of treatment, which bone marrow is then later reinfused. However, since the bone marrow is generally obtained from a donor who has a malignant condition, it has further been known to "purge" these bone marrow cells of any contaminating malignant cells by immunoselection or immunodepletion as this technique is also called.

In contrast, in the case of allogeneic or heterologous BMT, the bone marrow or stem cells are derived from a heterologous healthy

donor. Allogeneic BMT is particularly useful in patients exhibiting congenital defects which result in a defective immune or hematopoietic system. Thus, since the marrow is obtained from the healthy donor, the cells should not be contaminated by malignant cells. Instead, these cells contain allogeneic or heterologous normal T-lymphocytes, which may give rise to a condition known as graft-versus-host disease. Therefore, in the case of allogeneic or heterologous BMT, immunoselection has been utilized to selectively deplete or purge these normal T-lymphocytes from the donor bone marrow. Thus, it is clear that immunoselection comprises particular application in BMT, and more particularly in the selective recovery of healthy stem and progenitor cells. Moreover, cellular immunoselection comprises general applicability in the selective recovery of any desired cell wherein suitable immunological reagents are available, in particular, antibodies or antibody-like molecules which selectively bind the desired cell or the cells which are to be selectively depleted.

Cellular immunoselection may be generally classified into two types. Positive immunoselection implies the direct selection and recovery of cells bearing a given specificity from among a heterogenous population. Depending upon factors including the binding specificity and affinity of the particular immunoreagent (antibody or antibody-like molecule), labeling, and recovery methods these methods may give rise to cell compositions which are highly pure or merely somewhat enriched. The major limitation of positive cellular immunoselection has been the difficulty of physically labeling the cells so that they may be isolated without damaging the cells.

In order for a positive cellular immunoselection technique to be useful it should be (i) gentle, it should result in viable functional cells; (ii) rapid - the different operations should be performed with sufficient speed to allow recovery of viable cells; (iii) high capacity - a useful technique should at the minimum permit an input of 10^8 cells for laboratory-scale immunoselections, and ideally at least 10^9 - 10^{10} cells to be clinically relevant; and (iv) high-resolution - it should discriminate between the desired cells and the other cells in the sample with a high degree of reliability.

Heretofore, conventional positive immunoselection methods have not satisfied all these factors.

In contrast, negative cellular immunoselection (or immunodepletion as it is also referred) involves the specific elimination of undesired cells from a heterogenous population. This technique may obtain the desired cell type in highly concentrated form, however, it does not generally give rise to a pure cell sample. This is considered to be the most significant disadvantage of this technique. (For a general review of the subject of positive and negative immunoselection, see Basch et al. (1983), J. Immunol. Methods, 56:269.)

Both positive and negative immunoselection techniques have been used in BMT. Negative immunoselection techniques have, however, been utilized most often. These methods have generally involved treatment of the donor bone marrow with anti-tumor or anti-T-cell antibodies which have been incubated with complement, conjugated with toxins or radioisotopes, attached by various linkers or spacer systems to red blood cells or beads for rosetting and density fractionation, and similarly, coupled to plates or to fiber or beads of variable chemical composition which are packed in columns. (These techniques are, e.g., reviewed in Janossy (1984), Br. Med. Bull., 40:247; and Janossy (1987), Immunol. Today, 8:253.) Antibodies have also been coupled to magnetic particles or fluids of varying chemical composition or magnetic properties (reviewed extensively in Kemshead (1991), J. Hematotherapy, 1:35). These approaches to negative immunoselection share a number of significant limitations.

One limitation is that considerable antigenic heterogeneity exists among tumor cells, even from among those of the same type and patient origin. To overcome this, attempts have been made to assemble cocktails of different monoclonal antibodies specific for each antigenic variant cell type in order to ensure detection of all tumor cells (see e.g., Treleaven et al. (1984), Lancet, i:70; Reynolds et al. (1986), Cancer Research, 46:5882; Kemshead and Gibson (1987), Bone Marrow Transplantation, 2:84). However, developing and standardizing such panels

of antibodies can be difficult and costly. Moreover, it has been reported that some combinations of antibodies recognizing distinct antigens are inhibitory with respect to the efficiency of cell capture using magnetic particles, thus necessitating the testing of various different combinations of antibodies to determine which gives the best separation (Gee et al. (1991), J. Immunol. Methods, 142:127).

Another limitation of current purging techniques is that they often do not transition efficiently from laboratory-scale demonstrations (typically 10^{7-8} cell range) to large clinical scale separations (10^9 to over 10^{10} cell range). One consideration is that these techniques generally require large quantities or volumes of reagents to be effective with bone marrow, and therefore may be prohibitively expensive.

By far the greatest limitation of these purging techniques is that they are incompletely effective. For example, techniques capable of only 3-log (1000 fold) tumor cell reduction capability have been used to perform autologous BMT in patients (see Janossy (1987) Immunol. Today, 8:253; Bone Marrow Transplantation: Current Controversies, UCLA Symp. Molec. Cell Biol., Vol. 91 (1989) Gale and Champlin, eds). In the best current example of bone marrow tumor purging by negative immunoselection, 3-5 log purges have been achieved using cocktails of up to 5 different monoclonal antibodies directed against neuroblastoma cells coupled to immunomagnetic beads. (See e.g., Treleaven et al. (1984), Lancet, i:70, Reynolds et al. (1986), Cancer Research, 46:5882; Kemshead and Gibson (1987), Bone Marrow Transplantation, 2:84; Gee et al., in Magnetic Separation Techniques Applied to Cellular and Molecular Biology (1991), ed. Kemshead, p. 197). Similar approaches to purging of bone marrow have been employed in patients with breast carcinoma (Anderson et al. (1989), Cancer Research, 49:4659; O'Briant et al. (1991), Cancer, 68:1272), B-cell lymphoma (Funderud et al. (1990), Eur. J. Immunol., 20:201), myeloma (Rhodes et al. (1989), Brit. J. Haematol., 71:183), and common acute lymphoblastic leukemia (Kemshead and Gibson (1987), Bone Marrow Transplantation, 2:84). In some cases immunoselection has been

combined with pharmacologic purging ex vivo to increase overall purging effectiveness (see, e.g., O'Briant et al. (1991), Cancer, 68:1272; Shpall et al. (1992), J. Hematotherapy, 1:45). However, the agents used can also kill normal hematopoietic progenitors as well as delay engraftment (see e.g., Rowley et al. (1987), Blood, 70:271; Shpall et al. (1990), J. Clin. Oncol., 9:85).

To illustrate the inherent problems in the effectiveness of current purging methods, Table 1 is presented which depicts the possible number of tumor cells among normal bone marrow cells that could be reinfused in a transplant recipient. Assuming, e.g., a population of 1% tumor cells (7×10^7) in a bone marrow harvest of minimally 7×10^9 nucleated cells for an adult patient, a 4 log purge would theoretically result in 7×10^3 tumor cells remaining in the graft. This level of tumor cell contamination (1 tumor cell per 10^6 normal marrow cells) is not detectable by any current technology (Kemshead (1992), J. Hematotherapy, 1:35). Since malignant tumors typically develop from a single transformed cell at a time when a patient is not immunosuppressed as would be the case following cytoablative therapy preceding a transplant, the possibility of reinfusing any malignant cells is of concern.

In fact, these concerns have spurred a number of clinical marrow transplant protocols in which genetic markers have been inserted into the grafts (including tumor cells presumably in the grafts), in order to determine the extent to which tumor recurrence is attributable to reseeding of clonogenic tumor cells with the transplant, or to incomplete tumor cell killing in vivo (see e.g., Anderson (1992), Science, 256:808). Such findings will determine if greater emphasis should be focused on higher dose regimens in vivo or more effective purging procedures ex vivo. Despite the lack of a definitive answer to this question to date, evidence that purging may be associated with lower incidences of tumor relapse in many cases (see e.g., Shpall et al. (1992), J. Hematotherapy, 1:45), will ensure that such manipulations will continue for the future.

TABLE 1

TUMOR PURGING FROM BONE MARROW

% TUMOR CELL CONTAMINATION	LOG PURGE	# TUMOR CELLS PER $1x10^8$ BMC	# TUMOR CELLS PER 70 KG RECIPIENT*
1	-	1,000,000	70,000,000
0.1	1	100,000	7,000,000
0.01	2	10,000	700,000
0.001	3	1000	70,000
0.0001	4	100	7000
0.00001	5	10	700
0.000001	6	1	70
0.0000001	7	<1	7

* Patient reconstituted with $1X10^8$ BMC/KG

In light of the above discussed problems associated with negative immunoselection methods, recently methods for the positive immunoselection of targeted cells, e.g., human stem cells, as an alternative to negative immunoselection methods, have also been explored in the art.

While positive immunoselections have the potential of being able to obtain highly purified cell compositions, to date positive cell immunoselections have been subject to numerous problems which hinder the widespread use of these methods for purifying cells which are to be used therapeutically, e.g., bone marrow therapy.

A major disadvantage of currently available positive cell immunoselections is that the binding of antibodies to the targeted cell often adversely affects the functional characteristics of the cells and further makes removal of the cells from the immunological substrate difficult. For example, Pilling et al (1989), J. Immunological Methods 122:235 note the difficulties in removing positively selected cells from magnetic Dynabeads, and further acknowledge that such methods may be limited wherein the removal of beads is necessary (as would, of course, be necessary if the cells are to be used in therapy).

Another disadvantage of currently available positive immunoselection methods is that the purified cells are contaminated by non-

target cells, attributable, e.g., to non-specific binding of non-target cells to the immunological substrate, e.g., antibody bound magnetic beads. For example, Janssen et al (1989), J. Immunological Methods 121:289 disclose that while antibody-coated magnetic particles are efficient for purging neoplastic or immune competent cells from bone marrow prior to transplantation, they are unsuitable for positive immunoselection methods wherein the goal is to obtain a homogeneous population of cells.

Further, a recent literature reference by Manyonda et al (1992), J. Immunol. Methods, 149:1, confirms the current difficulties observed when utilizing positive immunoselection methods to purify CD4 bearing cells. Manyonda et al disclose the positive and negative selection of $CD45RO^+$ cells using a magnetic cell sorter, and teach an enriched fraction of greater than 98% purity from an initial population of about 50% antigen positive cells. However, they acknowledge that their cells are contaminated by non-target cells (monocytes), and that their method is further disadvantageous because it requires several separations, an initial optimization of all reagents utilized, and moreover obtains very low cell yields.

In order to develop an effective positive immunoselection of stem cells, or in particular, human pluripotent lympho-hematopoietic stem cells and progenitor cells, it is of course necessary to produce antibodies or antibody-like molecules which selectively bind stem and progenitor cells, and which do not bind mature cells. Potentially useful antibodies for this purpose include the anti-MY-10 antibody which reacts with the CD34 antigen, an antigen is expressed on the surface of stem and progenitor cells and which is not expressed on mature lymphoid or myeloid cells. (See, e.g., Civin et al. (1984), J. Immunol., 133:157; U.S. Patent Nos. 4,714,680; 4,965,204 and 5,035,994; and Civin et al. (1989), 2nd Intl. Symp. on Bone Marrow Purging and Processing, Cancun, Mexico, abstract). Stem cells or $CD34^+$ cells typically comprise 1-4% or less of bone marrow mononuclear cells, and a much lower fraction of normal blood mononuclear cells. Various other monoclonal antibodies with similar antigenic

specificities have also been developed (Tindle et al. (1984), Leuk Res., 9:1; Andrews et al. (1986), Blood, 67:842; Watt et al. (1987), Leukemia, 1:417; Kessler (1987), Fed. Proc., 46:1363).

U.S. Patent Nos. 4,714,680, 4,965,204 and 5,039,994 further contemplate using the disclosed monoclonal antibody for the positive immunoselection of stem cells. The described methods for the separation of antibody-bound stem cells from unbound cells include mechanical cell sorting (e.g., with a fluorescence activated cell sorter) and immobilization on supports such as agarose or polystyrene bead columns, hollow fiber membrane and petri dishes. These patents further contemplate two means for separating the bound cells from the column, specifically, vigorous agitation or enzymatic "nicking" or digestion of an enzyme cleavable spacer which intervenes the solid phase and the antibody. However, only small-scale demonstrations (1-5 $x10^8$ cells) are provided which methods use fluorescence activated cell sorting (FACS), immune rosetting followed by lysis of the red cells, and "panning" on petri dishes followed by agitation. However, given the state of the art, these methods cannot be practicably scaled-up to the cell numbers which are necessary for clinical BMT without substantial and prolonged manipulations of the marrow. Additionally, and perhaps more importantly, these patents further acknowledge, at least implicitly, that their methods obtain cell compositions which are contaminated by non-stem cells. This is clear from their disclosure that their method "provides a suspension of human cells comprising human lymph-hematopoietic stem cells substantially free of mature lymphoid and myeloid cells". Thus, despite the availability of immune reagents specific to stem cells, there is still a significant need in the art for improved positive immunoselections for recovering stem cells which are capable of both high capacity cell separations and which, moreover, yield stem cell compositions of high cell purities.

In order to increase the capacity of positive stem cell immunoselections, modifications to the above methods have been published by which CD34 monoclonal antibody, in particular anti-MY-10, has been

coupled to magnetic beads (Civin et al. (1989), In: Progress in Clinical and Biological Research, eds. Gross et al., p.387; Law et al. (1991), Blood, 78:223a). These demonstrations have been characteristically limited to small to large laboratory-scale separations, have required high bead to cell ratios (at least 1 bead per marrow mononuclear cell), and have required a pre-depletion step (negative selection) with magnetic beads to remove non-specifically binding cells. By these means CD34-positive cell purity was increased from approximately 36% without pre-depletion to 90-95% with depletions. In addition to the extended manipulations and relative expense of the magnetic beads and other components, this technique was further limited by virtue of the fact that the positively immunoselected cells were released from the beads using the proteolytic enzyme chymopapain. This limits such immunoselection procedures to those antigen-antibody complexes that are sensitive to enzymatic digestion, and, moreover, other cell surface antigens are removed so as to preclude subsequent analyses or immunoselections.

Toward this end, several publications and patents have described improvements in the efficiency of immunoselections using in particular biotin-derivatized antibodies in conjunction with immobilized avidin. The use of biotin and avidin in immunoselection techniques is well known in the art. Biotin, a natural vitamin found in trace amounts in all cells, combines with avidin, a glycoprotein derived from raw egg white with a very high affinity constant (Kd = 10^{-15}M). Because of this high affinity, the avidin-biotin complex has been used as a tool in molecular biology in methods including: isolation of biotin-derivatized material by affinity chromatography; affinity labeling and identification studies; affinity cytochemical labeling for localization studies in fluorescence and electro-microscopy; inhibition of bacteriophage; and study of cell surface molecular interactions.

The use of biotin and avidin complexes in the negative immunoselection of cells is also known in the art. For example, model negative immunoselection systems have been reported using biotinylated

anti-T cell antibodies to deplete splenic T cells on avidin coated petri plates (1984, J. Exp., 159:463 and similarly in U.S. patent No. 4,298,685). Additionally, biotinylated antibodies have been used with avidin-coupled sheep red blood cells to rosette antigen-positive cells and separate them on density gradients (Wormmeester et al. (1983), J. Immunol. Methods, 67:389; Furfang and Thierfelder (1987), J. Immunol. Methods, 91:123). Antigen negative as well as antigen positive cells could be recovered following disruption of the red cells; however, unfortunately these cells are coated with red membrane "ghosts" which is undesirable if the cells are to be used as a therapeutic.

Although the avidin-biotin complex is widely used for immunoassays and immunoseparations and has many attributes, one significant disadvantage is the fact that the avidin-biotin complex is essentially irreversible. For this reason, it is not easily utilized for positive cellular immunoselections. Therefore, in order to extend the versatility of the biotin-avidin system to positive as well as the negative immunoselections discussed above, several modifications have been described to obviate the irreversibility of this interaction. For example, one reference suggested derivatizing antibodies with biotin analogs exhibiting a reduced affinity for avidin, so that the cells immobilized on avidin coated matrices could be recovered by competition with free biotin (Basch et al. (1983), J. Immunol. Methods, 56:269-280). However, no data were presented to determine the feasibility of this method. Moreover, it should be noted that an analog with too low an affinity for avidin would result in inadequate selections.

U.S. Patent Nos. 4,253,996 and 4,276,206 further proposed the production of avidin-sepharose conjugates with reduced affinity for biotin, as a means for isolating biotin derivatized moieties by affinity chromatography. Additionally, Berenson et al. have proposed the use of biotinylated secondary antibodies produced against a first cell-specific antibody and the use thereof to immobilize cells onto polyacrylamide or agarose bead columns or to hollow fibers coupled with avidin. See Berenson et al. (1986), J. Immunol. Methods, 91:11; Berenson et al. (1986), Blood,

67:409; Berenson et al. (1987), Blood, 69:1363 and International Application Number PCT/0587/00101. The avidin is coupled at very low densities so as to reduce the affinity of the cells attached thereto. The cells are then recovered from the column by mechanical agitation of the column after washing free unbound cells.

Unfortunately, this technique also releases non-specifically bound or trapped cells which are comprised on the column. Thus, this does not result in very pure populations. In addition, the use of agitation during the separation procedure poses an undesirable risk of causing damage to the cells and in particular to the cell surface antigens. This procedure has also been specifically applied to the isolation of CD34-positive cells for clinical BMT, and a commercial purification device has become available for this purpose (CellPro, Inc., Bothell, WA). However, this purity of the recovered cells was diminished further as a consequence of scaling-up. Reported levels of cell purity obtained have ranged variously from 60 to 85% (Berenson et al. (1991), 2nd Intl. Symp. on Bone Marrow Purging and Processing, Cancun, Mexico, abstract), from 35 to 92% (Berenson et al. (1991), Blood, 77:1717) and from 50 to 90% (Heimfeld et al. (1991) Blood, 78:16a).

A published review of the prior art in regard to isolation of hematopoietic cells, including CD34-positive cells, has suggested that a magnetic matrix is more efficient at capturing and purifying CD34-positive cells than a column system as described above (Kemshead (1992), J. Hematotherapy, 1:35). Moreover, this review concluded the idea of selecting CD34-positive cells from bone marrow to avoid tumor cell contamination is unsound unless a very high purity of progenitor cells can be achieved. An illustration was provided showing that at a hypothetical 1% level of tumor cell contamination, only when 99% purity is possible do tumor cell depletions approach the levels theoretically possible with current purging methods. Such levels of purity have not been achieved with large scale immunoselections using the prior art.

Accordingly, given the above described state of the prior art, it is clear that improved methods for recovering targeted cell types by positive immunoselection are needed in the art. It is further clear that improved positive cellular immunoselection methods designed for the recovery of highly pure stem and progenitor cells, given their applicability in BMT, are needed in particular.

SUMMARY OF THE INVENTION

Accordingly, one object of the present invention is to provide improved methods for the positive immunoselection of desired cells from heterogenous compositions containing these cells, which methods attain levels of cell purity which substantially surpass the levels of cell purity obtained by conventional methods and which further do not disturb the cell surface membrane or membrane antigens.

Another object of the invention is to provide immunological kits which are suitable for said positive immunoselection of desired cells from heterogenous compositions containing the desired cells.

A further object of the invention is to provide methods of quantifying the purity of the resultant positively immunoselected cells which methods do not require the addition of foreign or unrelated cells to the pre-immunoselection mixture.

A further object of the invention is to provide methods for subjecting the resultant positively immunoselected cells to further multiple concurrent or sequential positive or negative immunoselections. These methods may be performed rapidly since the present methods do not disturb the cell surface membranes or membrane antigens.

A further object of the invention is to obtain methods for positive immunoselection of cells which use substantially less reagents and are therefore less expensive than conventional cellular immunoselections.

Another object of the present invention is to provide a means for assessing the purity of the purified immunoselected cells by further

reacting these cells with an immune reagent which binds a distinct antigenic epitope from the immune reagent used for positive immunoselection.

A more specific object of this invention is to provide a method for positive immunoselection of stem cells from heterogeneous compositions containing such cells, which results in stem cells virtually devoid of contaminating malignant or tumor cells or mature T-lymphocytes.

Another object of the invention is to provide highly pure stem cell compositions which are essentially devoid of contaminating malignant cells or mature T-lymphocytes and which consequently may be used therapeutically in BMT or for biomedical research.

Still another object of the present invention is to provide an immunological reagent (monoclonal antibody) which specifically binds lympho-hematopoietic stem cells and a hybridoma cell line which produces this monoclonal antibody.

Finally, another object of the present invention is to provide immunological kits for selectively recovering stem cells from heterogeneous compositions containing these cells.

DETAILED SUMMARY OF THE INVENTION

The present invention provides a significant advance in the art of positive immunoselection of cells, and in particular in the immunoselection of lympho-hematopoietic stem and progenitor cells.

However, prior to discussing this invention in more detail, the following terms will first be defined.

DEFINITIONS

As used herein, the following terms will have the definitions given below:

"Hapten" are those moieties which specifically react with antibodies specific thereto but which moieties themselves are not capable of causing the production of antibodies. Known haptens include, e.g., biotin, arsenilic-acid, dinitrophenol, fluorescein, oligopeptides and glycopeptides.

"Antibody" or "Antibody-like Molecule" are substances capable of specifically binding an antigen. Antibodies or antibody-like molecules in the context of this invention will include monoclonal and polyclonal antibodies, chimeric antibodies, and fragments thereof capable of specifically binding an antigen.

"Soluble hapten" is intended to mean a hapten which is sufficiently soluble to permit a solution containing said hapten to be capable of selectively dissociating target cells bound to a solid phase either directly or indirectly via a hapten-anti-hapten antibody complex.

"Monoclonal antibody" is an antibody produced by a clone or hybrid cell line. Methods of producing monoclonal antibodies are well known in the art.

"Immunoselection" means the selective recovery or removal of a moiety from a heterogeneous composition of the basis of its specific reactivity with an immunological binding partner, or immunological reagent, preferably an antibody or antibody-like molecule.

"Cellular Immunoselection" means the selective recovery of a target cell on the basis of the target cells 'or non-target cells' specific reactivity with an immunological binding partner, preferably an antibody or antibody-like molecule which is directed to an antigen contained on the surface of said target cell type or non-target cells.

"Positive Cellular Immunoselection" means the direct recovery of a target cell type from a heterogeneous cell composition on the basis of its specific reactivity with an antibody or antibody-like molecule which has been produced to an antigen on the surface of said target cell.

"Negative Cellular Immunoselection" or "Negative Cellular Immunodepletion" means the selective depletion of non-target cells from a heterogenous composition containing the target cells on the basis of the non-target cells' specific reactivity with one or more antibodies or antibody-like molecules directed to antigens contained on the surface of these non-target cells.

"Heterogeneous cell composition" means a composition containing the target cells which are to be recovered in admixture with non-target cells.

"Target Cells" are those cells which are to be selectively recovered on the basis of the inventive immunoselection procedure, e.g., stem cells and progenitor cells.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts an immunoblot analysis involving the CD34 antigen purified by immunoprecipitation which compares the immuno-reactivity of the K6.1 antibody, the anti-MY-10 antibody, and UPC-10 (a negative control myeloma protein of the same isotype as K6.1).

Figure 2 depicts an indirect immunofluorescence study involving FITC-avidin and flow cytometry comparing the effects of pre-binding either the K6.1 or anti-MY-10 antibody to the CD34 antigen or KG-1a cells relative to the subsequent binding of the other antibody.

Figure 3 depicts a flow cytometric analysis of CD34 antigen expression of cells positively immunoselected according to the invention.

Figure 4 depicts the results of immunofluorescence staining and flow cytometry analysis of immunoselected rhesus $CD34^+$ cells.

Figure 5 depicts engraftment as determined by peripheral blood neutrophil counts and platelet counts over time.

The present invention in its broadest embodiment comprises the immobilization of target cells contained in a heterogeneous composition to an insoluble solid phase matrix or support via an immune complex which contains a primary immune reagent capable of specifically binding the target cells, and an immune complex comprising a soluble hapten and an antibody which specifically binds the soluble hapten. Thereby, the target cells are immobilized in such a manner that they may be readily dissociated by the addition of a solution containing the soluble hapten. However, the invention is not limited thereby, and other more conventional methods of cell dissociation including agitation, high temperatures, and addition of enzymes

may be used as well. It should be emphasized, however, that dissociation by the addition of the soluble hapten containing solution is preferred. In the most preferred embodiment the soluble hapten will comprise biotin or a chemically derivatized biotin.

The aforementioned means of cell dissociation involving the addition of a solution of the soluble hapten essentially exploits a phenomenon known in the field of immunology as hapten competition. Although this general phenomenon is known in the art, it is novel in the context of the present invention relating to positive immunoselection of cells.

While hapten competition has been utilized in methods of positively immunoselecting cells, it has been limited to cells which are inherently hapten-specific (e.g., hapten-specific lymphocytes). This is in contradistinction to the subject invention wherein the target cells are not inherently hapten specific, but rather they are complexed with an immune reagent (as antibody) which is either directly or indirectly bound to the particular soluble hapten. Moreover, previous methods of immunoselection which have utilized hapten competition have also utilized other cell dissociation methods (e.g., agitation or elevated temperature). This is unlike the present invention wherein the immobilized target cells are dissociable using only hapten competition.

When selecting the hapten for use in the subject invention it is preferred to select haptens which are physiologically non-toxic at the concentrations required for cell dissociation. A higher hapten concentration in solution (e.g., in the millimolar range) is desirable because this will generally be in vast molar excess over the conjugated for mwhich is bound by antibody and will therefore favor greater kinetics (i.e., rates) of cell dissociation. Biotin is a particularly suitable hapten since it is a naturally occurring vitamin. This is an important consideration if the immunopurified cells are to be used as a therapeutic. However, more conventional haptens, such as arsenilic acid (Clark et al. (1982), J. Immunol. Methods, 51:167), dinitrophenols, or fluorescein (Basch et al. (1983), J. Immunol. Methods, 56:264) may be utilized if the cells are to be used in vitro, e.g., for

biomedical research. Other suitable haptens for use in the present invention include oligopeptides, glycopeptides, carbohydrates, etc.

In the preferred embodiment the target cells which are immunopurified will comprise human pluripotent lympho-hematopoietic stem cells and progenitor cells derived from human bone marrow or blood since these cells have a known applicability for BMT. However, the present invention is not intended to be limited to these target cells, but rather embraces the immunopurification of any target cells, cellular organelles or other components, or microorganisms to which immune reagents capable of specifically binding to a membrane antigen on the surface thereof are available.

When immunopurifying human pluripotent lympho-hematopoietic progenitor and stem cells, the preferred primary immune reagent capable of specifically binding these cells will be the K6.1 monoclonal antibody produced by the hybridoma cell line K6.1. This monoclonal antibody binds to an epitope on the CD34 antigen comprised on the surface of these cells. This monoclonal antibody was developed by the present inventor (see Kessler (1987), Fed. Proc., 46:136). This monoclonal antibody binds a distinct CD34 antigenic epitope from the MY-10 monoclonal antibody described, e.g., in U.S. Patent No. 4,714,680. This fact therefore enables the immunopurified human lympho-hematopoietic stem and progenitor cells to be immunoreacted with the MY-10 monoclonal antibody or other CD34 monoclonal antibodies which bind to CD34 epitopes distinct from the one to which the K6.1 antibody binds after the initial immunopurification using the K6.1 monoclonal antibody. Accordingly, the cells may be subjected to further immunoanalysis or additional immunopurification using the MY-10 monoclonal antibody or other CD34 monoclonal antibodies which bind to CD34 epitopes distinct from the epitope to which the K6.1 antibody binds. This further analysis and/or immunopurification is possible because of the inherent non-degradibility of the preferred dissociation method using soluble hapten, which method does not disturb the expression of either the target cell antigen (e.g., CD34) or of

other antigens comprised on the target cell. This is in distinction to more degradative dissociation methods, such as, e.g., agitation, proteolysis, or high temperatures which may adversely affect the expression of antigens comprised on the surface of the target cell.

Accordingly, this fact enables the cells purified by the subject invention to be subjected to additional positive and negative immunoselections. Moreover, this fact enables target cells which are immunopurified according to the subject invention to be essentially devoid of non-target cells.

This is a significant advantage of the inventive immunoselection which is particularly beneficial in the context of purification of human lympho-hematopoietic stem and progenitor cells if the cells are to be used for BMT wherein contaminant cells are especially problematic.

As discussed above, the present invention broadly relates to the selective immobilization of target cells contained in a heterogeneous cell composition to an insoluble solid phase matrix or support via an immune complex which contains a primary immune reagent which specifically binds the target cell, a soluble hapten, and an antibody specific to the soluble hapten.

This immobilization may in particular be accomplished by the following steps;

(a) reacting a heterogeneous composition containing target cells with a primary immune reagent which is capable of specifically binding said target cells and which immune reagent may optionally be conjugated to a soluble hapten to produce an immune complex of the target cell and the primary immune reagent;

(b) reacting the immune complex containing heterogeneous composition produced by step (a) with one or more secondary reagents at least one of which is capable of specifically binding the primary immune reagent and at least one of which is conjugated to the soluble hapten if the primary immune reagent has not been conjugated to the soluble hapten to

produce a complex consisting of the target cell--primary immune reagent--secondary reagents; and

(c) reacting the complex containing composition produced by step (b) with a solid phase matrix or support to which has been directly or indirectly bound an immune reagent capable of specifically binding the soluble hapten.

Preferably the heterogeneous cell composition will comprise cells obtained from blood, bone marrow, spleen, or other tissues which contain the target cells which are to be immunopurified. However, the invention broadly contemplates any heterogeneous cell composition containing target cells which are to be immunopurified.

The primary immune reagent used in step (a) will preferably be an antibody or antibody-like molecule capable of specifically binding an antigen comprised on the surface of the target cells. Both monoclonal and polyclonal antibodies may be utilized. As noted, this primary immune reagent may be conjugated to a soluble hapten, e.g., biotin. However, it may be preferable to conjugate one of the secondary immune reagents with the hapten since this will enable more of the soluble hapten to be conjugated to the target cells on a per cell basis. After the primary immune reagent has been complexed with the target cells, the unbound or excess antibody may be removed from the cells, e.g., by washing. However, this step is not critical to the invention and may be omitted if so desired.

The secondary reagents used in step (b) include, as stated above, at least one immune reagent which specifically binds the primary immune reagent used in step (a). Generally this will be an antibody or antibody-like molecule which specifically binds the primary immune reagent, e.g., immunoglobins which are species-specific (e.g., anti-mouse, anti-goat, anti-rabbit, etc.), class or isotype specific (e.g., anti-IgG, IgA, or IgM) or subclass-specific (e.g., anti-IgG1, IgG2a, IgG2b, IgG3). It may also be advantageous to remove the excess or unbound secondary antibody; however, this does not appear to be essential to the invention.

As noted, either the primary immune reagent or at least one of the secondary immune reagents is conjugated to the hapten, and preferably the secondary immune reagent. This conjugation may be effected by well known methods for conjugating haptens to antibodies. If the hapten is biotin, there are many commercially available biotin-derivatized antibodies, or they can be prepared by conjugation methods well known to those skilled in the art. A preferred biotin derivative, however, is biotin-e-aminocaproic acid -N-hydroxysuccinimide ester (biotin-X-NHS) which is available from Calbiochem in La Jolla, CA and other commercial sources.

The amount of hapten which is conjugated to the immune reagent may be varied so as to enable a greater or lesser amount to be bound per target cell. This enables control of the total amount of crosslinking between the immobilized target cell and the solid phase matrix or support and the avidity of the target cells to the solid phase.

If biotin in particular is utilized as the hapten, a preferred ratio has been found to be 6 to 8 biotin molecules bound per immune reagent (e.g., antibody). This avoids possible inactivation of the antibody which would prevent it from binding the anti-hapten antibody.

As noted above, it is preferable that the secondary immune reagents include an antibody directed against the primary immune reagent which is conjugated to the hapten. This results in the cells being immobilized via an indirect or "sandwich" technique. This is advantageous since it

(i) avoids the inconvenience of hapten-conjugating the primary immune reagent (since hapten conjugated antibodies are commercially available);

(ii) increases the sensitivity in immunoselections involving cell surface antigens which are comprised in low densities on the target cells since it amplifies the amount of hapten (e.g., biotin) molecules bound per target cell;

(iii) stabilizes the binding of a low avidity primary immune agent to the target cells; and

(iv) enables the positively immunoselected target cells to be subjected to further immunoselections (e.g., by selection of a secondary antibody with a restricted immunoglobin class or subclass binding specificity) using other primary immune reagents of alternative binding specificity.

As noted above, in step (c) the anti-hapten antibody may be directly or indirectly bound to the solid phase matrix or support. If the hapten is biotin, then of course anti-biotin antibodies will be bound. These antibodies may, e.g., be polyclonal or monoclonal, they may be specific species, may be fragments thereof, and may be used in purified (e.g., by affinity chromatography) or unpurified form. Anti-hapten monoclonal or polyclonal antibodies are commercially available or may be prepared by methods well known to those skilled in the art. Anti-biotin antibodies are in particular available from Vector Laboratories, Burlingame, California, and Bio-Technology Limited, Oxford.

The solid phase matrix or support used to immobilize the anti-hapten antibody may, e.g., be beads, fibers, mesh or tubing. These may be packed into various types of columns, cartridges or receptacles designed for extracorporeal cell flow-through or storage (e.g., flexible plastic blood bags and transfer pack units). Suitable columns for use in the immunoselection of cells are moreover well known to those skilled in the art.

In the preferred embodiment, the solid phase support will comprise paramagnetic beads or particles in suspension. Various types of magnetic beads suitable for immunoselection of cells are known in the art (see, e.g., Molday and Mackenzie (1982), J. Immunol. Methods, 52:353; Lea et al. (1985), Scand. J. Immunol., 22:207) and are available commercially, e.g., by Dynal, A.S. ("Dynabeads," Oslo, Norway through Dynal, Inc., Ft. Lee, NJ), Advanced Magnetics, Inc. ("BioMag" beads, Cambridge, MA), Immunotech Intl./AMAC, Inc. ("IObeads"), and Baxter/Fenwal (Deerfield, IL). The desirable characteristics of such magnetic beads includes high chemical stability for conjugation, high uniformity in size, shape, magnetic and surface properties. This list of

suitable solid supports is by no means intended to be limitative or exhaustive.

The direct or indirect binding of the anti-hapten antibodies to the support may be accomplished, e.g., by direct covalent attachment or by adsorption. Alternatively, the anti-hapten antibody may be attached via another antibody directly bound to the support. For example, as an illustrative embodiment of an indirect binding of anti-hapten antibodies an immunoaffinity purified $F(ab')_2$ anti-goat IgG antibody may be covalently bound to the surface of the magnetic beads which beads are then reacted with an immunoaffinity purified goat anti-biotin antibody. Alternatively, an anti-mouse Ig antibody may be covalently bound to the surface of the magnetic beads which beads are then reacted with a mouse monoclonal anti-biotin antibody. As an illustrative embodiment of direct binding the monoclonal anti-biotin antibody is attached covalently or by adsorption to the support.

When immobilizing the target cells to the anti-hapten antibody containing solid phase in step (c) it is necessary to contact the solid phase and the target cell complexes under conditions which permit the formation of the hapten-anti-hapten linkage. For example, the cell suspension containing the hapten-containing target cell complexes may be passed through or otherwise incubated with the anti-hapten antibody immobilized on the solid support which is in turn comprised in a suitable chamber. Optimal conditions for effecting this complexation will vary depending upon factors including the particular target cells, the concentration of the target antigen on the surface of said target cells, the amount of hapten molecules per antibody molecule, the target cell concentration, the avidity of the hapten for the anti-hapten antibody, and the amount of anti-hapten antibody bound to the solid support. However, it is well within the preview of the skilled artisan to vary parameters such as cell concentration, density, mass or surface area of the solid support, or the time or temperature so as to obtain optimal complexation of the target cells and the solid support.

Also embodied in the subject invention are alternative types of linkages involving a target cell and the solid phase matrix or support containing a hapten-anti-hapten antibody linkage. For example, it is also contemplated to couple the hapten, e.g., biotin, either directly or indirectly to a solid support and to react same with target cell complexes which contain an antibody specific to the hapten. This linkage of the hapten to the solid support may, e.g., be effected using a non-immunological spacer molecule. In addition, genetically engineered or chimeric antibodies derived from the fusion of two hybridomas (i.e., hybrid-hybridomas or tetradomas; Morrison, et al. (1988), Semin. Hematol. 25 (Suppl. 3):23; Chervonsky et al. (1988), Mol. Immunol. 25:913) may be used which exhibit binding specificities for both the hapten (e.g., biotin) and also for the cell-specific antigen on the target cells or for immunoglobulin species, class, or subclass antigens.

After the target cells have been immobilized to the solid support this support may then be separated from the heterogeneous cell composition. Depending upon the type of solid support this may be accomplished, e.g., by washing or flushing the chambers so as to selectively remove unbound cells. If magnetic beads are used, these beads may be drawn to and temporarily fixed to the walls of the container by juxtaposing a magnetic field such as that imposed by a rare-earth (e.g., samarium-cobalt) magnet (See, e.g., Treleaven et al. (1984), Lancet, 1:70), or a plurality of magnets, or an electromagnet, and then removing the non-target cells which remain free in suspension. These methods are well known to those skilled in the art. The purity of the immobilized target cells may further be enhanced by repetitive washing of the magnetic beads by removing the magnetic field, resuspending the beads with cells affixed thereto, and reimposing the magnetic field.

After the target cell containing solid support has been separated from the non-bound cells it is desirable to remove the target cells from the support. As noted previously, the preferred means for dissociating, the target cells will be to introduce a solution containing an amount of the soluble hapten, e.g., biotin, which is sufficient to dissociate the linkage

between the hapten and the anti-hapten antibody attached to the support. This hapten concentration may be varied as necessary. When biotin is utilized in particular as the hapten, an isotonic solution containing biotin in the range of 1-3 mM has been found to be especially suitable.

It is preferred that the dissociation temperature should be ambient or cooler than ambient (i.e., 0-4°C) rather than higher temperatures which favor cell detachment by antigen "capping" or shedding rather than via hapten competition. The time required for cell detachment will depend upon the aggregate affinity between the individual cells and the solid phase, which is affected, e.g., by the amount of crosslinking between the hapten and the anti-hapten antibody and the avidity of this complex. This dissociation time may be as short as several minutes to as long as 1-2 hours. However, it is contemplated that more rapid dissociation may be achieved with suitable monoclonal rather than polyclonal anti-hapten antibodies because of monoclonal antibodies' more uniform avidities.

After detachment the target cells may be separated from the solid support by a washing or flushing procedure, which may be repeated as necessary. If the solid support comprises magnetic beads then of course the magnetic field should be reimposed so as to remove the beads.

The purity and integrity of purified target cells recovered by the preferred method, hapten competition, are significantly improved relative to cells recovered by conventional methods. (See, e.g., Basch et al. (1983), J. Immunol. Methods, 56:269). This occurs in particular because this method detachment is specific, i.e., only cells immobilized by the hapten-anti-hapten antibody linkage are dissociated. Thus, non-target cells which are non-specifically bound or "trapped" on the solid support are not released by the hapten solution. This is in distinction to the conventional non-specific cell detachment methods such as, e.g., agitation, or enzyme digestion which may also release non-specifically bound cells.

Moreover, this method of target cell detachment is further advantageous since it preserves the integrity of all the cell surface membrane antigens, receptors, and other molecules contained on the target cell,

including the target cell antigen which binds the primary immune reagent. By contrast, nonspecific recovery methods such as agitation, proteolytic digestion, or exposure to chemical reducing agents (e.g., to cleave disulfide or mercury-sulfur bridges, as described in Bonnafous et al. (1983), J. Immunol. Methods, 58:93), oxidizing agents, or acidic or basic conditions may destroy membrane molecules or result in cells with the target antigen "ripped out" of the membrane. Moreover, cells may be obtained which comprise uniform or non-uniform configurations of antibodies, including various combination of primary, secondary, or even tertiary layers of antibody linking the cells to the solid phase.

In contrast, the inventive immunoselection results in target cells of higher purity and membrane integrity than had been obtained by conventional cellular immunoselection methods. In this regard, the present invention further relates to a method for testing the purity of the recovered cells and the integrity thereof. This method exploits the fact that "hapten-competition" cell detachment preserves the antigens and other molecules contained on the surface of the target cells. In the invention, after the initial immunoselection method, the target cells will be complexed with another antibody which binds a distinct antigenic epitope of the target antigen from that bound by the primary immune reagent used for the initial immunoselection. This other antibody may be utilized in a variety of standard immunoselections, e.g., direct immunostaining techniques. Alternatively, this other antibody may be detected by indirect or sandwich type assays provided that the antibody used does not cross-react with the primary immunoselecting antibody used in the initial immunoselection. This may be accomplished, e.g., by selecting two primary antibodies which are of different immunoglobulin species, class, or subclass.

Another and more sensitive method to analyze cell unity after the immunoselection has been effected is to include before immunoselection a subpopulation of labeled "indicator" cells. These may, e.g., be transformed or tumor cell lines, as described in Reynolds et al. (1986), Cancer Res., 46:5878.

However, there are some instances wherein the addition of such conventional "indicator" cells may be unsuitable. This is particularly so if the cells are to be used as a therapeutic wherein the introduction of transformed cells could have disastrous consequences. Therefore, the present invention further provides improved "indicator" cells relative to those utilized in conventional methods.

In particular, these improved indicator cells may be prepared by removing a small portion of the heterogeneous cell population from which the target cells are to be immunoselected (e.g., on the order of 5%) and labelling these removed cells with a known label. Methods of labelling cells are well known to those skilled in the art. However, it should be noted that labels which are physiologically safe in vivo are preferred.

In particular, the indicator cells (which will be referred to as autologous indicator cells) may be labelled, e.g., with the fluorescent DNA dye Bisbenzimide H 33342 (e.g., as described in Reynolds, et al. (1985), Transplantation Proc., XVIII (1):434 and Reynolds et al. (1986), Cancer Res., 46:5878). These labelled cells will then be reacted with an unhaptenlated form of the same primary immune reagent or primary plus secondary immune reagents used for immunoselection. This "neutralizes" or prevents these autologous indicator cells from being able to bind the anti-hapten antibody bound to the solid support.

These autologous indicator cells will then be combined with the remainder of the heterogeneous cell composition prior to their incubation with the anti-hapten antibody containing solid support. After immunoselection the purity of the recovered immunopurified cells may be determined by measuring to what extent these autologous indicator cells have been eliminated from the target cell composition.

The invention further contemplates subjecting the recovered immunoselected cells to additional immunoselections, which may be performed either sequentially or simultaneously. These methods are possible essentially because of the fact that the embodied cell dissociation method via

hapten competition retains the integrity of all or virtually all the membrane antigens comprised on the surface of the target cells.

In particular, the immunoselected cells may be reacted with one or more antibodies which are specific to the target cells or alternatively be reacted with antibodies specific to non-target cells. These procedures may be effected either concurrently or sequentially from the first immunoselection.

After the cells have been reacted with these particular antibodies the cells will preferably be washed to remove excess antibody, and then reacted with solid phase supports which bind only these additional antibodies. This can be accomplished, e.g., by designing solid phase supports to which are directly or indirectly bound antibodies which bind these other antibodies by virtue, e.g., of their binding distinct haptenic moieties, or immunoglobin species, classes or subclasses.

An illustrative example will be to subject the initial heterogeneous cell composition to a positive immunoselection using a biotinylated mouse IgG2a monoclonal antibody, followed by a negative immunoselection of the recovered cells using a mouse IgG1 monoclonal antibody which binds a non-target cell using a solid support containing anti-mouse IgG1 antibodies.

As noted above, a further purpose of the present invention is to provide kits for the embodied methods of immunoselection of target cells from heterogeneous cell compositions. These kits will typically comprise one or more of the following components:

(i) primary immune reagents which bind the target cells, which may or may not be conjugated to a soluble hapten;

(ii) one or more secondary reagents at least one of which specifically binds the primary immune reagent and wherein at least one of which is conjugated to a soluble hapten if the primary immune reagent is not conjugated to a hapten;

(iii) a solution containing the hapten in amounts which facilitate cell dissociation; and

(iv) a solid phase matrix or support contained in a suitable container or receptacle to which has been directly or indirectly bound an antibody capable of specifically binding the hapten.

These kits may further include immune reagents directed to additional primary immune reagents specific to the target cells so that these kits may provide for further positive or negative immunoselections.

These kits may further include conventional indicator cells (transformed or tumor cell lines) or the described autologous indicator cells. Such kits may be formulated as comprehensive systems for practicing the immunoselections described herein, or they may be formulated as separate but integrated components such as modules for performing positive immunoselections, for testing cell purity of the recovered target cells, or for performing further concurrent or sequential positive or negative immunoselections. These kits may incorporate pre-assembled sterilized units of different capacities, such as columns or cartridges.

If the solid phase in such kit comprises magnetic beads, these beads may be provided in flexible containers resembling plastic freezing or blood-type bags, along with supports or holders containing magnets or a plurality of magnets in the appropriate orientation, and pumping systems to facilitate cell transfer and washing.

Alternatively, the different elements of the kit may be provided separately or in various states of assembly. Typically, these kits will include a hapten in a form facilitating conjugation to an antibody, a hapten containing solution for cell dissociation, reagents for preparing the described autologous indicator cells, appropriately contained solid supports, anti-hapten antibodies or anti-hapten immunoglobin species-, class-, or subclass-specific antibodies, and the reagents for attaching them. These kits may further include the primary antibodies for selecting specific cell populations.

Given the described advantages of the inventive immunoselection techniques, these kits should afford improvements in speed,

simplicity, cost, and reproducability over conventional cellular immunoselection systems.

As has been described, in the particularly preferred embodiment the target cells which are positively immunoselected will comprise human lympho-hematopoietic stem and progenitor cells from humane bone marrow or blood. Conventional methods for positive immunoselection of these cells employ monoclonal antibodies specific for the MY-10/CD34 antigen comprised on the surface of cells. This monoclonal antibody is described, e.g., in U.S. Patent No. 4,714,680. Additionally, a number of other monoclonal antibodies which specifically bind the CD34 antigen have been described in the literature. (See, e.g., Tindle et al. (1984), Leukemia Res., 9:1; Andrews et al. (1986), Blood, 67:842; Watt et al. (1987), Leukemia, 1:417).

The present invention provides another monoclonal antibody for this use, which has been called the K6.1 monoclonal antibody and is produced by the K6.1 hybridoma cell line (Kessler (1987), Fed. Proc., 46:1363). The K6.1 hybridoma cell line has been deposited at the American Type Tissue Collection on July 20, 1992 according to the Budapest Treaty and is available under ATCC No. HB 11085.

The K6.1 antibody is of the mouse IgG2a subclass, and therefore may easily be purified by immunoaffinity chromatography using, e.g., staphylococcal protein A. Unlike the MY-10 antibody, this antibody is highly stable in solution in both the native and biotin-derivatized forms. Additionally, this antibody binds the CD34 antigen with higher avidity than the MY-10 antibody. Moreover, the epitope which is recognized by the K6.1 antibody is separate and distinct from the MY-10 antibody. Therefore, when the K6.1 antibody is bound to the CD34 antigen it does not prevent the binding of the MY-10 antibody thereto.

This particular CD34 epitope has been found to be conserved among all primate species tested (including monkeys and baboons). Accordingly, these described attributes of the K6.1 antibody render it particularly suitable for positive immunoselection procedures for the

recovery of lympho-hematopoietic stem and progenitor cells. Therefore, the present invention provides an improved immunoreagent for recovery of stem and progenitor cells relative to CD34 binding immunoreagents which have been available, in particular the anti-MY-10 antibody.

The following descriptions are provided to illustrate how the present invention provides an improved method for immunopurification of CD34 expressing cells and lympho-hematopoietic stem and progenitor cells in particular from bone marrow or blood. Although in the following descriptions a biotin-conjugated K6.1 antibody is utilized in particular, it should be emphasized that the present invention embraces the use of all anti-CD34 antibodies, and also antibodies which specifically bind stem cells or lymphatic or myeloid cells. Additionally, the invention embraces the use of any hapten which permits immobilization of the target cells to a solid phase via a hapten-anti-hapten antibody complex and which permits cell dissociation by hapten competition. Thus, the following description is intended to be only illustrative and in no way limitative of the present invention.

Typically, the heterogeneous cell composition containing lympho-hematopoietic stem and progenitor cells will be obtained by harvesting bone marrow from an allogeneic or autologous donor (e.g., by aspiration) by standard chemical protocols or from a cadaveric source such as vertebral bodies. This cell composition may be used for immunoselection directly or after cryopreservation and thawing.

Alternatively, blood may be withdrawn directly from the circulatory system or from the umbilical cord of a donor by connection through a catheter or shunt to a conventional cell centrifuge used for leukapheresis or hemapheresis. This collection may be performed continuously or stopped once a certain volume has been collected. Using either a blood marrow or blood source, differential centrifugation may be performed in order to separate the nucleated cells (i.e., the leukocytes) from other constituents, including red blood cells, platelets, and plasma. Additionally, downstream processing, e.g., by centrifugation, may be

effected to concentrate the mononuclear cell fraction which contains the desired stem cells and progenitor cells. These separation procedures are generally performed in chemical apheresis units.

The immunoselection steps will generally be carried out further downstream in a closed-in system. Preferably, this immunoselection will be conducted using the K6.1 monoclonal antibody as the primary immunoreagent and biotin as the soluble hapten. This separation procedure may further include re-infusion of the separated blood or marrow constituents depending upon the need of the particular donor. These steps may be effected using commercially available devices for delivering cells to columns or receptacles by, e.g., pumping, gravity-feeding or centrifugation.

It has been found that when cells are immunopurified according to the present invention, and CD34+ cells in particular, that various advantages over conventional cell immunopurification procedures are obtained. Some of these advantages are summarized below.

Foremost, it has been found that when CD34+ cells are purified according to the present invention that it is possible to obtain CD34+ cell compositions of significantly higher cell purities than had been obtainable using conventional methods. In fact, CD34+ cell compositions purified according to the methods taught herein comprise cell purities in excess of 99.95%. This exceeds the levels of cell purities obtainable by prior methods by two orders of magnitude or more. Additionally, if the object of immunopurification was to deplete or purge malignant cells from bone marrow (so that it may be used for autologous BMT), it has been found that the factor of depletion may equal or significantly exceed what is obtainable by specific negative immunoselection techniques. This factor is a product of the intrinsic purity of the CD34+ cells and the degree of enrichment of these marrow repopulating cells (e.g., a 50-fold enrichment of these cells should on average allow 50-fold fewer total cells/kg to be returned to the patient).

It is believed that these significantly enhanced cell purities are obtained by virtue of the increased specificity of the use of hapten for cell dissociation. However, increased cell purities may further be attributed to the use of magnetic beads as the solid phase and repeated washing cycles.

Secondly, it has been found that when CD34+ cells are prepared according to the present invention that the purity thereof may be evaluated simply and rapidly. This is essentially possible because the hapten mediated cell dissociation does not result in the loss or destruction of the CD34+ antigen. Accordingly, cell purity may be easily measured by assaying for CD34+ expression. This can be done, e.g., by labeling the immunopurified cells with commercially available CD34 specific antibodies, such as, e.g., the anti-MY-10/CD34 antibody (e.g., mouse IgG1 anti-HPCA-1, Becton-Dickinson), followed by staining with fluorescent anti-IgG1 antibody, which cells are then analyzed by microscopy or flow microfluorometry.

A third advantage of the present invention is attributable to the manner by which the cells are immobilized to the solid support. Specifically, because immobilization occurs via a linkage which recognizes only the hapten (e.g., biotin) conjugated form of the K6.1 antibody, it provides a means for selectively preventing immobilization of a portion of CD34+ cells, e.g., by preincubation with non-biotinylated K6.1 antibody. This allows a portion of the same or similar bone marrow cells which are used for immunoselection to be converted to "autologous indicator cells". Accordingly, this provides an alternative method for assaying CD34+ cell purity.

A fourth advantage of the present immunoselection is the fact that the recovered CD34+ cells should be less fragile than those CD34+ cells obtained by conventional methods. Accordingly, these cells should be capable of better withstanding further manipulations, such as cryopreservation and/or additional immunoselections. With respect to bone marrow transplantation, this is frequently performed following cryopreservation of cells in a fixed concentration of dimethylsulfoxide, and

the thawed cells are transplanted without washing. Therefore, the ability to obtain purified stem cells in highly concentrated form is advantageous in that smaller volumes could be administered than with purged or less purified cell preparations, thereby causing less discomfort or toxicity to the recipient.

A fifth advantage of the present immunoselection is attributable to the fact that it preserves the expression of all cell surface antigens, and not just CD34. Accordingly, the purity of the CD34+ cells may be increased further, or any subset within this population may be enriched or depleted by effecting further immunoselections with another antibody or plurality of antibodies. This may result in cell purities approaching 100% or even absolute purity with respect to antigen expression. In the case of autologous BMT, residual contamination of CD34+ cells by malignant cell expression, e.g., of a known differentiation or tumor associated antigen "X" may be eliminated by labeling with anti-"X" antibody and then immobilizing on a solid phase containing an antibody that binds anti-"X" antibody. In practice, the anti-"X" antibody may be of different immunoglobulin isotype or subclass from the CD34 antibody used for the initial immunoselection or may be conjugated with a different hapten. Therefore, this will provide the capability of removing malignant cells which are CD34 antigen positive, as in the case of hematologic malignancies such as certain leukemias and lymphomas. This would extend the range of capability of malignancies treatable by autologous BMT. However, in these situations, there may still be a risk of co-purifying malignant cells, particularly given the antigenic variation of malignant cells even from the same donor and cell type. Accordingly, BMT in such situations may still be preferred. However, in any event, it should be at least possible to increase the magnitude of immunodepletion of malignant cells which are CD34 positive from what can be achieved by conventional direct negative immunodepletion of malignant cells.

This method should also provide a means for the purification of cell subsets within the CD34+ population, such as, e.g., the totipotent stem cell, the oligopotent lymphoid, or myeloid progenitor cell, or the bone

marrow repopulating stem cell using suitable primary immune reagents with specificity to these CD34+ subsets. This should be possible because of the fact that this method does not disturb the cell surface antigens expressed on the surface of the immunopurified CD34 cells.

A sixth advantage of the present immunoselection involves economics. In particular, when CD34 cells are positively immunopurified according to the subject invention and then subjected to further secondary negative immunoselections, the quantity of reagents used (e.g., antibodies and magnetic beads) are significantly less than required in conventional methods. In the above example of purging CD34+ cells of residual malignant cells for autologous BMT, reagent savings using magnetic beads for example may exceed tenfold.

An seventh advantage obtained by the present invention is attributable to the K6.1 antibody. In particular, because this antibody binds a CD34 epitope which is conserved in other primates, it should be possible to utilize this antibody for the immunopurification of CD34+ cells from non-human primates. This would result in alternative sources of CD34+ for research and for therapeutic investigative analysis, e.g., the optimization of genetic therapy procedures which may not be desirable in humans.

Given the above described advantages of the present invention, it is contemplated that any method which relies upon the use of highly purified cell populations containing lympho-hematopoietic stem and progenitor cells will be significantly enhanced.

One noteworthy example involves the production of monoclonal antibodies which recognize specific antigens on lympho-hematopoietic stem and progenitor cells. By using the highly pure and antigenically intact stem and progenitor cells provided by the present invention as immunogens and for subsequent screening for binding specificities it should be possible to obtain monoclonal antibodies which distinguish between different stem and progenitor cell subsets.

Another example involves the development and use of various in vitro bioassays to identify and test the actions of growth factors and other

drugs (e.g., anti-neoplastic agents, immune modulators) directly on normal lympho-hematopoietic cells. Prior art isolation methods have been limited to 1-2 week clonal assays, rather than large population sampling assays because of the problem of ruling out indirect or secondary effects caused by contaminating cells. Results with large population sampling, however, may be obtained more quickly than with clonal assays. Accordingly, because of the highly pure stem and progenitor cells provided by the present invention, it should be possible to screen the effects of such drugs and growth factors on stem and progenitor cells much more rapidly than had been possible heretofore.

Another example involves the purification of these stem and progenitor cells with the object being expanding their number or directing their maturation in vitro with the aid of specific growth factors or suitable agents. The purified stem and progenitor cells obtained by the present invention will facilitate the design of tissue culture media and culturing methods which promote their amplification and/or maturation. Thus, these methods could provide larger quantities of purified stem and progenitor cells for reinfusion into BMT patients, an obvious concern of conventional bone marrow treatment protocols.

Moreover, the isolation and expansion of totipotent stem cells may reduce the amount of bone marrow or blood collected for autologous BMT, and may permit their use as "universal donors" for allogeneic BMT.

Another example involves the use of the present invention to obtain other cell types to accompany purified stem and progenitor cells in BMT, in particular for alleviating the severity of the immunodeficient state which accompanies this procedure. This immunodeficiency occurs in both autologous and allogeneic BMT when the donor marrow is purged of T-cells. Only a small number of donor T-cells can sustain engraftment, possibly attributable to the suppression of residual host immunity or the inhibition of the production of hematopoietic growth factors. Conventional methods of obtaining CD34+ cells, in particular stem and progenitor cells, have been variably contaminated by either T-cells or tumorigenic cells. In

contrast, this disclosure provides improved composition for BMT which are virtually devoid of T-cells or tumorigenic cells. Therefore, this invention provides methods for the positive immunoselection of cells other than CD34+ selected cells (e.g., T-cell subsets such as CD4+ helper cells) from donor blood or marrow using commonly available monoclonal antibodies. Accordingly, it should be possible to infuse these cells along with the stem or progenitor cells or CD34+ cells in BMT in order to provide immunocompetent cells earlier during recovery. This is particularly attractive since the patients undergoing BMT (e.g., cancer patients) tend to be immunocompromised. Thus, a more immediate means of compensating for this immunocompromised state should be beneficial.

It should further be possible to positively immunoselect cells other than stem and progenitor cells according to the inventive method for the purpose of generating or expanding such populations ex vivo and using the same as a therapeutic. In particular, the present method may be utilized to obtain cells commonly known to practitioners in the field of immunology as tumor-infiltrating lymphocytes (TIL cells), lymphocyte activated killer cells or cytotoxic lymphocytes. These cells may be administered alone as an anti-tumor or anti-infection therapeutic, or in combination with stem and progenitor cells. For example, large numbers of cytotoxic lymphocytes could be generated to viruses, such as cytomegalovirus (CMV) to which BMT patients are susceptible, or to human immunodeficiency virus (HIV).

Finally, it is noted that the disclosed positive immunoselections employing a hapten-anti-hapten antibody linkage system are not limited to the use of cell specific antibodies. For example, it is envisaged that lymphocyte populations binding to virus particles, other microorganisms, or parasitic organisms labelled with a soluble hapten (e.g., biotin) may be isolated for study and expanded if desired.

Another example involves the use of these purified stem and progenitor cells for gene therapy. In such methods known transformation vehicles and methods (e.g., retroviral vectors, membranous vesicles, electroporation) may be utilized to introduce heterologous genetic material

(e.g., genes for repair of inherited metabolic defects, or resistance to drugs or viral infections) into stem cells in vitro. These heterologous genes will then be expressed in the progeny of these cells when they are infused into a patient. The cells purified by the present invention will be particularly suitable for this purpose since membrane structural integrity, receptor expression, and cell viability are important for efficient gene transfer. In addition, the present techniques will enable directing or targeting the carrier vehicles used for genetic transformation to the membrane bound hapten (e.g., biotin) on positively immunoselected cells.

Additionally, methods for making monoclonal antibodies could be improved by using a hapten (biotin) labeled antigen to preselect antigen binding lymphocytes prior to fusion with immortal cells to produce hybridomas. This would significantly reduce or possibly eliminate the complexity of screening large numbers of hybridomas to identify antigen specific clones. Further, positive immunoselection could replace the subcloning procedures which are necessary to preserve the specificity of hybridoma lines.

The following examples are provided to illustrate specific embodiments of the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended to in any way limit the scope of the invention.

EXAMPLES

EXAMPLE 1: Development of K6.1 anti-CD34 monoclonal antibody.

The monoclonal antibody K6.1 was produced by fusing SP-2/0-AG14 plasmacytoma cells (American Type Culture Collection, Rockville, MD) with splenocytes from a BALB/cByJ mouse (Jackson Laboratory, Bar Harbor, ME) which had been hyperimmunized with viable KG-1a cells (ATCC). Injections containing 10 to 20 million KG-1a cells washed in saline were performed approximately monthly for a period of 6 months; the first and last immunizations were intravenous and the other immunizations were intraperitoneal. The last injection was performed 3 days prior to fusion. Cell hybridization and selection in HAT medium were performed according to the techniques of Kohler and Milstein (1975), Nature, 256:495, as modified by Fazekas de St. Groth and Scheidegger (1980), J. Immunol. Methods, 35:1, and Lane et al. (1984), J. Immunol. Methods, 72:71.

Culture supernatants collected approximately 2 weeks after fusion were screened for antibody activity against MY-10/CD34 antigen in KG-1a cell lysates by immunoblot ("Western blot") analysis. Earlier workers had identified a major 120 kD surface membrane glycoprotein characteristic of primary acute myelogenous leukemia cells and cell lines, including KG-1a (Andersson et al. (1979), Int. J. Cancer, 23:306, Koeffler et al. (1980), Blood, 56:265, and Gahmberg et al. (1982), Biochim. Biophys. Acta, 651:65). This is probably identical to the 115 kD glycoprotein antigen identified by Civin et al. as p115/MY-10 (1984; J. Immunol., 133:157, U.S. Patent No. 4,714,680). Initially, pools of about 10 growth positive hybridoma wells were screened, and individual wells of antibody positive pools were then screened. Antibody positive wells were subcloned by limiting dilution (Oi and Herzenberg, in Selected Methods in Cellular Immunology (1980), Mishell and Shigii, eds., pp. 351-72), and clones were screened the same way.

KG-1a cells were solubilized at $1x10^8$ cells/ml in Laemmli sample buffer (0.0625 M Tris-HCl, pH 6.8; (1970) Nature, 227:680), containing 0.5% Triton X-100 and 2mM PMSF, and centrifuged (30,000 xg, 30 min), and the supernatants were reduced in the presence of 50mM DTT, 4% SDS, and 10% glycerol (60 min, 37°C). Electrophoresis was performed on 8-16% pore-gradient, SDS polyacrylamide gels according to the method of Laemmli (1970; Nature, 227:680), as modified by Jones (in Selected Methods in Cellular Immunology (1980), Mishell and Shigii, eds., pp.398-440). Proteins were then transferred to nitrocellulose membranes for immunoblot analysis (Towbin et al. (1979), Proc. Nat. Acad. Sci. USA, 76:4350, and Burnette (1981), Anal. Biochem., 112:195), using alkaline phosphatase conjugated goat anti-mouse IgG antibody (BioRad Labs, Richmond, CA) for detection with BCIP/NBT as substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

The hybridoma clone K6.1 was identified as producing a monoclonal antibody of the IgG2a isotype, as determined with an isotype screening ELISA kit (Zymed Laboratories, S. San Francisco, CA) on immobilized KG-1a cells (Cobbold and Waldmann (1981), J. Immunol. Methods, 44:125). The hybridoma was expanded in roller bottles in Iscove's Modified Dulbecco's Medium (IMDM, MA Bioproducts, Walkersville, MD) containing fetal calf serum (Hyclone). After supernatant harvesting, the K6.1 antibody was purified by hydroxylapatite chromatography (Stanker et al. (1985), J. Immunol. Methods, 76:157), followed by pH-gradient elution from protein A-Sepharose (Ey et al. (1978), Immunochemistry, 15:429). The yield of antibody was 40-45 mg/liter of supernatant. This was concentrated on ultrafiltration membranes (Amicon YM-10, Danvers, MA), and dialyzed into normal saline. Analysis of antibody purity was performed on 30-40 ug reduced and unreduced samples by SDS-polyacrylamide gel electrophoresis under Laemmli conditions, followed by Coomassie blue staining; no detectable contamination was noted.

EXAMPLE 2: Reactivity of K6.1 antibody with CD34/MY-10 antigen.

Immunochemical identity between the antigens recognized by K6.1 and anti-MY-10 antibodies was established by showing that anti-MY-10 reacted in immunoblot analysis with antigen purified by immunoprecipitation with K6.1 antibody. About 50 million KG-1a cells prewashed in saline were solubilized in 1 ml of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 0.5% Triton X-100 detergent and 2 mM PMSF. After centrifugation (30,000 xg, 30 min, 4°C), immunoprecipitation was performed from the supernatant essentially as described by Kessler (in Methods in Enzymology (1981), Langone and Van Vunakis, eds., Vol. 73, pp.442-59). Fifty ug of purified K6.1 antibody was incubated with the supernatant for 2 hr at 4°C, followed by mixing for 15 min with 200 ul of a 10% suspension of protein A-Sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ) prewashed in lysis buffer. The Sepharose was washed 5 times by centrifugation in washing buffer containing 0.05% Triton, and the pellet was then divided into 3 equal parts. In part a the pellet was suspended in 100 ul of Laemmli sample buffer containing 2% SDS and 25 mM DTT, and incubated for 60 min at 22°C to elute the CD34 antigen. In part b the pellet was suspended in buffer containing the enzyme endoglycosidase F (Genzyme Corp., Cambridge, MA) to digest off asparagine-linked oligosaccharides and incubated according to published procedures references supplied by the company. Part c was the incubation control for part b and lacked the enzyme. After this treatment the pellets from parts b and c were suspended in Laemmli sample buffer as above. After centrifugation to recover the supernatant, containing the antigen along with separated K6.1 antibody, aliquots corresponding to 5 million cell-equivalents were loaded into individual lanes of a 7.5% SDS-polyacrylamide gel and electrophoresed under Laemmli conditions. The separated molecules were then transferred onto nitrocellulose membranes for immunoblot analysis as described above.

Sections of the nitrocellulose membrane containing the antigen immunoprecipitated by K6.1 antibody were incubated with the following monoclonal antibodies or immunoglobulins at a concentration of 5 ug/ml:

K6.1 antibody (positive control for antigen retention), anti-MY-10 antibody (purchased from Becton Dickinson Immunocytometry Systems, Mtn. View/San Jose, CA, under the trade name HPCA-1), UPC-10 (negative control myeloma protein of the same isotype as K6.1, purified by protein A-Sepharose chromatography of ascites from BALB/c mice bearing the tumor). Detection with alkaline phosphatase-goat anti-mouse IgG and BCIP/NBT was as described above.

FIGURE 1 depicts the results of this analysis. This shows that the patterns of reactivity of K6.1 and MY-10 antibodies to the untreated (lane "a"), enzymatically treated (lane "b"), or control antigen (lane "c") immunoprecipitated by K6.1 antibody were the same. The intense band coinciding with the p115 marker in the K6.1 and anti-MY-10 sections is the CD34/MY-10 antigen. The lower minor band coinciding with the γ-symbol is the reduced γ-chain of the K6.1 antibody originally involved in the immunoprecipitation, which is also detected by the alkaline phosphatase-anti-IgG. The more intense staining with K6.1 indicates that this antibody reacts with the antigen more strongly than anti-MY-10.

EXAMPLE 3. Reactivity of K6.1 and anti-MY-10 antibodies with distinct and non-overlapping epitopes on CD34 antigen.

The design of this analysis was to determine if pre-binding of either K6.1 or anti-MY-10 antibody to CD34 antigen on KG-1a cells would interfere with or block the subsequent binding of the other antibody. Absence of inhibition or blocking is interpreted as indicating that the respective epitopes recognized by the antibodies are distinct and non-overlapping. In order to tell the 2 antibodies apart on the cells, the antibody to be tested after the preincubation step was pre-conjugated with biotin, and was detected by indirect immunofluorescence with FITC-avidin (Becton Dickinson) and flow cytometry with a Becton Dickinson FACS II.

The antibodies were conjugated with biotin as follows: Purified K6.1 antibody from Example 1 was mixed with $NaHCO_3$ to give a solution containing 3 mg antibody/ml in 0.1 M $NaHCO_3$. Biotin-N-

hydroxysuccinimide ester (Calbiochem, La Jolla, CA) was dissolved in dimethylsulfoxide (DMSO) at a concentration of 12 mg/ml, and 5.0 ul of this was added to each ml of antibody solution. After 1 hr at room temperature, NH_4HCO_3 was added to 50 mM final concentration to stop the reaction. The mixture was then passed through a Sephadex PD-10 column (Pharmacia) equilibrated in phosphate-buffered saline (PBS, 6.7 mM Na phosphate, pH 7.2, 137 mM NaCl) to desalt and exchange the buffer.

Preservation of binding activity with biotinylation of anti-MY-10 required a moderately low conjugation ratio and the presence of carrier IgG. Anti-MY-10 (purchased from Becton-Dickinson as HPCA-1) was dialyzed into normal saline. 100 ug of this antibody was mixed with 900 ug of carrier MOPC-21 myeloma protein and $NaHCO_3$, to give a 1 mg/ml solution of IgG in 0.1 M $NaHCO_3$. This myeloma protein has no known binding specificity, and was previously purified by DEAE-cellulose chromatography from ascites of BALB/c mice bearing the MOPC-21 plasmacytoma (gift of Dr. M. Potter, NIH, Bethesda, MD). To one ml of this solution 2.3 ul of the 12 mg/ml BNHS/DMSO was added, and the mixture was reacted, stopped, and desalted as described above. From previous studies performed by the present inventor, these two conjugation conditions were known to give products with a mean of 6 biotin molecules bound/molecule of immunoglobulin.

In preliminary studies, each of the 4 reagents (K6.1 and anti-MY-10 in biotinylated and non-biotinylated forms) was titered by indirect immunofluorescence staining of KG-1a cells, using FITC-avidin or FITC-anti-mouse IgG antibody (Becton-Dickinson), to determine the minimum amount of primary antibody needed to give saturation staining of CD34. In the cross-blocking study, KG-1a cells were preincubated with 10-20 times the saturating amount of either non-biotinylated antibody. Next, the minimum saturating amount of either biotinylated antibody was added to each sample (4 combinations), and incubation was continued. The cells were then washed, stained with FITC-avidin and analyzed on the FACS.

The results of this analysis are depicted in the histograms in FIGURE 2, in which cell number on the y-axis is plotted against linearly increasing channels of fluorescence intensity on the x-axis. Binding of biotinylated-K6.1 antibody (left panels) was blocked by preincubation with excess non-biotinylated K6.1 (positive blocking control; lower panel), as expected, but was unaffected by preincubation with anti-MY-10 (upper panel). Similarly, binding of biotinylated-anti-MY-10 (right panels) was blocked by preincubation with excess non-biotinylated anti-MY-10 (upper panel), but not with K6.1 (lower panel). By similar methods another CD34 monoclonal antibody, 12.8 (Andrews et al. (1986), Blood, 67:842), was tested and found to crossblock with K6.1, indicating that these 2 antibodies identify similar or adjacent epitopes. The 12.8 antibody was obtained as a gift of ascites fluid from Dr. R.G. Andrews (Fred Hutchinson Cancer Research Center, Seattle, WA) and is now available from CellPro, Inc. (Bothell, WA).

EXAMPLE 4: Positive immunoselection of CD34+ cells from bone marrow.

Biotinylated K6.1 antibody stock. Purified K6.1 antibody from Example 1 was mixed with $NaHCO_3$ to give a solution containing 5 mg antibody/ml in 0.1 M $NaHCO_3$. Biotinyl-e-aminocaproyl-N-hydroxysuccinimide ester (B-X-NHS; Calbiochem, La Jolla, CA) was dissolved in dimethylsulfoxide at a concentration of 16 mg/ml, and 10.0 ul of this was added per ml of antibody solution. After 1 hr at room temperature, NH_4HCO_3 was added to 50 mM to stop the reaction, and the mixture was passed through a Sephadex PD-10 column equilibrated in sterile PBS for desalting and buffer exchange. From previous studies performed by the present inventor, these conjugation conditions were known to give a product with a mean of 8 molecules biotin bound/antibody molecule. The antibody solution was sterile filtered through a 0.2 um membrane and stored at 4°C.

Preparation of stock immunomagnetic beads. Dynabeads M-450 Uncoated (product 14002), consisting of uniform, magnetic polystyrene beads with diameter 4.5 um (c.v. <5%), were purchased from Dynal, Inc. (Fort Lee, NJ, manufactured by Dynal A.S., Oslo, Norway). Ten ml (300 mg) of Dynabeads, containing $4x10^8$ beads/ml, were washed sequentially into 30%, 50%, and 70% 1,4-dioxane in water, and then into 100% dioxane, using rare-earth magnets (samarium-cobalt, approx. 1.5 x 0.5 x 0.5 in., Edmund Scientific Corp., Barrington, NJ). The beads were then suspended in 10 ml dioxane containing 100 mg carbonyldiimidazole (Pierce Chemical Co., Rockford, IL, or Polysciences, Inc., Warrington, PA), and shaken vigorously for 30 min at 22°C. Then, the beads were washed back into water by reversal of the above sequence. Finally, the beads were suspended in 20 ml 0.1 M $NaHCO_3$ solution containing 1.5 to 2.0 mg affinity purified rabbit $F(ab')_2$ anti-goat IgG antibody, and the suspension was tumbled (approx. 30 rpm) for 16-20 hr at 22°C. At the end of this period, 5 mg of clinical reagent grade bovine serum albumin was added (Bovuminar, Armour Pharmaceutical Co., Kankakee, IL; note: it was assumed that pharmaceutical grade human serum albumin also could be substituted for use in BMT) and the beads were tumbled for another 3-4 hr. Then, Tween 20 detergent was added (from 10% stock) to 0.1% final concentration, along with ethanolamine-HCl (from 5 M stock, pH9) to 0.1 M final concentration, and the beads were mixed for another 2 hr. Finally, the beads were washed twice in sterile-filtered Tris-buffered saline (20 mM Tris-HCl, pH7.4, 0.137 M NaCl) containing 0.1% Tween-20 and 0.01% merthiolate, and thrice washed in Tris-buffered saline containing merthiolate. The beads were then stored at 4°C.

A stock of affinity purified rabbit $F(ab')_2$ anti-goat IgG antibody was made in preparation for the above coupling reaction. This was done by immunizing rabbits with DEAE-cellulose-purified goat IgG, and subjecting the serum to $(NH_4)_2SO_4$ precipitation, pepsin digestion, passage over protein A-Sepharose, and affinity chromatography on goat IgG-Sepharose (with $MgCl_2$ as eluant) according to published methods (Stanworth

and Turner, in Handbook of Experimental Immunology, Vol 1 (1978), ed. Weir, pp.12.1-46; Goding (1978), J. Immunol. Methods, 20:241; and Kessler et al. (1978), J. Immunol., 127:1674).

Harvest of bone marrow. Human bone marrow was obtained as surgically resected vertebral bodies harvested from cadaveric donors immediately following the removal of kidneys for donation (Sharp et al. (1984), J. Immunol. Methods, 69:187; Melaragno et al. (1984), NIH Technology Assessment Meeting; Noga et al. (1986), J. Immunol. Methods, 92:211). A standard "bone marrow protocol" has been developed at the U.S. Navy Tissue Bank, Bethesda, MD. This protocol is described in a manual entitled The Procuring and Processing of Human Cadaveric Bone Marrow, Naval Medical Research Institute Publication No. 90-62 (T.R. Faloon, ed.) and is available as AD# A226538 from the Defense Technical Information Center, Cameron Station, Alexandria, VA on request. Briefly, individual vertebral bodies were disarticulated aseptically from a vertebral log (lumbar 1-5), and bone marrow matrices were exposed by removal of cortical bone with the aid of osteotomes. The bone marrow matrices were then sectioned and processed through a tissue grinder, and the cell suspension was passed through sieving screens to remove sediment. The cells were suspended and washed in a citrate-phosphate buffered saline dextrose solution, and then washed and incubated in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% fetal calf serum and DNAse (usually 1000 units/ml). In other studies, smaller amounts of iliac crest bone marrow were obtained by aspiration from volunteer donors.

Bone marrow debulking and mononuclear cell enrichment. To improve the specificity of the immunoselection and economize on reagents, red cells were removed and mononuclear cells were enriched prior to positive immunoselection. This was accomplished by any of the following methods:

a. Density gradient centrifugation. Diluted bone marrow cells were centrifuged over Percoll (d. 1.077, Pharmacia) or Ficoll-Hypaque or equivalent at d. 1.077, e.g., Lymphocyte Separation Medium (LSM,

Litton Bionetics, Charleston, SC) or Ficoll-Paque (Pharmacia), and "light density" mononuclear cells were collected from the interface.

b. Negative immunomagnetic selection of Fc receptor bearing cells. Bone marrow cells were first depleted of red cells by NH_4Cl lysis using ACK Lysis Buffer (MA Bioproducts). BioMag anti-human IgG immunobeads (M4320, Advanced Magnetics, Inc., Cambridge MA) were preincubated with heat-inactivated pooled human AB serum (#34004-1, Pel-Freez Clinical Systems, Brown Deer, WI) to generate immobilized magnetic human immune complexes. Similarly, immobilized magnetic mouse immune complexes were made by incubating BioMag anti-mouse IgG immunobeads (M4400) with ascites fluid from BALB/c mice previously inoculated with the IgG2a hybridoma D3-2H2-9-21 (anti-Dengue complex, ATCC HB 114) or the plasmacytoma P1.17 (ATCC TIB 10). Neither the human serum immunoglobulins nor the two mouse IgG2a proteins had antibody specificity for human cells, but could bind to Fc receptor-bearing cells. To a suspension of bone marrow cells at 25 x 10^6/ml, each of the washed magnetic bead complexes was mixed at a ratio of 10-20 beads/cell (assuming 1.3 x 10^9 beads/mg) for 60 min at 4°C on a rotator (approx. 30 rpm). Cells reacting with the magnetic beads were removed by holding the mixture next to a samarium-cobalt magnet (Treleaven et al. (1984), Lancet 1:70) for several minutes and collecting the cells remaining in suspension.

c. Negative immunomagnetic selection of surface immunoglobulin positive B cells and CD11b (Mac-1) positive cells. BioMag anti-human IgG beads were used without human serum pre-incubation. BioMag anti-mouse IgG beads were preincubated with ascites fluid from BALB/c mice inoculated with the plasmacytoma OKM1 (ATCC CRL 8026) producing anti-human CD11b monoclonal antibody. They were then incubated with a suspension of human bone marrow cells as described in method b above.

Positive immunoselection of CD34+ cells. All cell washing, incubation, and selection steps were performed at 4°C (unless noted otherwise) in 0.2 um sterile filtered "immunoselection washing buffer."

This consisted of Hanks' balanced salt solution containing 12.5 mM HEPES buffer, 1000 units/ml DNAse 1 (Calbiochem), and 5% heat-inactivated pooled human AB serum (#34004-1, Pel-Freez Clinical Systems, Brown Deer, WI). The human serum was previously dialyzed extensively (40 volumes x 5 changes) against PBS to remove traces of biotin. This was included as a source of human IgG to saturate Fc receptors and minimize cytophilic binding of the cell specific antibody (i.e., K6.1); for therapeutic immunoselection purposes, it was assumed that substitutes such as dialyzed serum from the marrow donor or pharmaceutically approved gamma globulins for injection would be used.

Bone marrow mononuclear cells were washed and adjusted to a concentration of 50 x 10^6/ml. Biotinylated-K6.1 antibody, prepared as described above, was added at a ratio of 6-10 ug/ml of cell suspension and incubated with occasional mixing for 30 min at 4°C. The cells were then washed by centrifugation 3-4 times, and set to a concentration of 25 x 10^6/ml.

Dynabeads M-450 coated with anti-goat IgG antibody as described above were activated with goat anti-biotin antibody. The number of Dynabeads used was proportional to the total number bone marrow mononuclear cells, using a ratio of 1 bead/10 cells (the underlying objective was to provide at least 3 beads/target cell contained in this population, but not to provide an overabundance of beads). It is to be noted that manufacturer's specifications and prior art use of these beads for negative immunoselections of tumor cells from bone marrow commonly employ a ratio of 10-20 beads/total number of cells. Thus, positive immunoselection as described here reflects a 100-fold or greater savings in bead consumption. Anti-goat IgG Dynabeads were washed magnetically 4-5 times with a rare-earth magnet. The beads were then suspended at a concentration of 1 x 10^8/ml in washing buffer containing 2.5 ug/ml affinity purified goat anti-mouse biotin antibody (#SP-3000, Vector Laboratories, Burlingame, CA), mixed vigorously for 30 min at room temperature, and then washed magnetically twice, and resuspended to 1 x 18^8/ml. This concentration of

anti-biotin was optimized in preliminary titration studies using as an endpoint the kinetics of cell dissociation from the magnetic beads (described below). The binding of anti-goat IgG to goat anti-biotin does not reach equilibrium under these conditions; varying the anti-biotin provided a convenient way to compensate indirectly for different cell surface antigen densities, by controlling the amount of biotin-anti-biotin crosslinking.

The bone marrow mononuclear cells, containing biotinylated-K6.1 antibody coated target cells, were incubated with the anti-biotin Dynabeads for 30 min on a rotator (approx. 30 rpm). The magnetic CD34+ cells were then selected by attraction to a samarium-cobalt magnet, and removal of non-target cells free in suspension. These cells were purified by a further 4-5 washing cycles in which the magnet was removed, the cells resuspended, and the magnet reopposed, with removal of non-target cells free in suspension each time. Finally, the magnetic CD34+ cells were suspended in medium (e.g., IMDM) containing 2.5 mg/ml biotin, put on a rotator for 1-2 hr, and free CD34+ cells were recovered from magnetically immobilized Dynabeads. In more than 300 CD34+ immunoselection experiments performed this way, these recovery conditions were observed to result in the complete detachment of beads from over 90% (usually well over 95%) of the immunoselected cells.

The principle of this recovery method was that free biotin provided in vast molar excess replaced the biotinylated-K6.1 antibody bound by the anti-biotin Dynabeads, as a function of normal antibody-ligand dissociation kinetics. These factors, along with the total amount of antibody crosslinking between cell and magnetic bead, influenced the kinetics of cell detachment from beads. So long as vigorous agitation was avoided (to prevent membrane or linkage shearing) and warming was avoided (to prevent antigen capping and modulation) during dissociation, the recovered CD34+ cells uniformly retained CD34 antigen bound by biotinylated-K6.1 antibody. These cells have been used routinely in growth studies in vitro. They are not affected by the bound antibody, which is retained only transiently. By exploiting the cross-species reactivity of this antibody, similar preparations

of CD34+ cells have been made by the present inventor from rhesus monkey bone marrow. Preliminary studies in which these cells have been used for autologous BMT in lethally irradiated animals have shown that residual K6.1 antibody does not affect CD34+ cell homing and engraftment in vivo.

EXAMPLE 5: Yields of CD34+ cells from human cadaveric bone marrow.

An important test of the practicality of an immunoselection method is whether it can be scaled up to the cell numbers normally encountered in clinical settings, such as in BMT. The data provided in TABLE 2 address this point by providing cell yields from positive immunoselections of CD34+ cells from 4 cadaveric bone marrows. The total number of white blood cells from 5 vertebral bodies approximates the number obtained from 0.5 to 1 liter of aspirated marrow from BMT donors. The debulking procedures described in Example 4 to enrich for mononuclear cells (examples shown in this table employed OKM1 anti-CD11b depletion) typically remove 60-75% of the cells. The yields of CD34+ cells are well into the theoretical useful range for BMT, since it is believed that 10^6 CD34+ cells may have the marrow repopulating capacity of 10^8 unfractionated bone marrow cells (normally provided at 10^8 cells/kg body wt) based on their normal frequency in marrow. Considerable variation occurs in yields of CD34+ cells from cadaveric marrows due to differences in donor age, medications received before patient death, and the amount of time passed between death and marrow harvesting.

EXAMPLE 6: Measurement of CD34+ cell purity by analysis of CD34 antigen expression.

The retention of CD34 antigen expression of cells which are positively immunoselected as described in Example 4 provides a unique capability for measuring cell purity as soon as the cells are recovered, rather than after prolonged cell culture (e.g., overnight) during which the antigen may be reexpressed. To accomplish this, the recovered cells are stained for

flow cytometry analysis with another monoclonal antibody that recognizes a separate epitope on the CD34 molecule. This second CD34 antibody is distinguishable from the K6.1 antibody that resides on the cells by virtue of belonging to a different immunoglobulin isotype or class, or being directly conjugated with a fluorochrome or other hapten. Example 3 showed that anti-MY-10 fulfills this property. To illustrate, CD34+ cells purified from bone marrow as in Example 4 were stained with anti-MY-10 (anti-HPCA-1, Becton-Dickinson) or with isotype-matched MOPC-21 IgG1 myeloma protein as a negative control. After washing, they were then stained with phycoerythrin-conjugated goat anti-mouse IgG1 antibody (Fisher Biotech Antibodies, Fisher Scientific) and then analyzed by flow cytometry with an Ortho Cytofluorograf (Ortho Diagnostic Systems, Inc., Westwood, MA). The results are depicted in FIGURE 3a, in which cell number is plotted on the y-axis against channels of logarithmically increasing fluorescence intensity on the x-axis. Analyses of several other commercially available CD34 mouse monoclonal antibodies on purified CD34+ cells revealed at least 2 that fulfilled the above criteria. To illustrate, cells were stained with TUK3 mouse monoclonal antibody (Civin et al., in Leucocyte Typing IV. White Cell Differentiation Antigens (1989), ed. Knapp et al., pp. 818-25, available from DAKO Corp., Carpenteria, CA), or the negative control myeloma protein J606 belonging to the IgG3 isotype. After washing, the cells were then stained with phycoerythrin-conjugated goat anti-mouse IgG3 antibody (Fisher Biotech). Other cells were stained with directly phycoerythrin-conjugated anti-HPCA-2 (Landsdorp et al. in Leucocyte Typing IV. White Cell Differentiation Antigens (1989), ed. Knapp et al. pp. 826-27, available from Becton-Dickinson), or with a phycoerythrin-conjugated IgG1 isotype control. The cells were then analyzed with a Coulter Epics Elite flow cytometer. The results are depicted in FIGURE 3b (TUK3) and 3C (anti-HPCA-2) with the same channel parameters as above. Staining with the specific antibodies is represented by the solid lines, and staining with the negative control immunoglobulins is depicted by the broken lines. The discrimination (i.e., peak separation) between the CD34+ and

negative control histograms is a reflection of the avidities of the different antibodies. Nevertheless, this method shows the cells are minimally 98-99% CD34+, which is at the upper limit of detection by this approach. To the knowledge of the present inventor, except for flow cytometry sorting, this exceeds the levels of CD34+ cell purity reported by others using other separation methods.

EXAMPLE 7: Measurement of CD34+ cell purity by ""rare event analysis" with normal labeled or "autologous indicator" cells.

A more sensitive measurement of CD34+ cell purity was obtained with a modification of a technique used for "rare event analysis" (Reynolds et al. (1986), Cancer Res. 46:5878). Labeled "indicator" cells were seeded into a bone marrow cell suspension at a known low frequency before positive immunoselection, and the frequency of these cells was later measured in the purified CD34+ population. Light density, Ficoll-separated, peripheral blood mononuclear cells were incubated at 1 x 10^7/ml in washing buffer (Example 4) with the fluorescent DNA stain Bisbenzimide H-33342 (Calbiochem) at 4 ug/ml for 45 min at 37°C. Peripheral blood cells were used in preference to bone marrow cells because they contained a lower frequency of CD34+ cells; they were otherwise assumed to be equivalent to debulked marrow cells as described in Example 4. Nevertheless, the possibility remained that stained CD34+ cells could be immunoselected and complicate measurements. To "nullify" these cells so that they would be unrecognizable to the anti-biotin Dynabeads, they were reacted with non-biotinylated K6.1 antibody (incubation conditions as described above) after DNA staining. They were then mixed with the remaining bone marrow before incubation with the Dynabeads (note: if the antigen on indicator cells was not blocked with K6.1 antibody, the possibility existed that it could be bound by biotinylated-K6.1 antibody dissociating from bone marrow target cells). After positive immunoselection was performed, as described in Example 4, a sample of the recovered CD34+ cells was examined with a Leitz fluorescence microscope.

TABLE 3 shows the results of a series of analyses performed by this method. The data are interpreted as follows:

"H342+ PRESEL" is the actual percentage of Bisbenzimide H-33342 stained indicator cells in the bone marrow suspension before positive immunoselection (a minimum of 500 total cells were counted).

"#H342+/TOTAL ($X10^5$)" is the number of stained indicator cells per number of CD34+ cells counted. In BM1993, for example, there were 2 fluorescent cells per 470,000 CD34+ cells.

"PURGE FACTOR" is the magnitude of indicator cell depletion, obtained by dividing the percentage of fluorescent cells before selection by the percentage after selection. In BM1993, for example, there was a 4277-fold depletion of indicator cells.

"%CD34+" is the per centum purity of recovered CD34+ cells, obtained directly from the "purge factor." In BM1993, for example, 4276 of every 4277 cells were CD34+.

"LOG PURGE" is the logarithmic conversion of the "purge factor."

"BMT EQUIVALENT LOG PURGE" is the clinically equivalent "purge factor" that would be realized if these cells were to be used for bone marrow transplantation. It is 100-fold higher than the "log purge," because it assumes that 1 CD34+ cell has the marrow repopulating capacity of 100 unfractionated bone marrow cells.

To the knowledge of the present inventor, the efficiency of cell depletion (of non-CD34+ cells) obtained by positive immunoselection of CD34+ cells equals or exceeds the capabilities of any negative immunoselection system (e.g., for bone marrow tumor cells or T cells). In addition, this substantially exceeds the capabilities of any known positive immunoselection system (including fluorescence activated cell sorting, which has a much lower cell throughput).

EXAMPLE 8: Expression of other surface membrane antigens on purified CD34+ cells.

The preservation of CD34+ cell surface membrane integrity by immunoselection as described in Example 4 enabled these cells to be analyzed for expression of other membrane antigens. Analysis was further facilitated by the ability to recover CD34+ cells in large quantity (Example 5), so that many different antigens could be studied on the same cell population, and by the high degree of cell purity (Examples 6 and 7), so that only single-color analyses were necessary in these studies. However, the use of simultaneous, multiple-color staining and analyses for particular combinations of antigens to delineate various cell subsets would also be facilitated.

The results of staining and flow cytometry analyses (on an Ortho Cytofluorograf) with a large panel of different monoclonal antibodies are summarized in TABLE 4. These data are provided primarily for illustrative purposes for the present invention, and not to include or exclude particular cell subsets within the CD34+ population. For simplification, the frequencies of cells which express the different antigens were ranked as follows: +4, 90-100%; +3, 60-89%; +2, 30-59%; +1, 5-29%; -, <5%. Monoclonal antibodies with the specificities indicated were obtained from several commercial suppliers, including Becton-Dickinson (Mountain View, CA), Coulter (Hialeah, FL), Hybritech (La Jolla, CA), Ortho Diagnostic Systems (Raritan, NJ) and Sera-Lab (Accurate Chemical & Scientific Corp., Westbury, NY), and were used according to the suppliers' directions. Antibodies MHM24 and H52 were a gift of Dr. J. Hildreth (Johns Hopkins Univ., Baltimore, MD). As a general rule, antibodies that were available as FITC or phycoerythrin conjugates were used for direct immunostaining, along with negative control immunoglobulins matched wherever possible for isotype, conjugation method, concentration, and supplier. Antibodies that were available as unconjugated proteins were used in indirect immunostaining with FITC or phycoerythrin conjugated, immunoglobulin isotype or subclass-specific secondary antibodies (Fisher Biotech), along with

isotype or subclass-, concentration-, and source-matched negative control immunoglobulins.

EXAMPLE 9: Sequential immunoselections: Positive immunoselection of CD34+ cells followed by negative immunoselection of tumor cells.

The capability to subject purified CD34+ cells to further immunoselection procedures was desirable. These could be used, e.g., to further increase CD34+ cell purity, or to enrich or deplete any cell subset or lineage within this population. Studies using "rare event analysis" were performed according to the protocol described in Example 7, but with the following modifications: "Indicator" cells were from the human T cell leukemia line Jurkat Clone E6-1 (catalog no. 177, obtained from the AIDS Research and Reference Reagents Program, NIAID, NIH), instead of normal light density mononuclear cells. The logistics of depleting these cells from bone marrow were considered similar to clinical BMT situations in which contaminating tumor cells may be present. After Bisbenzimide H-33342 staining, Jurkat cells were seeded into the bone marrow at a higher frequency than the previous example, and they were not pre-blocked with non-biotinylated-K6.1 (they are CD34 negative).

TABLE 5 shows the results of studies with several bone marrow preparations. The format of this table for the CD34+ immunoselection phase is the same as described in Example 7. As was shown with peripheral blood mononuclear indicator cells in the previous example, the clinically equivalent factor of tumor cell purging that may be realized in BMT with a single round of positive immunoselection of CD34+ cells was on the order of 5-6 logs.

Residual Jurkat cells contaminating the recovered CD34+ population were further depleted by negative immunoselection. During the interval of biotin-induced dissociation of CD34+ cells from the magnetic beads, the monoclonal antibody G19.4 (gift of Dr. J. Ledbetter, Oncogen, Seattle, WA) was added (diluted ascites equivalent to 5-10 ug antibody/ml). This IgG1 antibody is specific for the CD3 T cell antigen which is expressed

on Jurkat cells (Weiss et al. (1984), J. Immunol. 133:123), but generally not on CD34+ cells (TABLE 4). CD34+ cells recovered from positive immunoselection were washed and adjusted to a concentration of 2.5 x 10^6/ml. A suspension of Dynabeads M-450 coated with sheep anti-mouse IgG1 (Dynal product no. 11004) was then added at a ratio of 1 bead/2 cells (approx. 1.25 x 10^6/ml), and the mixture was rotated (approx. 30 rpm) for 30 min. The magnetic Jurkat cells were then removed with a samarium-cobalt magnet, and the CD34+ cells free in suspension were collected.

Examination of Jurkat-depleted CD34+ cells was performed by thorough scanning with a Leitz inverted fluorescence microscope. The cells were concentrated by centrifugation and counted, and a known quantity (15-25 x 10^6) was placed in slide-mounted culture flasks (Lab-Tek Flaskette, No. 4820, Miles Scientific, Naperville, IL). A series of previous observations determined that a single fluorescent Jurkat cell could be detected among 20 x 10^6 other cells in these chambers.

The results of these analyses are also presented in TABLE 5 (last column). "DEPLETION #H342+/TOTAL ($X10^7$)" is the number of fluorescent Jurkat cells per total number of cells counted. In 2 of the 3 experiments (BM2002 and BM2003), the depletion of fluorescent Jurkat tumor cells was absolute (0 per 29 and 46 million CD34+ cells, respectively), and in the third experiment only 2 tumor cells were seen among 40 million CD34+ cells. By extension to clinical BMT situations, the present methods of positive immunoselection (e.g., of CD34+ cells), followed by negative immunoselection of tumor cells, may result in tumor cell depletions in vitro that exceed prior art depletions by 100- to 1000-fold or greater. These may lead in certain cases to the total elimination of tumor cells from bone marrow in vitro. Particular examples of malignancies that may contaminate CD34+ cell preparations, and against which antibodies to tumor-associated antigens are available or may be obtained for this purpose include breast cancer, neuroblastoma, multiple myeloma, lymphoma, and leukemias.

By similar procedures uncommitted CD34+ progenitor cells, which are CD38- and lack lineage-specific antigens such as CD5, CD10, CD33, and CD71, could be separated from CD34+ cells that are lineage-committed and express the CD38 antigen in high density (Terstappen et al. (1991) Blood 77:1218). Similar procedures could also be used to purify multipotent CD34+ stem cells by virtue of expression of Thy-1 antigen and non-expression of lineage-specific antigens (Baum et al. (1992) Proc. Natl. Acad. Sci. USA 89:2804).

EXAMPLE 10: In vitro growth of recovered CD34+ cells.

The growth capabilities of CD34+ cells immunoselected as described in Example 4 were analyzed in a variety of cell culture systems.

For example, hematopoietic, clonogenic, lineage-restricted (granulocyte-macrophage colony forming units, CFU-GM; erythroid burst-forming units, BFU-E) and multipotential (mixed colony forming units, CGU-Mix) progenitors were assayed in methylcellulose cultures using an adaptation of standard methods (Iscove et al. (1974), J. Cell. Physiol., 83:309 and Lu et al. (1986), Cancer Res., 46:4357). Cells were plated in 35 mm diameter gridded tissue culture plates (Nunc Inc., Naperville, IL) in IMDM supplemented with 1.1% methylcellulose (4000 CP; Fluka, Buchs, Switzerland), 30% (vol/vol) FBS, 2.5 U/ml final concentration human recombinant erythropoietin (Amgen, Thousand Oaks, CA), and 10% (vol/vol) giant-cell tumor conditioned media (GCT, Gibco). Cultures were incubated at 37°C with 5% CO_2 and 100% humidity, and colonies were counted using an inverted phase microscope and 40x magnification at 14-21 days. CFU-GM, hemoglobinized BFU-E, and mixed erythroid-nonerythroid colonies (CFU-Mix) were recognized using standard criteria of clonal morphology (Lu et al., ibid.); in addition, colonies containing approximately 50-500 cells were distinguished from very large, high proliferative potential (HPP) colonies that contained >500 cells and appear to represent particularly immature subsets of clonogenic progenitors (McNiece et al. (1986), Exp. Hematol., 14:856).

Typical results of this analysis are shown in Table 5 (adapted from La Russa et al. (1992), Exp. Hematol., 20:442). In numerous studies of this type clonogenic progenitors constituted 15-20% more of the CD34+ cells, and were enriched in progenitors from over 20-fold to over 70-fold compared to unseparated marrow (depending on the source of the cells and particularly the percentage of CD34+ cells before immunoselection).

In related studies it was demonstrated that the immunoselected CD34+ cell population could be further manipulated to obtain selected subsets that could also grow in tissue culture. For example, CD34+ cells depleted of cells which coexpress the CD33 antigen have been obtained by treating them with an anti-CD33 immunotoxin (anti-CD33 blocked ricin; LaRussa et al., ibid.). CD33 is expressed by most normal clonogenic myeloid progenitors (CFU-GM, BFU-E, CFU-Mix), as well as most acute myelogenous leukemia cells, but not on stem cells or more primitive progenitors (Andrews et al. (1989), J. Exp. Med., 169:1721; Sutherland et al. (1989), Blood, 74:1563). Recovery of clonogenic progenitors from CD34+ cells treated this way was reduced by >85% for CFU-GM and 20-40% for CFU-Mix and BFU-E. In contrast, the capacity of the remaining CD34+CD33- cells to form HPP CFU-GM, BFU-E, and CFU-Mix or to initiate hematopoiesis in long term marrow cultures (an in vitro functional assay for multipotent stem cells) was preserved or enhanced.

In other studies, it was demonstrated that new types of cells could be derived from the immunoselected CD34+ cells in tissue culture. For example, human CD34+ cells cultured over agarose surfaces or cocultured with mouse 3T3 fibroblast stromal cells in the presence of recombinant human IL-3 alone or combined with recombinant human stem cell factor gave rise to a population of human mast cells (Kirshenbaum et al. (1991), J. Immunol., 146:1410; Kirshenbaum et al., (1992), J. Immunol., 148:772). Mast cells are of interest, among other reasons, because of their role in allergies. By use of a distinguishing surface membrane marker on these cells (e.g., the expression of receptors for IgE) a haptenylated ligand (e.g., biotinylated IgE) could be used to immunoselect them for further

study. This illustrates the feasibility of this immunoselection approach to obtain desired blood cells or other products from previously immunoselected CD34+ cells or other cells.

EXAMPLE 11: Immunoselection and analysis of CD34+ cells from nonhuman primates.

The crossreactivity of K6.1 monoclonal antibody with CD34 antigen of nonhuman primates (Kessler et al. (1990), Exptl. Hematol., 18:677a (abstract); Wieder et al. (1991), Blood, 77:448; Donahue et al. (1991), Blood, 78:79a (abstract)) makes possible the immunoselection of CD34+ cells from these species for preclínical experimental studies. To demonstrate this, bone marrow (30-100 ml) was harvested surgically from iliac crests and ischial tuberosities into heparinized syringes of anesthetized rhesus macaques (M. mulatta). The bone marrow was diluted with HBSS and passed through nylon screen or wire mesh (Baxter) to remove clumps, and mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque (Pharmacia), and washed. Immunoselection of CD34+ cells was then performed as described in Example 4.

Immunoselected rhesus CD34+ cells were subjected to immunofluorescence staining and flow cytometry analysis as described in Examples 6 and 8. Illustrative results are depicted in Figure 4.

In Figure 4a these cells were stained with CD34 monoclonal antibody TUK3 (solid line; described in Example 6) or the negative isotype control J606 (broken line). This shows the immunoselected rhesus cells were virtually all CD34+, comparable to results with human CD34+ immunoselections. An important distinction between staining of human versus nonhuman primate CD34 antigen is that most CD34 antibodies that are available do not crossreact with nonhuman primate CD34 (e.g., anti-MY-10), or they bind with a substantially lower avidity (e.g., ICH3 from CalTag Laboratories, Inc., So. San Francisco, CA, and possibly to a smaller extent (TUK3) which may preclude staining of 100% of the immunoselected CD34+ cells. Monoclonal antibody 12.8 (described in Example 3) also

crossreacts with nonhuman primate CD34, but because the binding of 12.8 antibody is blocked by K6.1 antibody (Example 3) this cannot be used for reanalysis of cells immunoselected with K6.1.

In Figure 4b these cells were stained with CD38 monoclonal antibody OKT10 (Ortho Diagnostic Systems, Raritan, NJ) or the isotype control MOPC-21, followed by phycoerythrin-conjugated goat anti-mouse IgG1 antibody (Fisher Biotech). The histogram shows that similar to human CD34+ cells (Example 8 and Terstappen et al. (1991), Blood, 77:1218) there is a small population of CD34+, CD38- cells. This population is noteworthy because it represents very primitive progenitor cells lacking lineage commitment or possibly stem cells (Terstappen et al., ibid.).

In Figure 4c these cells were stained with anti-human Thy-1 monoclonal antibody (Seeger et al. (1982), J. Immunol., 128:983, gift of R.C. Seeger, Childrens Hospital, Los Angeles, CA) or the isotype control J606, followed by phycoerythrin-conjugated goat anti-mouse IgG3 antibody (Fisher Biotech). The histogram shows that there is a small population of CD34+,Thy-1+ cells. This population is noteworthy because in humans and mice it is reported to represent multipotent CD34+ stem cells (Baum et al. (1992), Proc. Natl. Acad. Sci. USA, 89:2804). In the present example this population may be more prominent than the published report with human cells (Baum et al. (1992), ibid.) because prior to marrow harvest the donor was injected with a bolus of 5-fluorouracil to kill cycling cells and increase the frequency of stem cells and immature progenitors (Wieder et al. (1991), ibid.).

EXAMPLE 12. Transplantation of CD34+ cells.

To determine the efficacy of immunoselected CD34+ cells for bone marrow transplantation, marrow was harvested from normal rhesus macaques and CD34+ were purified as described in Example 11. Twenty-four hours after harvest the monkeys were lethally irradiated with 10.0 Gy (0.4 Gy/min) gamma total body irradiation and transplanted with 1.5-2.8x10^6 autologous CD34+ cells/kg (Kessler et al. (1990), Exptl. Hematol.,

18:677a (abstract)). Positive control animals received $50x10^6$ marrow mononuclear cells/kg. Engraftment as determined by peripheral blood neutrophil counts and platelet counts over time is shown in Figure 5. The 3 CD34+ cell recipients (2N6, 474D, and n34) engrafted with faster kinetics of myeloid cell recovery (neutrophil counts $>1000/mm^3$ by day 21; platelet counts $>50,000/mm^3$ by day 23) than the mononuclear cell controls (exemplified by 79n). These findings show that residual K6.1 antibody, if any, that may be associated with the CD34+ cells does not interfere with engraftment. It is noteworthy that others have reported the results of CD34+ cell immunoselections from baboons and clinical patients for transplant purposes using a commercial immunoselection device (CellPro, Inc., Bothell, WA). However, the levels of CD34+ cell purity they obtained were low compared to the present invention, ranging from 35 to 92% (Berenson et al. (1988), J. Clin. Invest., 81:951; Berenson et al. (1991), Blood, 77:1717; Heimfeld et al. (1991), Blood, 78:16a (abstract)).

TABLE 2

YIELDS OF CD34+ CELLS FROM HUMAN CADAVERIC BONE MARROW

MARROW NO.	INITIAL WBC (approx.)	WBC POST DEBULK	YIELD CD34+ CELLS
1	$1.1x10^{10}$	$2.4x10^{9}$	$1.53x10^{8}$
2	$1.8x10^{10}$	$5.2x10^{9}$	$1.01x10^{8}$
3	$2.4x10^{10}$	$9.6x10^{9}$	$2.25x10^{8}$
4	$1.7x10^{10}$	$6.6x10^{9}$	$3.14x10^{8}$

TABLE 3

CD34+ CELL PURIFICATION AND PURGING EFFICIENCY

MARROW NO.	%H342+ PRESEL[1]	#H342+ TOTAL ($x10^{6}$)	PURGE FACTOR	%CD34+	LOG PURGE	BMT EQUIV LOG PURGE[2]
1	1.82	2/4.7	4277	99.98	3.6	5.6
2	0.50	2/8.8	2228	99.96	3.3	5.3
3	1.46	8/10.4	1896	99.95	3.3	5.3
4	0.49	2/23.2	5675	99.98	3.8	5.3

[1] PBL were prestained with Hoechst 33342 and seeded into the marrow cell suspension before positive selection.

[2] Assuming 10^{6} CD34+ cells/kg would be equivalent to 10^{8} marrow MNC/kg in a BMT recipient.

TABLE 4

IMMUNOPHENOTYPES OF PURIFIED CD34+ CELLS

MAb SPECIFICITY	CD#	DISTRIBUTION	FREQUENCY
PAN-HEMOPOIETIC PRECURSOR			
K6.1, MY-10	CD34		+4
Leu-17 (OKT10)	CD38		+3
MULTI-LINEAGE			
HLA-DR, I2	–		+3
HLA-DQ (Leu-10)	–		+1
HLA-DP	–		+1
CALLA, J5	CD10	pre-B, cALL	+1
MHM24	CD11a	LFA-1α chain	+2
H52, 60.3	CD18	CD11ß chain	+2
ALB-9	CD24	G, B, non-B & T ALL	-
IL-2 Receptor	CD25		+1
Transferrin Receptor	–		+2
4B4	CDW29	M, B, T, null	+2
4F2	–	M, act-B & T	+2
Leu-8, TQ-1	–	B, T, & null ss	+1
HLe-1	CD45	T200	+4
MYELOID LINEAGES			
ALB-6, Ba-2, p24	CD9	M, pre-B, Plt	+2
Mo.1, OK-M1, CR3 (Mac-1, Leu-15)	CD11b	G, M (C3BiR)	-
Mo.2, Leu-M3, MY-4, 63D3	CD14	M(G), DC	-
Leu-M1	CD15	G(M)	-
Leu-M2 (Mac-120)	–		-
MY-7	CD13	G, M	+3
MY-9	CD33	myelog. leuk.	+2
CR1 (C3bR)	CD35	G, M. DC	-
5F1 (Gp1b)	CDw42	Plt	+1
PM81	–	G, M, AML, ALL	-
PLT-1 (69)	–	Plt, MK	+1

MAb SPECIFICITY	CD#	DISTRIBUTION	FREQUENCY
B LINEAGE			
Leu-12, B4	CD19		-
Leu-16, B1	CD20	B, DC	-
B2 (C3dR)	CD21	B, DC	-
BL14	CD37		-
PCA-1	–		-
T LINEAGE			
Leu-1	CD5	pan-T, pan-Thy	-
Leu-2, T8	CD8	T ss	-
Leu-3, T4	CD4	T ss	-
Leu-4, T3	CD3	pan-T	-
Leu-5, T11	CD2	pan-T	+/-
Leu-6, T6, BL6	CD1	Thy	-
Leu-9	CD7	pan-T	+/-
SPV-L14	CD6	pan-T	-
Ta-1	CDw26	act-T	-
NK ASSOCIATED			
Leu-7	–	NK, T ss	-
Leu-11a	CD16	NK & G (IgG FcR)	-
Leu-19, NKH-1, MY-31	–	NK & T ss	-

TABLE 5

CD34+ CELL POSITIVE IMMUNOSELECTION FOLLOWED BY IMMUNODEPLETION

MARROW NO.	%H342+ PRESEL[1]	#H342 TOTAL (x10⁶)	PURGE FACTOR	%CD34+	LOG PURGE	EMT EQUIV LOG PURGE	DEPLETION[2] 3H342+ TOTAL (x10⁶)
1	2.30	2/4.7	7360	99.99	63.9	5.9	0/2.9
2	3.62	0/9.4	>34,028	>99.997	>4.5	>6.5	0/4.6
3	1.54	6/5.0	1278	99.92	3.1	5.1	2/4.0

[1] Jurkat cells were prestained with Hoechst 33342 and seeded into the marrow suspension before positive selection.

[2] Residual Jurkat cells were immunodepleted from the CD34+ cell suspension with a CD3 monoclonal antibody.

TABLE 6

RECOVERY OF HPP PROGENITORS FROM CD34+ MARROW CELLS

Progenitor Cells	Colonies*
CFU-GM (total)	1116+118
CFU-GM (HPP)	186+41
BFU-E (total)	429+69
BFU-E (HPP)	51+3
CFU-Mix	711+11

* Progenitor cells/10⁴ CD34+ marrow cells plated in clonal assay; mean values + standard error of the mean (SEM) for three replicate studies.

While the invention has been described in terms of various preferred embodiments, the skilled artisan will appreciate that various modifications, substitutions, omissions, and changes may be made without departing from the spirit thereof. Accordingly, it is intended that the scope of the present invention be limited solely by the scope of the following claims, including equivalents thereof.

WHAT IS CLAIMED IS:

1. A method for immobilizing target cells to a solid phase matrix or support comprising the following steps:

(i) reacting a heterogeneous composition of cells containing target cells with a primary immune reagent which is capable of specifically binding said target cells and which primary immune reagent is optionally conjugated to a soluble hapten to produce primary reagent-target cell complexes;

(ii) reacting the complex containing heterogeneous composition produced by step (i) with one or more secondary immune reagents, at least one which is capable of specifically binding to the primary reagent and wherein at least one of said secondary reagents is conjugated to a soluble hapten if the primary reagent has not been conjugated to soluble hapten so as to produce complexes of secondary reagents with first reagent-target cell complexes; and

(iii) reacting the complex containing composition of step (ii) with a solid phase or matrix to which has been directly or indirectly bound an antibody capable of specifically binding to said soluble hapten and resulting in the immobilization of the immune complexes of step (ii) to the solid phase or matrix via an immune complex which comprises a hapten-anti-hapten antibody complex.

2. The method of claim 1 wherein the primary immune reagent comprises an antibody or antibody fragment or chimeric antibody capable of binding to an antigen contained on the surface of the target cells.

3. The method of claim 2 wherein the primary immune reagent is a monoclonal antibody or fragment thereof capable of binding to an antigen contained on the surface of the target cells.

4. The method of claim 1 wherein the target cells comprise stem or progenitor cells.

5. The method of claim 1 wherein the target cells comprise human pluripotent lympho-hematopoietic progenitor and stem cells.

6. The method of claim 5 wherein the primary immune reagent is the monoclonal antibody K6.1 produced by hybridoma cell line K6.1 and deposited under ATCC No. HB 11085.

7. The method of claim 1 wherein the soluble hapten comprises biotin, fluorescein, digoxigenin, nitrophenol compounds, arsenilic acid, or any construction or derivatization of these molecules which affects or enhances their solubility or conjugation to the materials in claims 1.

8. The method of claim 1 wherein the solid phase comprises paramagnetic beads or particles.

9. The immobilized target cell containing matrix or support produced by the method of claim 1.

10. The immobilized target cell containing matrix or support produced by the method of claim 5.

11. The immobilized target cell containing matrix or support produced by the method of claim 6.

12. A method for positive immunoselection of target cells contained in a solid phase matrix or support comprising the following steps:

(i) reacting a heterogeneous composition of cells containing target cells with a primary immune reagent which is capable of specifically binding said target cells and which primary reagent is optionally conjugated to a soluble hapten to produce primary reagent-target cell complex;

(ii) reacting the complex containing heterogeneous composition produced by step (i) with one or more secondary immune reagents, at least one of which is capable of specifically binding the primary reagent and wherein at least one of said secondary reagents is conjugated to a soluble hapten if the primary reagent has not been conjugated to soluble hapten so as to produce complexes of the secondary reagents and the first reagent-target cell complexes;

(iii) reacting the complex containing composition of step (ii) with a solid phase matrix or support to which has been directly or indirectly bound an antibody or antibody fragment capable of specifically binding to said soluble hapten and resulting in the immobilization of the immune complexes of step (ii) to the solid phase or matrix via an immune complex which comprises a hapten-anti-hapten antibody complex;

(iv) separating the resultant target cell containing solid phase matrix or support from the heterogeneous cell composition; and

(v) dissociating the immobilized target cells from the solid phase matrix or support via hapten competition by a method comprising contacting said solid phase matrix or support with a solution containing a molar excess of the soluble hapten.

13. The method of claim 12 further comprising the following step:

(vi) recovering said dissociated cells.

14. The method of claim 12 wherein the primary immune reagent comprises an antibody ør antibody fragment or chimeric antibody capable of binding to antigen contained on the surface of the target cells.

15. The method of claim 14 wherein the primary immune reagent comprises a monoclonal antibody or antibody fragment or chimeric antibody capable of binding to an antigen contained on the surface of the target cells.

16. The method of claim 12 wherein the target cells comprise stem or progenitor cells.

17. The method of claim 16 wherein the target cells comprise human or nonhuman primate pluripotent lympho-hematopoietic progenitor or stem cells.

18. The method of claim 17 wherein the primary immune reagent comprises the monoclonal antibody K6.1 produced by hybridoma cell line K6.1 and deposited under ATTC No. HB 11085.

19. The method of claim 12 wherein the soluble hapten comprises biotin or a chemically derivatized biotin.

20. The method of claim 12 wherein the solid phase comprises paramagnetic beads or particles.

21. The immunoselected cells resulting from claim 12.

22. The immunoselected cells resulting from claim 13.

23. The method of claim 13 which further includes the following step:

(vii) reacting the target cells of step (vi) with a labeled immune reagent which binds to a distinct antigenic epitope from the primary immune reagent.

24. The method of claim 12 which further comprises subjecting the recovered target cells of step (vi) to additional positive or negative immunoselections, wherein these immunoselections are effected simultaneously or sequentially.

25. A purified cell composition comprising purified human pluripotent lympho-hematopoietic stem cells which is virtually devoid of mature lymphoid and myeloid cells.

26. The composition of claim 25 which further comprises human lympho-hematopoietic progenitors, colony-forming cells and/or immature precursor cells.

27. A purified cell composition comprising cells capable of specifically binding to the monoclonal antibody produced by the hybridoma K6.1 which composition is substantially devoid of cells which do not have a cell surface antigen capable of being bound by said monoclonal antibody.

28. The method for positive immunoselection of target cells of claim 13 which further comprises including in the heterogeneous cell composition used in step (i) autologous or heterologous indicator cells which are labelled with an immune reagent distinct from the primary immune reagent used with (i) and which does not comprise the soluble hapten; and

quantifying after step (vi) the extent of elimination of said indicator cells.

29. A kit for positive immunoselection of target cells from a heterogeneous cell composition comprising:

(a) a primary immune reagent which specifically binds to the target cells and may optionally be conjugated to a soluble hapten;

(b) one or more secondary immune reagents at least one of which is capable of specifically binding the primary immune reagent and wherein at least one secondary immune reagent is conjugated to a soluble hapten if the primary immune reagent is not conjugated to a soluble hapten;

(c) a solid phase matrix or support to which is directly or indirectly bound an immune reagent capable of specifically binding to said soluble hapten; and

(d) a solution comprising an amount of said soluble hapten which is sufficient to dissociate target cells bound to said solid phase matrix or support via an immune complex containing a hapten-anti-hapten antibody linkage.

30. The kit of claim 30 wherein the soluble hapten comprises biotin or fluorescein.

31. The kit of claim 30 wherein the solid phase matrix or support comprises magnetic beads or particles.

32. The kit of claim 30 wherein the solid phase matrix or support is contained in a reservoir, a plastic culture flask, a chromatography column or an extracorporeal flow-through cartridge.

33. An improved method of BMT which comprises the infusion of healthy stem cells and progenitor cells to a recipient in need thereof wherein the improvement comprises administering a therapeutically effective amount of the purified cell composition of claim 25.

34. The method of claim 34 wherein the recipient is autologous.

35. The method of claim 34 wherein the recipient is heterologous or allogeneic.

36. The method of claim 34 wherein the purified cell composition further includes tumor-infiltrating lymphocytes (TIL cells),

lymphocyte activated killer cells (LAK cells), cytotoxic lymphocytes (CTLs), or CD4+ helper cells.

37. A monoclonal antibody K6.1 or a monoclonal antibody having the identifying characteristics thereof which is produced by hybridoma cell line K6.1 which has been deposited under ATCC No. HB 11085.

38. The hybridoma cell line K6.1 or cell lines having the identifying characteristics thereof which has been deposited under ATCC No. HB 11085.

39. A method of using the monoclonal antibody of claim 38 for the positive immunoselection of stem and progenitor cells from non-primate bone marrow or blood containing compositions.

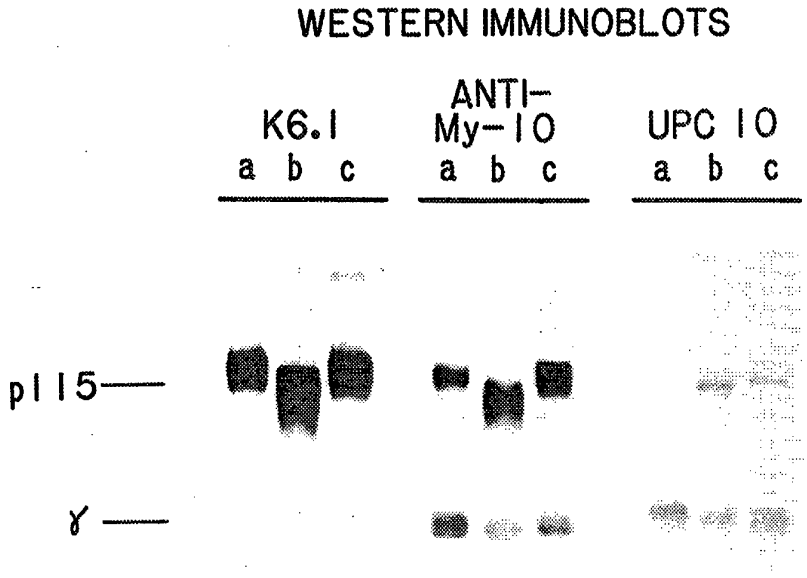


FIG. 1

FIGURE 2A

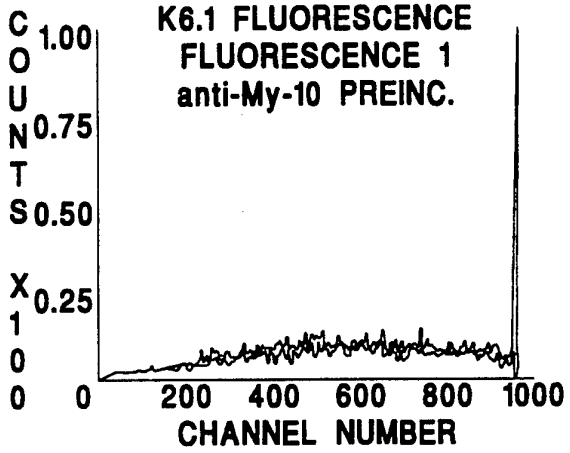
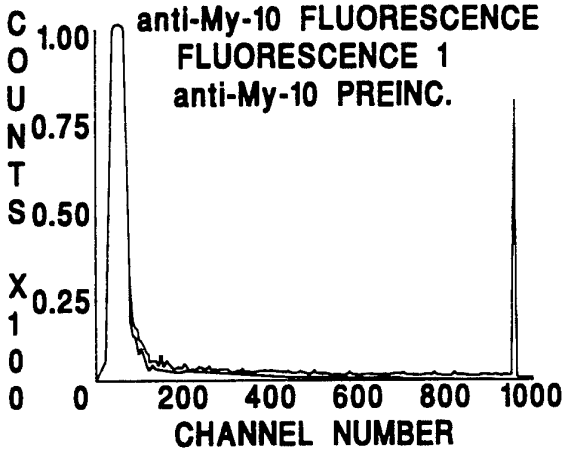


FIGURE 2B



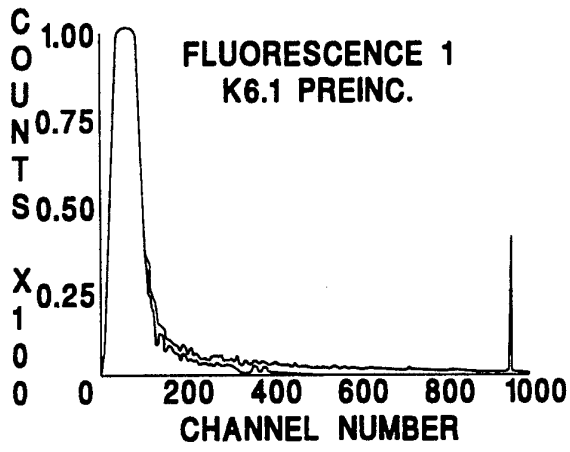


FIGURE 2C

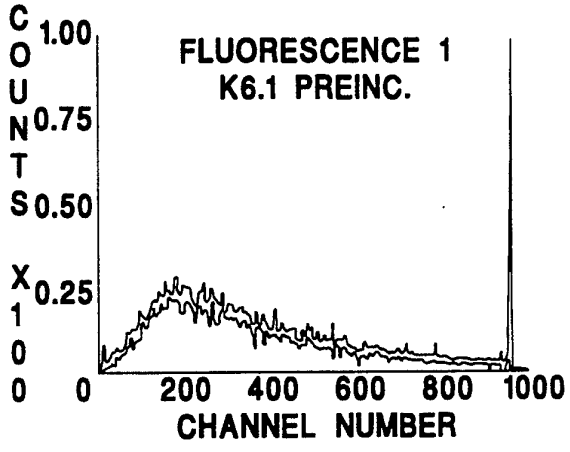


FIGURE 2D

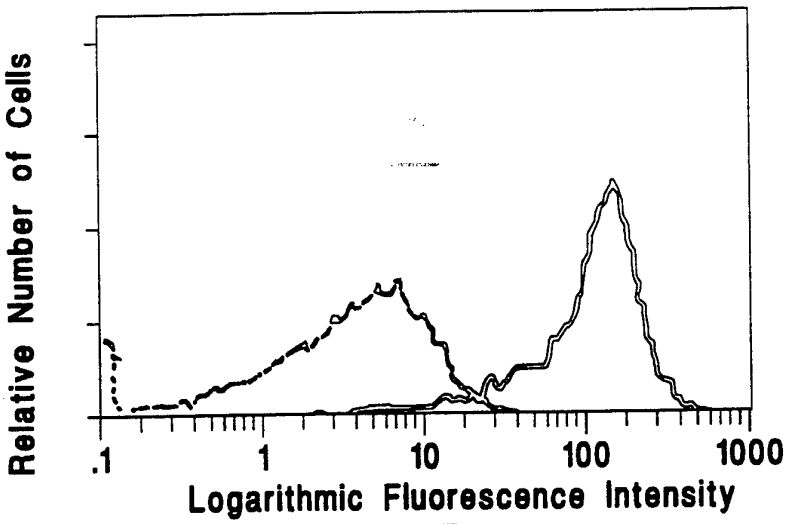


FIGURE 3A

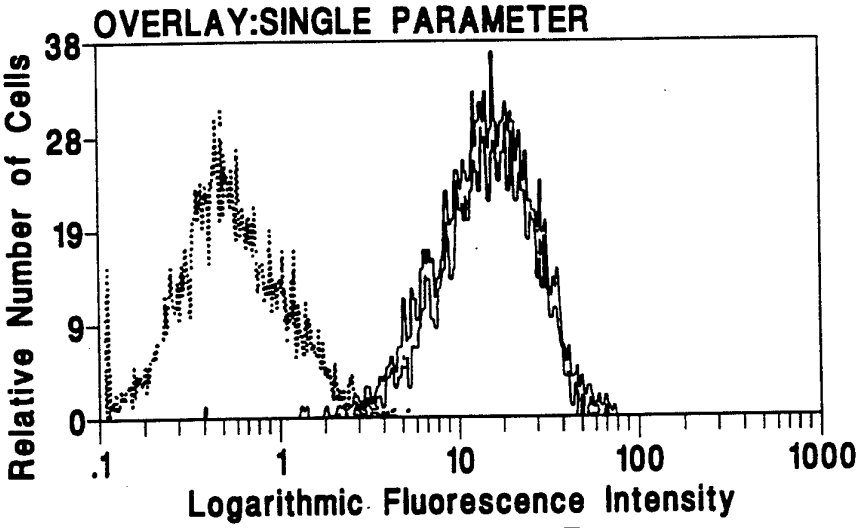


FIGURE 3B

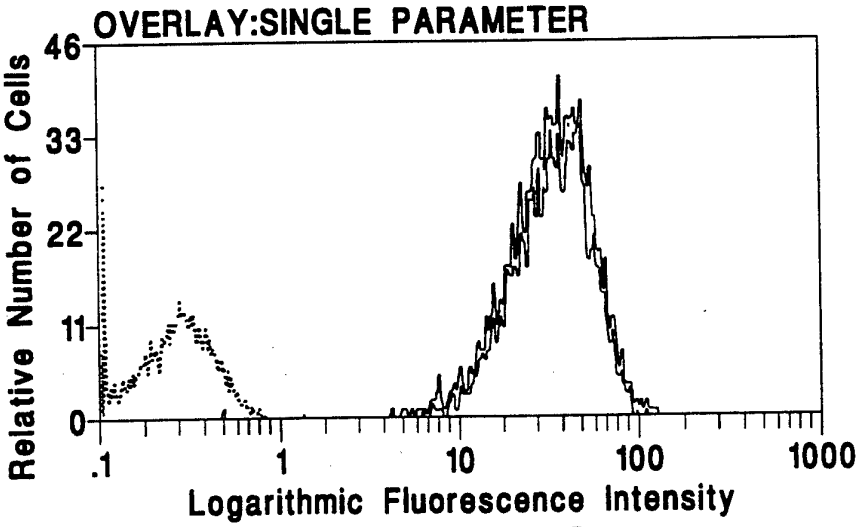


FIGURE 3C

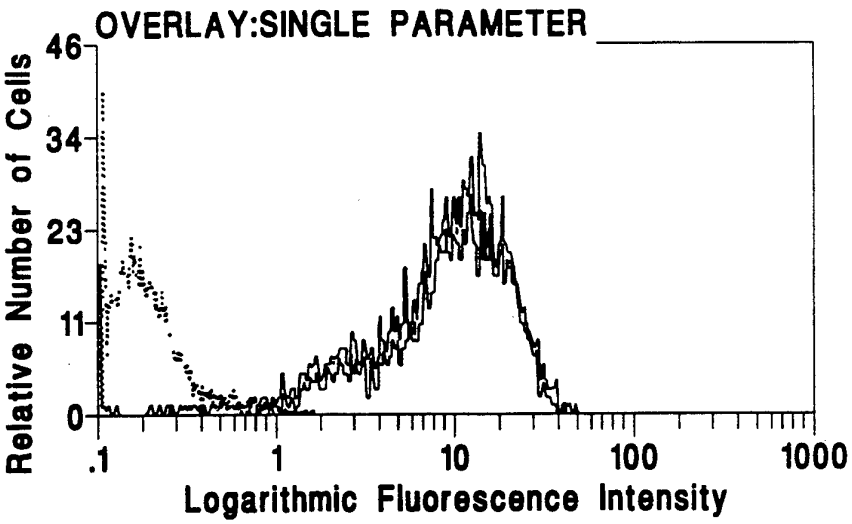


FIGURE 4A

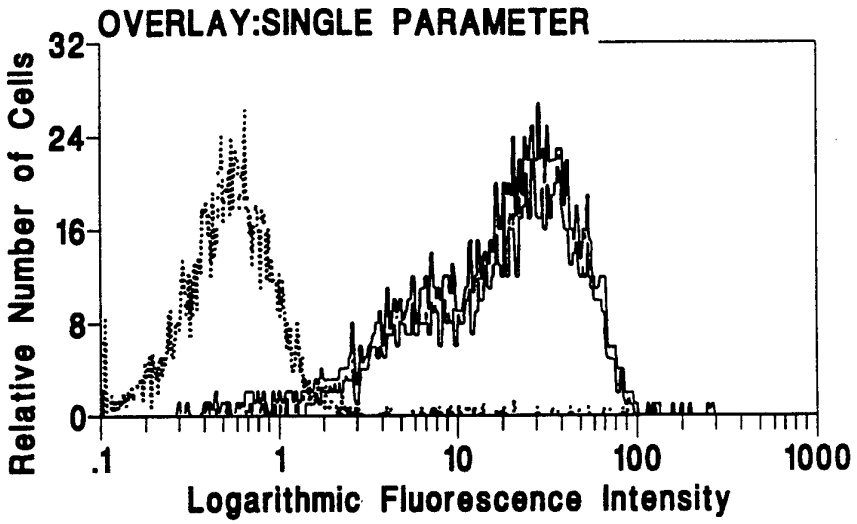


FIGURE 4B

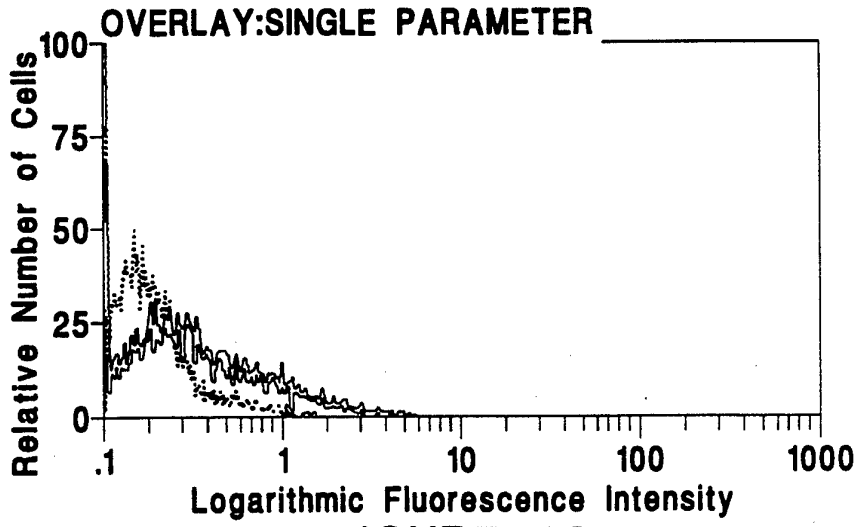
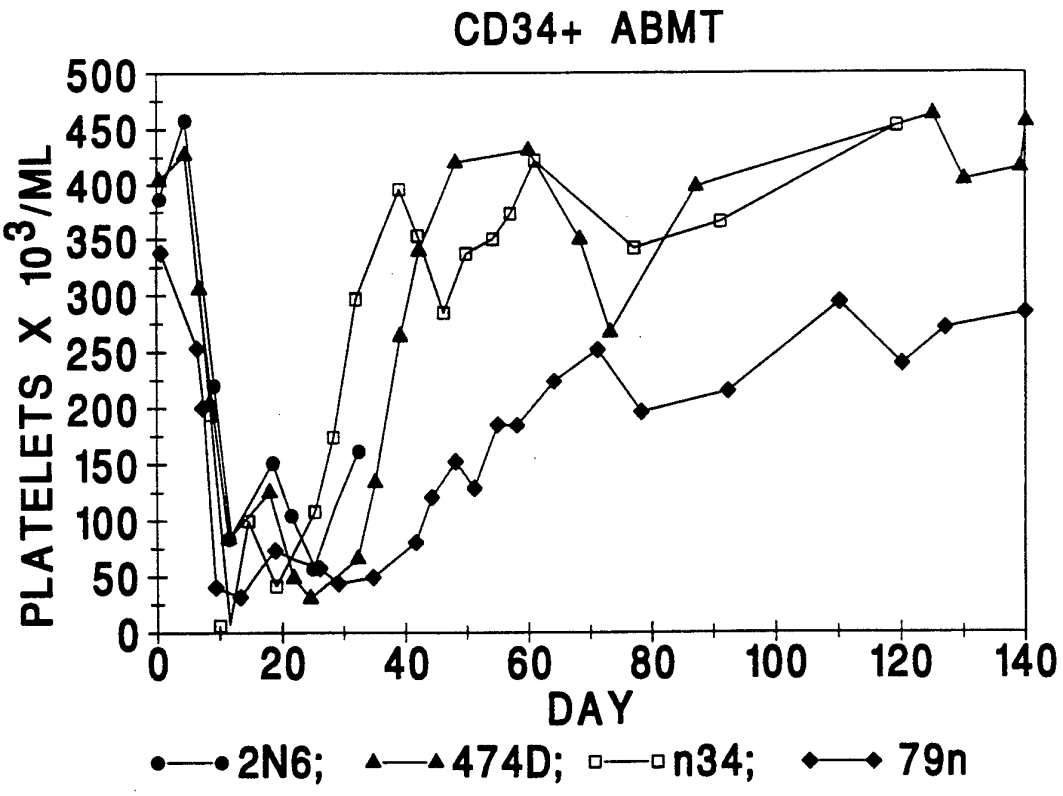


FIGURE 4C

FIGURE 5A



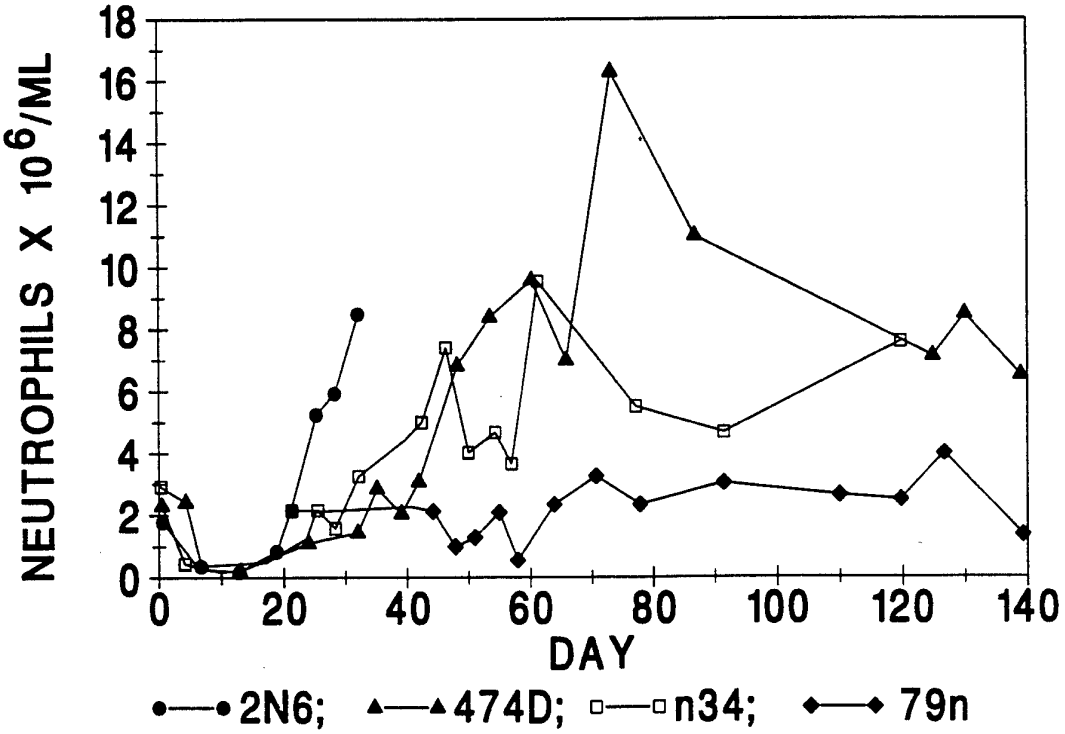


FIGURE 5B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07005

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93U, 93V; 435/2, 7.21, 7.24, 7.5, 172.2, 174, 176, 240.2, 240.21, 240.27, 975; 436/519, 524, 526, 528; 530/388.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, search terms: stem cell, cell? (separat? or isolat? or immunoselect? or select?), CD34?, My-10, anti-biotin, hapten? (elution or dissociat? or disassociat? or release?)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 87/04628 (BERENSON ET AL) 13 August 1987, see entire document, especially pages 4, 6-7, 9, 11, 16, and 27, and Claims 1-12.	1-36 and 39
Y	JOURNAL OF IMMUNOLOGICAL METHODS, Volume 56, issued 1983, R.S. Basch et al, "Cell Separation Using Positive Immunoselective Techniques", pages 269-280, see especially pages 271-274.	1-32 and 39
X Y	JOURNAL OF IMMUNOLOGY, Volume 146, No. 5, issued 01 March 1991, A.S. Kirshenbaum et al, "Demonstration of the Origin of Human Mast Cells from CD34+ Bone Marrow Progenitor Cells", pages 1410-1415, see especially page 1411.	21-22, 25-27, 37-38 1-20, 23-24, 28-36, 39

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 22 OCTOBER 1993	Date of mailing of the international search report 04 NOV 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer JAMES L. GRUN, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07005

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,778,751 (EL SHAMI ET AL) 18 October 1988, see especially Col 21 and Claims 1, 2, 10, and 13.	1-20, 23-24, 28-32, 39
X	US, A, 4,714,680 (CIVIN) 22 December 1987, see entire document, especially Col 7-8.	21-22, 25-27, 33-35
X Y	US, A, 5,035,994 (CIVIN) 30 July 1991, see entire document, especially Col 5, 6, 7-8, 14, and 17, and Claims 1-3 and 6.	21-22, 25-27, 33-35 1-20
X Y	US, A, 5,081,030 (CIVIN) 14 January 1992, see entire document, especially Col 4 and 5-7.	21-22, 25-27, 33-35 1-20
X	SCIENCE, Volume 242, issued 11 November 1988, T.M. Folks et al, "Infection and Replication of HIV-1 in Purified Progenitor Cells of Normal Human Bone Marrow", pages 919-922, see especially page 919, Table 1, and page 920, Col 2.	21-22, 25-27, 37-38
X Y	FEDERATION PROCEEDINGS, Volume 46, No. 4, issued 05 March 1987, S.W. Kessler, "Epitope Diversity and Structure of the CD34 (p115) Hematopoietic Progenitor Cell Antigen", page 1363, Abstract No. 6109, see entire document.	37-38 37-39
Y	JOURNAL OF CLINICAL INVESTIGATION, Volume 81, issued March 1988, R.J. Berenson et al, "Antigen CD34+ Marrow Cells Engraft Lethally Irradiated Baboons", pages 951-955, see entire document, especially page 951, Abstract.	33-35, 39
X Y	BLOOD, Volume 70, Supplement 1, issued 1987, S.W. Kessler et al, "Large-Scale Purification and Characterization of CD34-Positive Hematopoietic Progenitor Cells", page 321a, see entire document.	21-22, 25-27, 37-38 33-36, 39
X,O Y,O	BONE MARROW PURGING AND PROCESSING, THIRD INTERNATIONAL SYMPOSIUM, held 1991, Kessler et al, "'Reverse Purging' of Marrow by CD34+ Cell Positive Immunoselection", see entire document.	21-22, 25-27, 37-38 33-35, 39
Y	BLOOD, Volume 77, No. 8, issued 15 April 1991, R.J. Berenson et al, "Engraftment After Infusion of CD34+ Marrow Cells in Patients With Breast Cancer or Neuroblastoma", pages 1717-1722, see entire document.	33-36
X	US, A, 5,061,620 (TSUKAMOTO ET AL) 29 October 1991, see entire document, especially Col 1-2.	21-22, 25-27, 33-35

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US[illegible]07005

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF HEMATOTHERAPY, Volume 1, issued 1992, J.T. Kemshead, "Immunomagnetic Manipulation of Hematopoietic Cells: A Review of Current Technology", pages 35-44, see entire document.	1-39

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07005

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A01N 1/02; A61K 35/14, 35/28; C07K 15/28; C12N 5/06, 5/08, 5/20; G01N 33/544, 33/551, 33/553, 33/554

A. CLASSIFICATION OF SUBJECT MATTER:
US CL : 424/93U, 93V; 435/2, 7.21, 7.24, 7.5, 172.2, 176, 240.2, 240.21, 240.27; 436/519, 524, 526, 528; 530/388.7