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(54) Title: INTRACELLULAR MARKER FOR PURIFICATION OF STEM CELLS		
(57) Abstract An intracellular enzyme, cytosolic aldehyde dehydrogenase, expressed at high levels in primitive stem cells, appears to play an important role in allowing these cells to be resistant to cyclophosphamide. A fluorescent cell-permeable, non-polar aldehyde oxidized intracellularly by ALDH is used to identify human stem cells that are then isolated. For instance, a non-polar aldehyde, such as dansyl aminoacetaldehyde, serves as a substrate for an intracellular ALDH. Bone marrow progenitor stem cells treated with DAAA proliferate normally. Bone marrow cells expressing high levels of dansyl fluorescence after incubation with DAAA and sorted based upon dansyl fluorescence, preferably using an automated fluorescence cell sorting technique such as FACS, are substantially enriched for pluripotent hematopoietic progenitor stem cells. By this method, an enriched cellular composition comprising human lymphohematopoietic stem cells can be obtained. The cell population is capable of self-regeneration in a co-culture medium and of differentiation into various lymphoid hematopoietic lineages.		

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INTRACELLULAR MARKER FOR PURIFICATION OF STEM CELLSStatement as to Federally Sponsored Research

- 5 This invention was partially supported by NIH Grants # PO1 CA15396 and RO1 HL46533. The United States Government may have an interest in the subject matter of this invention.

Field of the Invention

- 10 This invention relates generally to enriched pluripotent stem cell compositions, methods and compounds for producing these populations, and the use of these compositions in the treatment of various disorders in which hematopoietic reconstitution is desirable, such as by bone marrow transplantation (BMT).

Background of the Invention

- 15 All the cells circulating in the blood are descendants of a very small number of pluripotent stem cells. These ancestral cells, which comprise less than 0.01 percent of the nucleated cells in the bone marrow, are capable of restoring normal hematopoiesis in individuals in need of reconstitution of their bone marrow. The pluripotent stem cell has the unique capacity for self-renewal and the potential for growth and differentiation along granulocytic, monocytic, erythroid, megakaryo-
20 cytic, and lymphoid lineages. Some stem cells divide and give rise to progeny that lose their ability to differentiate along multiple pathways and become committed to a specific hematopoietic lineage. These committed progenitor cells continue to proliferate and differentiate into morphologically identifiable precursor cells which then undergo terminal maturation, thereby developing highly specialized functions
25 and lose their ability to proliferate. Techniques have been developed which support the growth and differentiation of hematopoietic progenitor cells *in vitro*. Using these techniques, hematopoietic colonies of mixed and single lineages have been identified and characterized with respect to the factors required for their growth.

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Mammalian blood cells provide for an extraordinarily diverse range of activities. The blood cells are divided into several lineages based on function. The lymphoid lineage, comprising B cells and T cells, provides for the production of antibodies, regulation of the cellular immune system, detection of foreign agents in the blood, detection of cells foreign to the host, and the like. The myeloid lineage, which includes monocytes, granulocytes, megakaryocytes, as well as other cells, monitors for the presence of foreign bodies in the blood stream, provides protection against neoplastic cells, scavenges foreign materials in the blood stream, produces platelets, and the like. The erythroid lineage provides the red blood cells, which act as oxygen carriers.

As noted above, the stem cell population constitutes only a small percentage of the total number of leukocytes in bone marrow. At the present time it is not known how many of the markers associated with differentiated cells are also present on the stem cell. One marker which has been indicated as present on stem cells, CD34, is also found on a significant number of lineage-committed progenitors. Another marker that provides for some enrichment of progenitor activity is Class II HLA (particularly a conserved DR epitope recognized by a monoclonal antibody designated J1-43). However, these markers are found on numerous lineage-committed hematopoietic cells. In particular, B cells (CD19+) and myeloid cells (CD33+) make up 80-90% of the CD34+ population. Moreover, a combination of CD 3, 8, 10, 15, 19, 20 and 33 will mark $\leq 90\%$ of all CD34+ cells. Therefore, in view of the small proportion of the total number of cells in the bone marrow which are stem cells, the uncertainty of the markers associated with stem cells, as distinct from more differentiated cells, and the general inability to conduct a definitive biological assay for human stem cells, the identification and purification of stem cells has been elusive.

Aldehyde dehydrogenase (ALDH) is an enzyme responsible for oxidizing intracellular aldehydes and plays an important role in metabolism of ethanol, vitamin A, and cyclophosphamide. Substrates for these enzymes include acetaldehyde and biogenic amines produced during catecholamine catabolism. Further, ALDH appears to be a crucial step in the conversion of vitamin A to its active metabolite, retinoic acid (J. Labrecque, *et al.*, *Biochem Cell Biol*, 71:85, 1993; A. Yoshida, *et al.*, *Enzyme*, 46:239, 1992). Of the four ALDH isozymes identified (A. Yoshida, *et*

al., *Enzyme*, 46:239, 1992), liver cytosolic ALDH appears to be the specific isozyme involved in metabolism of vitamin A (J. Labrecque, *et al.*, *Biochem Cell Biol*, 71:85, 1993; A. Yoshida, *et al.*, *Enzyme*, 46:239, 1992) and cyclophosphamide resistance (J.E. Russo *et al.*, *Cancer Res* 48:2963, 1988). Both hematopoietic progenitors and intestinal crypt stem cells display high levels of cytosolic ALDH, and are accordingly relatively resistant to cyclophosphamide. Although all hematopoietic progenitors are known to express relatively high levels of cytosolic aldehyde dehydrogenase, both mouse and human HSC appear to express significantly higher levels than less primitive hematopoietic progenitors. Primitive hematopoietic progenitors are more resistant to 4HC than are later progenitors, and this appears to result in large part from their high expression of cytosolic ALDH rather than from their non-cycling status. However, there is no difference in the sensitivity to 4HC analogues of primitive hematopoietic progenitors that are not inactivated by aldehyde dehydrogenase (E.A. Sahovic *et al.*, *Cancer Res*, 48:1223, 1988).

Increased NAD-dependent aldehyde dehydrogenase (ALDH, Enzyme Commission 1.2.1.3) activity has been identified as a mechanism of antitumor drug resistance to the alkylating agent cyclophosphamide (CPA). CPA, a nitrogen mustard derivative incorporating an oxazaphosphorine, is a prodrug, requiring activation by the cytochrome P-450 mixed function oxidase system to produce 4-hydroxycyclophosphamide (4HC). A spontaneous beta elimination from aldophosphamide liberates the active DNA alkylating agent, phosphoramidate mustard, plus acrolein.

CPA is currently used clinically in the treatment of a diverse group of solid and hematologic malignancies, and is a key component of the cytoreductive regimens prior to bone marrow transplantation. A major factor in the success of CPA has been the high therapeutic index observed (Mullins and Colvin, *Cancer Chemother Repts*, 59:411, 1975). This high therapeutic index is due in part to a "sparing" effect that CPA exerts on normal hematopoietic stem cells (Fried, *et al.*, *Cancer Research*, 37:1205, 1977) and intestinal crypt stem cells. While damage to these rapidly proliferating stem cell renewal systems is the dose-limiting toxicity of other alkylating agents, these tissues are not profoundly effected by CPA. The diminished toxicity is specific for CPA (and its activated congeners) among alkylating

agents. Thus, elevated ALDH levels have been postulated as a mechanism for the relative resistance of bone marrow and intestinal stem cells to CPA. *In vivo* studies in mice have shown that a cytosolic ALDH isozyme found in murine tumor
5 tissue is responsible for conferring cyclophosphamide resistance (J.E. Russo *et al.*, *Enzymology and Molecular Biology of Carbonyl Metabolism*, 2:65-79, 1989).

Elevated levels of ALDH have been best characterized as a mechanism of cellular resistance to CPA in the L1210 murine lymphocytic leukemia model (DeWys, 1973), in which a 200-fold higher cytosolic ALDH activity was measured in the cell
10 line resistant to CPA (L1210/CPA) compared to the sensitive, wild-type line.

By definition human hematopoietic stem cells (HSC) are progenitor cells and are pluripotent in that they have the ability to repopulate lymphohematopoietic lineages on a long-term basis. The isolation of human HSC remains a goal particularly difficult to achieve, since prior to the present invention no single cell
15 characteristic has been found specific for HSC which could be used to prepare an enriched cellular composition. Therefore, the isolation and/or identification of HSC suitable for re-introduction into a host requires an eventual *in vivo* approach, yet prior art techniques for HSC detection, namely measurement of substrate oxidation in whole cell lysates (J.E. Russo *et al.*, *Cancer Res* 48:2963, 1988; J.E.
20 Russo *et al.*, *Enzymology and Molecular Biology of Carbonyl Metabolism* 2:65-79, 1989) or reaction of fixed cells with antibodies (M.B. Kastan *et al.*, *Blood* 75:1947, 1990; J.E. Russo *et al.*, *Enzymology and Molecular Biology of Carbonyl Metabolism* 2:65-79, 1989) are lethal to the cells being studied. By the same token, the applicability of animal models has been limited because analogues of many well-
25 characterized HSC markers have not been identified in other species (G.J. Spangrude *et al.*, *Science*, 241:58, 1988; C.T. Jordan, *et al.*, *Cell*, 61:953, 1990). Thus, there is a need for a stem cell enriched composition which is produced using essentially non-toxic methodology thereby allowing safe and effective reconstitution of a host in need of such therapy. The present invention answers
30 this need.

Summary of the Invention

Recognizing the importance of an enriched stem cell composition, the inventors developed a method and compounds for producing a pluripotent stem cell composition which can be used as an *in vivo* source of hematopoietic cell lineages in reconstitutive therapy. This composition advantageously provides a heretofore unattained percentage of uncommitted stem cells which are isolated under essentially non-toxic conditions to thereby enhance the utility of the composition for therapeutic purposes.

Brief Description of the Drawings

Figure 1A is a histogram analysis of dansyl fluorescence from four leukemia cell lines treated with dansylaminoacetaldehyde (DAAA). These results are from one representative experiment of human leukemia cell lines treated with DAAA. Gray peaks represent unstained cells and black peaks represent stained cells.

Figure 1B is a histogram showing corrected mean fluorescence intensity (MFI) for dansyl fluorescence of human and mouse leukemic cell lines treated with DAAA. CPA cells were treated with DEAB or DG. Each data point represents the mean $\pm$ S.E.M. of 3-5 separate experiments for each cell line. CPA = L1210/CPA; DG = dansyl glycine.

FIGURE 2 shows the chemical structures of DAAA and its conversion to dansyl glycine.

FIGURE 3 is a histogram showing corrected MFI for dansyl fluorescence of the indicated selected bone marrow cell subsets treated with DAAA. Each data point represents the mean $\pm$ S.E.M. of 3 separate experiments.

FIGURES 4A and 4B are graphs showing the effect of 4HC on clonogenic leukemia cells. Each data point represents the mean $\pm$ S.E.M. of 3-5 separate experiments for each cell line. Figure 5A shows the results obtained with human leukemia cells, and Figure 5B shows the results obtained with mouse leukemia cell lines.

Description of the Preferred Embodiments

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In one embodiment, the present invention provides a method for indirectly identifying intact, viable cells within a cell mixture that express an intracellular marker, for instance an enzyme such as cytosolic ALDH. The intracellular marker reacts with a cell-permeable fluorescent aldehyde to render the fluorescent aldehyde polar, and, hence, non-permeable to the cell membrane. Accordingly, the method of this invention comprises the steps of contacting a cell mixture with a cell-permeable, non-polar fluorescent aldehyde that is rendered polar by contact with the intracellular marker, for instance by oxidation. Once rendered polar, the fluorescent aldehyde is no longer permeable to the cell membrane and, hence, is trapped within only those cells in the cell mixture that express the intracellular marker. Cells containing the trapped polar, non-permeable fluorescent aldehyde so formed are identified by fluorescence using techniques and equipment well known to those of skill in the art. Cell populations enriched in cells containing the polar fluorescent aldehyde can be obtained using cell sorting techniques, preferably automated fluorescence cell sorting techniques that separate cells containing or having attached thereto a fluorescent marker, such as Fluorescence Activated Cell Sorting (FACS). Such fluorescence cell sorting techniques are well known to those of skill in that art and do not form a part of this invention.

In the preferred embodiment, the fluorescent cell permeable aldehyde is a substrate for aldehyde dehydrogenase (ALDH), and is oxidized by contact with intracellular ALDH to a non-permeable polar fluorescent molecule. When the fluorescent polar molecule is contacted by a light beam having the requisite wavelength to excite the molecule, the fluorescent light emitted as the molecule drops back to its ground state is detected, thereby indicating the presence of a cell or cell population containing intracellular ALDH. Most preferably, the fluorescent aldehyde is the novel compound, dansylaminoacetaldehyde (DAAA), a substrate for aldehyde dehydrogenase, or analogs thereof. Dansyl fluorescence is excited at both 351.1 nm and 363.8 nm and is detected at about 521 nm. A description of the synthesis of this novel compound is found in Example 2 of this application. Alternatively, the method of this invention can be used to detect intracellular phosphokinases. For example, 2-deoxy-2-dansylaminoglucose (2, 2-DDG) should be a fluorescent substrate for hexokinase (a phosphokinase). Phosphorylation of this compound by hexokinase will produce

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phosphorylated glucose (dansylaminoglucose-6-phosphate) which will be trapped in the cell. Thus, 2, 2-DDG would represent a reagent to detect the presence of hexokinase in cells.

- 5 Fluorescent cell permeable and non-polar derivatives of DAAA have also been synthesized and are contemplated within the scope of this invention. For instance, dansyl aminoacetaldehyde diethyl acetal is a non-polar DAAA precursor that can be converted to DAAA by exposure to acid before being exposed to cells. Alternatively, fluorescent derivatives of naturally occurring aldehydes can also be
- 10 used in the practice of this invention. For instance, a fluorescent derivative of retinaldehyde can be synthesized and used to identify cells active in retinoic acid synthesis.

- The general method for producing a non-polar fluorescent aldehyde for use in the practice of this invention, *i.e.*, a substrate for aldehyde dehydrogenase, is by
- 15 reacting a physiologically compatible fluorescent molecule bearing an electrophilic group and a protected aldehyde, such as a methyl or ethyl acetal having a nucleophilic group. For example, the fluorescent electrophile may be a sulfonyl chloride, such as dansyl chloride or Texas Red sulfonyl chloride; an isothiocyanate, such as fluorescein isothiocyanate; an N-hydroxysuccinimide,
- 20 such as N-hydroxy succinimidorhodamine; or a thiol-reactive fluorescent derivative, such as 5-iodoacetamidofluorescein. The protected aldehyde may contain one of a number of nucleophilic groups, such as an amino, hydroxyl, phenolic, or thiol group. The teachings of the art regarding fluorescent compounds are well known.

- 25 In another embodiment, the method for identifying cells containing cytosolic ALDH in intact, viable cells is used as a technique for providing a composition enriched in hematopoietic stem cells, preferably a cell suspension of pluripotent hematopoietic stem cells (pluripotent HSC), that is substantially free of lineage-committed cells. By definition "pluripotent" hematopoietic stem cells are those
- 30 progenitor cells having the ability to repopulate lymphohematopoietic lineages on a long-term basis.

Optionally, a large proportion of differentiated cells may be removed by initially using a "relatively crude" separation. The source of the cells may be the bone marrow, fetal, neonate, or adult or other hematopoietic cell source, *e.g.*, fetal liver or blood. For example, magnetic bead separations may be used initially to remove large numbers of lineage committed cells, namely major cell populations of the hematopoietic systems, including such lineages as T cells, B cells (both pre-B and B cells), myelomonocytic cells, or minor cell populations, such as megakaryocytes, mast cells, eosinophils and basophils. Preferably, at least about 70%, usually at least 80%, of the total hematopoietic cells will be removed. It is not essential to remove every dedicated cell class, particularly the minor population members at the initial stage. Usually, however, the platelets and erythrocytes will be removed prior to fluorescence sorting. Since there will be positive selection in the protocol, the dedicated cells lacking the positively selected marker will be left behind. However, it is preferable that negative selection is done for all of the dedicated cell lineages, so that in the final positive selection, the number of dedicated cells present is minimized.

The methods of this invention have therapeutic utility. For instance, the suspension of HSC and/or pluripotent HSC obtained by the method of this invention can be used for performing hematopoietic reconstitution of a recipient using an enriched pluripotent HSC composition derived from the recipient (autologous reconstitution) or derived from an individual other than the recipient (non-autologous reconstitution) in the treatment or prevention of various diseases or disorders such as anemias, malignancies, autoimmune disorders, and various immune dysfunctions and deficiencies, as well as recipients whose hematopoietic cellular repertoire has been depleted, such as recipients treated with various chemotherapeutic or radiologic agents, or recipients with AIDS. Other therapeutic uses of the compositions of the invention are well known to those of skill in the art.

Since high levels of cytosolic ALDH produce cyclophosphamide-resistance in certain tumor cell lines, in another embodiment the method of the present invention for identifying cells containing cytosolic ALDH can be used to study the phenomenon of cyclophosphamide resistance in tumor cells. Further, the methods of this invention can be used to recover and study subpopulations of

viable cells from a cell mixture, such as a bone marrow aspirate, containing leukemia cells or other malignant cells.

- In yet another embodiment of the invention, the method of providing a
- 5 substantially homogeneous composition of human pluripotent hematopoietic stem cells (pluripotent HSC) can be used to separate the progenitor stem cells useful in a bone marrow transplant from the other healthy cells in a bone marrow aspirate provided by a healthy bone marrow donor. Alternatively, when the donor is the patient, the method of this invention can be used prior to treatment of the patient
- 10 with chemotherapeutic or radiologic agents, to separate the pluripotent HSCs for eventual reintroduction into the patient after therapy has been completed. In the latter case, the tumor cells can be removed from the cell mixture comprising the bone marrow aspirate by tagging the tumor cells with markers that are independent of the intracellular aldehyde-derived fluorescence described, such as
- 15 tumor-specific cell surfaces markers. One skilled in the art will appreciate that alternative methods well known in the art, such as *ex vivo* magnetic cell sorting can also be employed to remove the tumor cells from the cell mixture before subjecting the cell mixture to cell sorting utilizing the intracellular fluorescent aldehyde method of this invention.
- 20 In another aspect, the invention also provides an enriched cellular composition of human lymphohematopoietic stem cells (pluripotent HSC), believed to be the earliest lymphohematopoietic stem cell (pluripotent HSC). The term "substantially homogeneous" as used herein means that the composition contains no more than about 5% of lineage-committed cells. The pluripotent HSC are phenotypically and
- 25 biologically distinct from the pluripotent HSC described by others and characterized by the following attributes: (a) having a small size, generally from about 8 to 10 μm ; (b) expressing levels of ALDH from about 10 to about 30 nanomoles aldehyde oxidized/mg protein/min; (c) being substantially free from expression of markers specific for committed lymphohematopoietic lineages, such
- 30 as CD19, CD33 and CD5; and (d) being negative for expression of c-kit and Thy. Preferably, no more than about 30% of the cells in the enriched cellular composition express CD34, as determined by flow cytometry using anti-CD34 antibody. As a result, the use of CD34, c-kit and Thy as markers for the identification and isolation of the pluripotent HSC cells herein is precluded. This

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analogous cell population in the mouse has been shown to be the pluripotent HSC. 5-10 of these marrow cells (small, lineage-negative, ALDH⁺) will reconstitute all bone marrow-derived lineages of a mouse for the life-time of the mouse. These
5 cells are also capable of extensive self-renewal as shown by their ability to continue to reconstitute all bone marrow-derived cells upon serial bone marrow transplantation.

The substantially homogeneous composition of human lymphohematopoietic stem cells provided herein is capable of self-regeneration in a coculture medium.
10 Exemplary methods for expansion and/or providing for the optimization of human hematopoietic progenitor cell cultures are known in the art, for instance, in U.S. Patent No. 5,399,493, which is incorporated herein by reference in its entirety. As one skilled in the art will also appreciate, the pluripotent HSCs of this invention can be directed to differentiation as a lymphoid, myeloid, or erythroid-lineage
15 population by incubation with the appropriate hematopoietic growth factor or combination of growth factors. Representative growth factors known in the art that can be used for these purposes are IL-1, IL-6, SCF, EPO, and IL-3. Other growth factors for stimulating proliferation of hematopoietic lineages from the stem cell composition of the invention are known to those of skill in the art (see, for
20 example, Harrison's Principles of Internal Medicine, Isselbacher, *et al.*, eds., pp 1714-1717, McGraw Hill, 1994, incorporated herein by reference). As used herein, the term "a suitable hematopoietic growth factor" means one or more hematopoietic growth factors known in the art to direct a progenitor stem cell to the desired lymphoid, granulocytic, monocytic, megakaryocytic, myeloid, or
25 erythroid-lineage cell population.

The human stem cells provided herein find a number of uses, for instance: (1) in regenerating the hematopoietic system of a host deficient in stem cells; (2) in treatment of a host that is diseased and can be treated by removal of bone marrow, isolation of stem cells, and treatment of the host with therapeutic agents
30 such as drugs or irradiation prior to re-engraftment of stem cells; (3) as a progenitor cell population for producing various hematopoietic cells; (4) in detecting and evaluating growth factors relevant to stem cell self-regeneration; and (5) in the development of hematopoietic cell lineages and screening for factors associated with hematopoietic development.

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The enriched cellular composition of human lymphohematopoietic stem cells provided herein can be obtained by isolating cells that express an intracellular enzyme which hydrolyzes a fluorescent non-polar substrate. Preferably, the enzyme is ALDH and the substrate is DAAA as described in the Examples herein. In a preferred embodiment of the method of the invention, therefore, the cell sorting step is performed using automated cell sorting, such as fluorescence activated cell sorting (FACS), a high speed method of sorting fluorescent cells.

The following examples illustrate the manner in which the invention can be practiced. It is understood, however, that the examples are for the purpose of illustration and the invention is not to be regarded as limited to any of the specific materials or conditions therein.

EXAMPLE 1

Synthesis of N-dansyl aminoacetaldehyde (DAAA).

DAAA diethyl acetal was synthesized by adding triethylamine (28 μ L, 0.2 mmol) and then aminoacetaldehyde diethyl acetal (29 μ L, 0.2 mmol, Sigma Chemical Co.) via syringe to a solution of dansyl chloride (54 mg, 0.2 mmol) in acetone (1 mL) and under nitrogen. After stirring overnight, the reaction mixture was dissolved in water (2 mL) and the solution pH was adjusted (1 M NaOH) to 6-8 using pH paper. The solution was then extracted with ethyl acetate (3x5 mL) and the organic layers were stirred for 10 min with $MgSO_4$ and decolorizing carbon. The dried, decolorized extracts were concentrated on a rotary evaporator and the resultant oil was chromatographed on silica gel (J. T. Baker Chemicals, 60-200 mesh, 7 mm x 6 cm column, ethyl acetate eluant). The product was obtained as an oil [53 mg, 0.14 mmol, 70% yield, R_f 0.86 (ethyl acetate)] which was crystallized from ether/hexane to afford a pale green, microcrystalline solid (m.p. 64.5-66.5 $^{\circ}C$). In some repeat procedures, the product was somewhat resistant to crystallization (greater concentrations of impurities still present); however, further purification on silica gel using ether/hexane at a 1:1 ratio, (R_f 0.420) provided a product which would crystallize, with a typical final yield ca. 30%. Elemental analysis of DAAA diethylacetal: [found (theory)]: C, 58.90 (58.97); H, 7.29 (7.15); and N, 7.55 (7.64). Molar extinction coefficient (acetone) = 4480 at 335 nm. 1H NMR (500 MHz, $CDCl_3$) δ : 8.55 (d, J = 8.4 Hz, 1H, aromatic), 8.27 (d, J = 8.5 Hz,

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1H, aromatic), 8.25 (d, $J = 6.8$ Hz, 1H, aromatic), 7.58 (t, $J = 8.2$, 1H, aromatic), 7.53 (t, $J = 7.9$, 1H, aromatic), 7.19 (d, $J = 7.5$ Hz, 1H, aromatic), 4.92 (br t, $J = 5.6$ Hz, 1H, NH), 4.22 (t, $J = 5.6$ Hz, 1H, NCH_2CHO), 3.55 - 3.45 and 3.30 - 3.20 (m, 4H, two CH_2CH_3), 2.99 (d, $J = 6.0$ Hz, 2H, NCH_2CH), 2.89 (s, 6H, two NCH_3), and 1.06 (t, $J = 7.1$ Hz, 6H, two CH_2CH_3). DAAA diethyl acetal was dissolved in acetone and aliquots were transferred to a test tube and concentrated under a stream of nitrogen. The tubes were then capped and stored at 5°C. DAAA diethyl acetal (5 μmoles) was activated to DAAA by incubation with 0.1 ml 1N HCl at 4°C for 12 hours. DAAA solution was neutralized with 0.9 ml of 0.133N NaOH and 0.056M HEPES.

EXAMPLE 2

Flow cytometry with DAAA.

In an attempt to examine cytosolic ALDH on a single-cell basis, leukemia cell lines and bone marrow cells were incubated with the fluorescent substrate DAAA, washed, and subjected to flow cytometry. The cell lines and plastic nonadherent mononuclear marrow cells were incubated with 50 μM DAAA at 10^6 cells/ml for 30 minutes at 37°C. After washing twice, some mononuclear marrow cells were treated with fluorescent monoclonal antibodies (Becton-Dickinson, Mountain View, CA), as previously described (M.B. Kastan et al., *Blood* 74:1517, 1989) to study cytosolic ALDH activity of CD34^+ , CD19^+ , and CD5^+ marrow cells. The cells were sorted on a Epics 753 Dual Laser Flow Cytometer (Coulter Electronics, Hialeah, FL) equipped with two 90-5 water cooled argon lasers (Coherent, Palo Alto, CA). Dansyl fluorescence was excited at both 351.1 nm and 363.8 nm using a multiline ultraviolet filter, and was detected at 521 nm. Cytosolic ALDH activity was estimated by the corrected mean fluorescence intensity (MFI) (M.B. Kastan et al., *Blood* 75:1947, 1990; Kastan, 1989, *supra*) of dansyl staining, which equals the mean fluorescence of unstained cells subtracted from the mean fluorescence of cells treated with DAAA. The mean fluorescence level represents the average channel value for a given sample on the flow cytometer. To confirm that cellular dansyl fluorescence was the result of the activity of cytosolic ALDH, L1210/CPA cells were incubated with 50 μM 4-(diethylamino) benzaldehyde (DEAB) for 15 minutes at 37°C prior to the DAAA incubations to inhibit cytosolic ALDH activity. DEAB is a specific, competitive inhibitor of cytosolic ALDH that is nontoxic to cells

in vitro and *in vivo*. (J.E. Russo *et al.*, *Biochem Pharmacol* 37:1639, 1988)

L1210/CPA cells were also incubated with 50 μ M dansyl glycine (DG) alone (Sigma, St. Louis, MO) at 10^6 cells/ml for 30 minutes at 37°C.

- 5 The histogram analysis of the four human leukemia cell lines treated with DAAA is shown in Figure 2A. This assay is based on the diffusion of hydrophobic DAAA into cells, where subsequent oxidation by ALDH generates dansyl glycine, as shown in Figure 3. Dansyl glycine contains a carboxylate group that is charged at physiological pH and therefore cannot cross plasma membranes. As shown in
- 10 Figure 2B, L1210/CPA cells incubated with dansyl glycine rather than DAAA exhibited no detectable fluorescence. As shown in Figure 4, CD34⁺ progenitors also expressed relatively high levels of cytosolic ALDH by DAAA, while lymphocytes, both B (CD19⁺) and T lineage (CD5⁺), expressed low levels.

- The levels of cytosolic ALDH activity in leukemic cells lines and normal bone
- 15 marrow cell populations determined by DAAA mirrored the results obtained using cytosolic ALDHh antibodies and flow cytometry (Kastan, 1990, *supra*). These results indicate that cytosolic ALDH is the enzyme responsible for DAAA oxidation.

- DAAA treatment did not appear to harm cells; human bone marrow cells, treated
- 20 with DAAA and processed by flow cytometry (but not sorted), maintained normal progenitor growth as shown in Table 1 below.

TABLE 1

Growth of bone marrow hematopoietic progenitors
isolated by DAAA and flow cytometry

5		<u>Colonies/10⁵ cells</u>
	Human CFU-GM	
	Unstained MNMC	163 ± 5
	Stained MNMC*	155 ± 5
	Dansyl ⁺ MNMC	216 ± 13
10	Dansyl ⁻ MNMC	67 ± 20
	Mouse CFU-GM	
	Dansyl ⁺	800 ± 185
	Dansyl ⁻	250 ± 60
	Mouse CFU-S (day 8)	
15	Dansyl ⁺	23.5 ± 1
	Dansyl ⁻	7 ± 1.3

MNMC - mononuclear marrow cells

* Incubated with DAAA and processed (but not sorted) by flow cytometry

+ 5% of cells with the highest fluorescence

20 - 5% of cells with the lowest fluorescence

The data in Table 1 show the results of flow cytometry sorting of human and mouse marrow cells based on their fluorescence intensity after incubation with DAAA. The assays of progenitor content is also shown. Isolation of bone marrow cells with high ALDH expression by fluorescence sorting based on DAAA was enriched for hematopoietic progenitors capable of both *in vitro* and *in vivo* growth. The 5% of human bone marrow cells with highest dansyl fluorescence produced substantially more CFU-GM than the 5% of cells with lowest dansyl fluorescence (p=0.007, T-test). Similarly, using DAAA-derived fluorescence, it was possible to

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obtain a composition that is enriched for CFU-GM ($p=0.03$) and the more primitive CFU-S ($p=0.01$) from mouse bone marrow.

5 The foregoing description of the invention is exemplary for purposes of illustration and explanation. It should be understood that various modifications can be made without departing from the spirit and scope of the invention. Accordingly, the following are intended to be interpreted to embrace all such modifications.

EXAMPLE 3

IN VIVO STEM CELL RECONSTITUTION OF 10 HEMATOPOIETIC REPERTOIRE

The unique murine LHSC, that represents about 0.02 - 0.05% (i.e., 1/2000 - 1/5000) of mouse bone marrow cells, was isolated. In order to distinguish engraftment due to the transplanted LHSC from engraftment resulting from residual host stem cells, male bone marrow was used as a source of LHSC and female
15 mice were used as the recipients. Small bone marrow cells (about 8-10 μ m in diameter) were isolated from bone marrow removed from male mice by counterflow centrifugal elutriation (flow rate 25 ml/min, 3000 rpm-1260 g) or flow cytometry. Cells expressing lineage-specific markers are excluded by fluorescence activated cell sorting (FACS) using a combination of antibodies that
20 recognize these cell surface markers. These cell surface markers included, but were not restricted to the following: CD5 (T-cells), B220 (B-cells), Gr-1 (granulocytes), Mac-1 (monocyte/macrophages), Ter-119 (erythroid). Finally, the small cells lacking these lineage-specific markers and expressing ALDH by DAAA using the technique described above in Example 2 were collected. Ten purified
25 LHSC, along with 20,000 female bone marrow cells that were sorted by counterflow centrifugal elutriation to only be able to provide a transient, early wave of engraftment (Jones, *Nature*, 347:188, 1990), were injected into lethally irradiated (1100 cGy) "syngeneic" female mice via their tail veins. Peripheral blood or bone marrow was removed from the recipients at various times after
30 transplantation (usually 3, 6, 12, and 18 months after transplantation) and the various lymphohematopoietic lineages were assayed for the Y chromosome (i.e., originating from the transplanted, isolated male LHSC) using a Y chromosome-

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specific probe and fluorescence *in situ* hybridization. Bone marrow was isolated from some mice surviving 6-12 months and retransplanted into secondary female recipients as described above. Engraftment in these secondarily transplanted mice was also studied as described above.

The 10 purified male LHSC cells repopulated all lymphohematopoietic lineages of the lethally irradiated female mice for their entire lifetime. In addition, LHSC self-renew as demonstrated by their ability to reconstitute lethally irradiated mice upon secondary serial transplantation. The LHSC are slowly proliferating; they will not form assayable progenitors (CFU-GM, day 8 CFU-S, or day 12 CFU-S) upon primary culture or radio protect (produce early engraftment that will rescue mice from lethal marrow aplasia after total body irradiation). Initial engraftment (occurring in the first month after transplantation) originated from the 20,000 accessory female bone marrow cells capable of only transient, early engraftment.

An early wave of engraftment can also be provided by incubating the purified LHSC in a variety of known growth factors *in vivo* for 7-14 days before transplantation. Later engraftment (3 months and beyond) occurred primarily from the 10 isolated male LHSC. The LHSC did not express high levels of other presumed stem cell surface markers (c-kit, Sca-1, Thy-1, AA4.1).

What is claimed is:

1. An enriched cellular composition comprising human pluripotent hematopoietic stem cells (pluripotent HSCs), wherein the cells of the composition are characterized by:
 - a) having a size from about 8 to 10 μm ;
 - b) expressing levels of aldehyde dehydrogenase from dansyl aminoacetaldehyde from about 10 to about 30 nanomoles aldehyde oxidized/mg protein/min
 - c) being substantially free from expression of markers specific for committed lymphohematopoietic cells; and
 - d) being negative for expression of c-kit and Thy.
2. The cellular composition of claim 1, wherein no more than about 70% of the pluripotent HSC are CD34 positive and wherein the cells are capable of self-regeneration in a coculture medium and of differentiation to members of the lymphoid hematopoietic lineage.
3. The cellular composition of claim 1, wherein the composition is a cell suspension substantially free of lineage-committed cells.
4. The cellular composition of claim 3, wherein the composition contains fewer than 5% of lineage-committed cells.
5. The cellular composition of claim 1 prepared by preparing a composition enriched in cells that express an intracellular enzyme comprising contacting a cell mixture with a cell-permeable, fluorescent non-polar substrate for the enzyme, wherein the activation of the substrate by the enzyme yields a polar substrate identifying cells in the cell mixture that emit fluorescence derived by activation of the substrate by the enzyme, and isolating the identified cells by fluorescent cell sorting.
6. A method for identifying cells in a cell mixture that express an intracellular marker comprising:

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- a) contacting a cell mixture with a cell-permeable, fluorescent non-polar product that reacts upon contact with the intracellular marker so as to form a biocompatible fluorescent product that is polar and non-permeable to the cell membrane, and
 - b) identifying cells in the cell mixture that contain the polar fluorescent product.
7. The method of claim 6, wherein the marker is aldehyde dehydrogenase (ALDH), the fluorescent non-polar aldehyde is a substrate for ALDH, and the identifying is by detection of fluorescence.
8. The method of claim 6, wherein the marker is a phosphokinase.
9. The method of claim 6, wherein the product is an aldehyde.
10. The method of claim 7, wherein the non-polar fluorescent aldehyde is a fluorescent derivative of retinaldehyde.
11. The method of claim 6, wherein the fluorescent non-polar product is the product of the reaction between a fluorescent molecule bearing an electrophilic group and a protected aldehyde containing a nucleophilic group.
12. The method of claim 11, wherein the fluorescent molecule is selected from the group consisting of a sulfonyl chloride, an isothiocyanate, an N-hydroxysuccinimide, or a thiol-reactive fluorescent derivative.
13. The method of claim 11, wherein the protected aldehyde contains a nucleophilic group selected from the group consisting of amino, hydroxyl, phenolic and thiol.
14. A method for identifying cells containing cytosolic aldehyde dehydrogenase (ALDH), the method comprising contacting a cell mixture with a diagnostic amount of a cell-permeable fluorescent substrate for

cytosolic ALDH and identifying cells in the cell mixture that emit fluorescence derived by activation of the substrate by the cytosolic ALDH.

15. The method of claim 14, wherein the cell-permeable fluorescent substrate is a dansylaldehyde having an aliphatic or aromatic aldehyde moiety.
16. The method of claim 14, wherein the fluorescent substrate is dansyl aminoacetaldehyde (DAAA).
17. The method of claim 14, wherein the cells identified are hematopoietic stem cells (HSC).
18. The method of claim 17, wherein the HSC are from about 8 μm to 10 μm , express aldehyde dehydrogenase from dansyl aminoacetaldehyde from about 10 to about 30 nanomoles aldehyde oxidized/mg protein/min, are substantially free from expression of markers specific for committed lymphohematopoietic cells, and are negative for expression of c-kit and Thy.
19. The method of claim 14, wherein the cells identified are lymphohematopoietic stem cells (pluripotent HSC).
20. The method of claim 14, wherein the cell mixture comprises bone marrow extract.
21. The method of claim 14, wherein the bone marrow extract is derived from a healthy donor.
22. A method for preparing a composition enriched in cells that express an intracellular enzyme comprising contacting a cell mixture with a cell-permeable, fluorescent non-polar substrate for the enzyme, wherein the activation of the substrate by the enzyme renders the substrate polar, identifying cells in the cell mixture that emit fluorescence derived by activation of the substrate by the enzyme, and isolating the identified cells by fluorescent cell sorting.

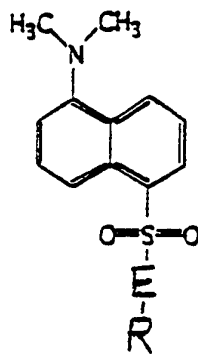
-20-

23. The method of claim 22, wherein the enzyme is aldehyde dehydrogenase.
24. The method of claim 22, wherein the enzyme is a phosphokinase.
25. The method of claim 22, wherein the fluorescent non-polar substrate is the product of the reaction between a fluorescent molecule bearing an electrophilic group and a protected aldehyde containing a nucleophilic group.
26. The method of claim 25, wherein the fluorescent molecule is selected from the group consisting of a sulfonyl chloride, an isothiocyanate, an N-hydroxysuccinimide, or a thiol-reactive fluorescent derivative.
27. The method of claim 25, wherein the protected aldehyde contains a nucleophilic group selected from the group consisting of amino, hydroxyl, phenolic and thiol.
28. A method for preparing a composition enriched in viable cells containing cytosolic aldehyde dehydrogenase, the method comprising contacting a cell mixture with a diagnostic amount of a cell-permeable fluorescent non-polar substrate for cytosolic aldehyde dehydrogenase (ALDH), identifying cells in the cell mixture that emit fluorescence derived by activation of the substrate by the ALDH, and isolating the identified cells by fluorescence of the cell.
29. The method of claim 28, wherein the fluorescence is dansyl fluorescence.
30. The method of claim 28, wherein the cell-permeable fluorescent substrate is dansyl aminoacetaldehyde (DAAA), or a fluorescent derivative thereof.
31. The method of claim 28, wherein the enriched cells are hematopoietic stem cells.
32. The method of claim 28, wherein the fluorescence of the cell is detected by fluorescent cell sorting.

33. The method of claim 32, wherein the fluorescent cell sorting is by cell cytometry.
34. The method of claim 28, wherein the cell mixture comprises bone marrow extract.
35. The method of claim 34, wherein the bone marrow extract is non-autologous.
36. A method for *in vitro* propagation of a lineage-differentiated hematopoietic cell line comprising culturing a pluripotent hematopoietic cell line of claim 1 with a suitable hematopoietic growth factor.
37. The method of claim 36, wherein the growth factor is selected from the group consisting of IL-1, IL-6, SCF, EPO, and IL-3.
38. A method of treating a subject in need of pluripotent hematopoietic stem cell (HSC) therapy which comprises administering to the subject a therapeutically effective amount of the enriched cellular composition of claim 1.
39. The method of claim 38, wherein the enriched cellular composition is autologous.
40. The method of claim 38, wherein the enriched cellular composition is non-autologous.
41. The method of claim 38, wherein the subject is human.
42. The method of claim 38, wherein the enriched cellular composition is human.

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43. A chemical compound having the formula:



wherein E is an electrophilic moiety, R is an aldehyde containing 1-6 carbons.

44. The compound of claim 43, wherein E is selected from the group consisting of amino, hydroxyl, phenolic, and thiol moiety.
45. The compound of claim 43, wherein R is methyl or ethyl acetal and E is an amino moiety.
46. The compound of claim 43, wherein the compound is dansylaminoacetaldehyde.

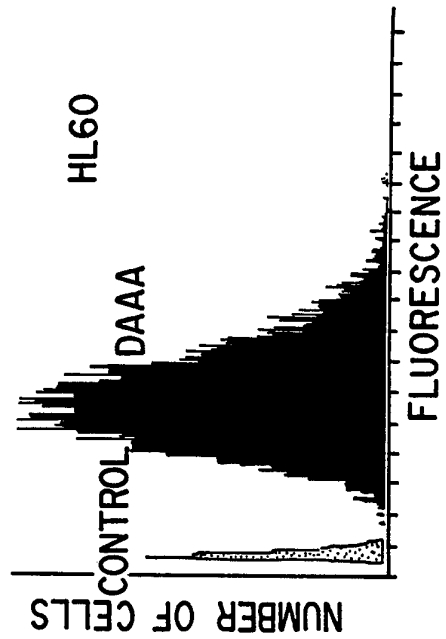


FIG. 1B

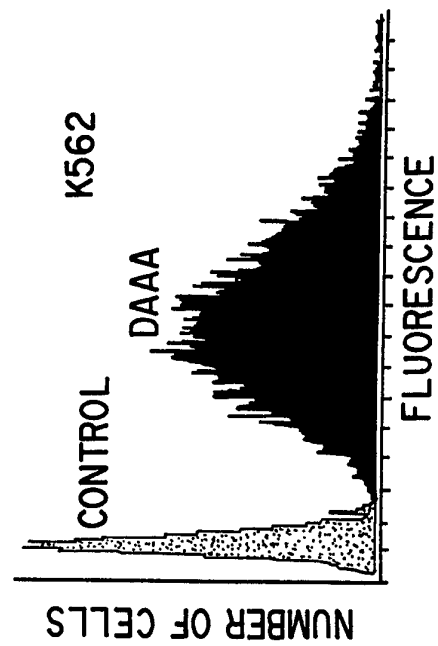


FIG. 1D

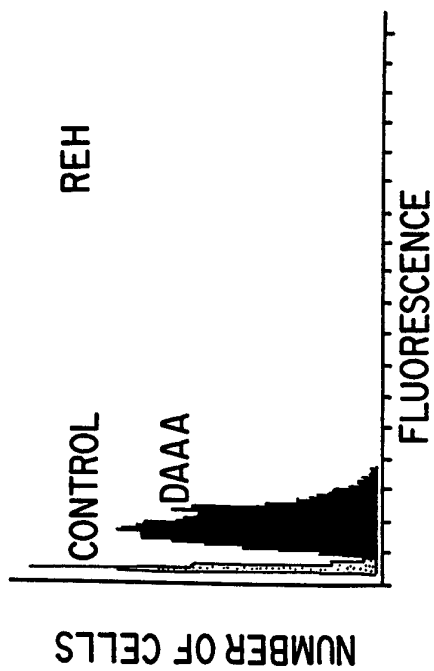


FIG. 1A

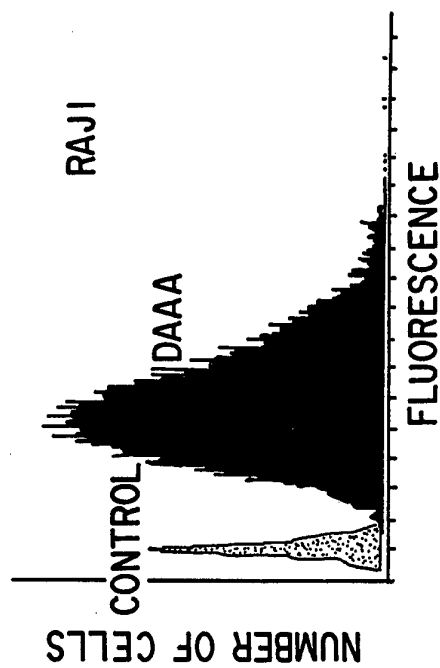


FIG. 1C

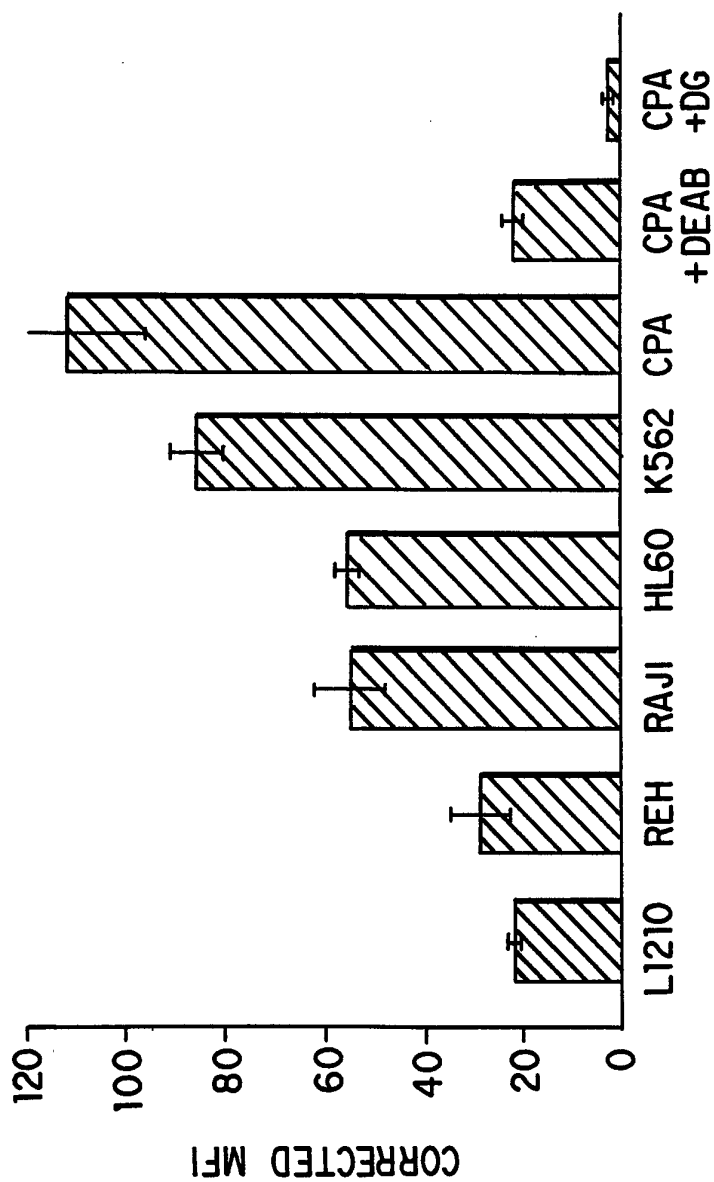
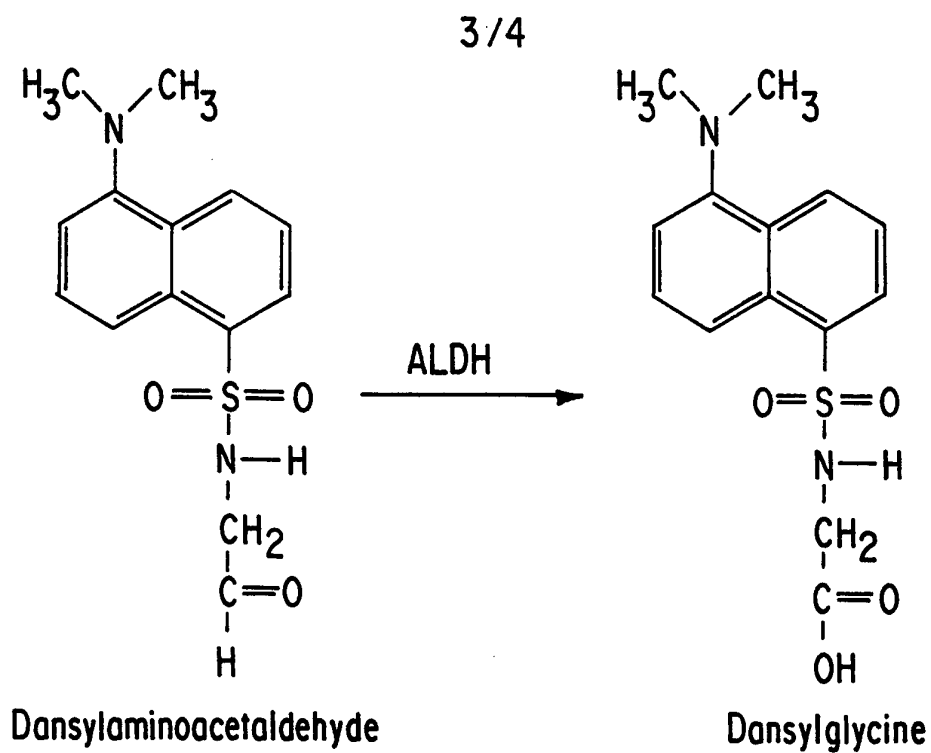
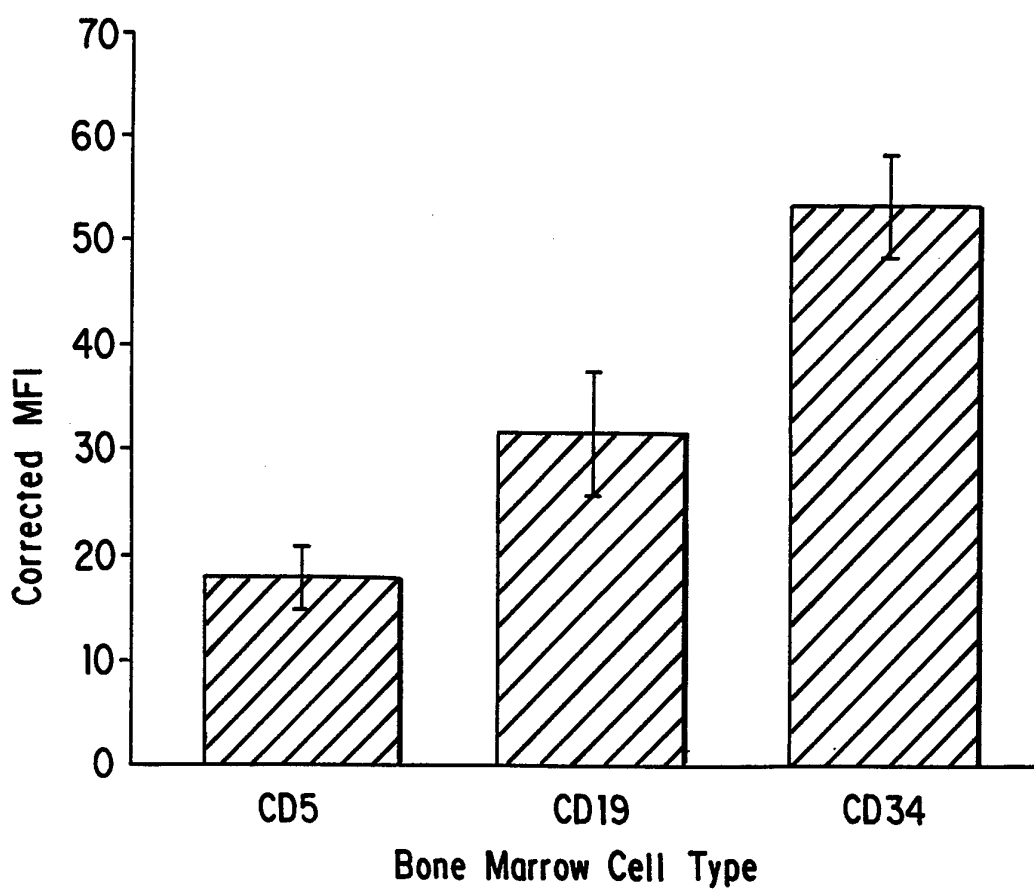


FIG. 1E

**FIG.2****FIG.3**

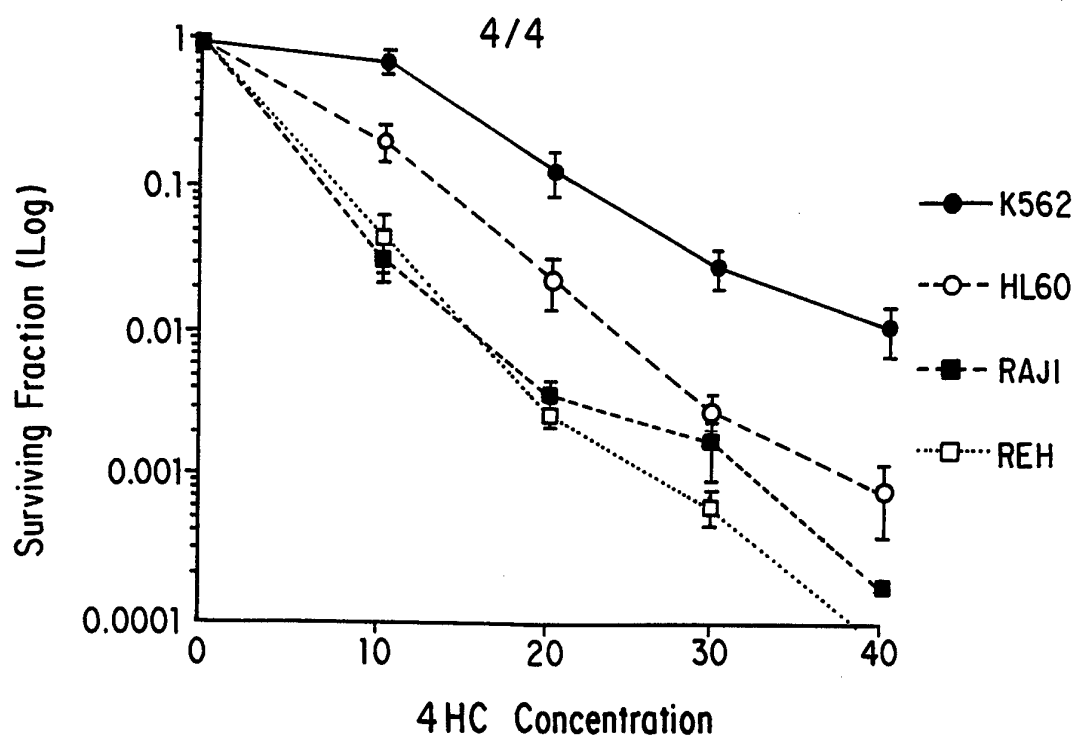


FIG. 4A

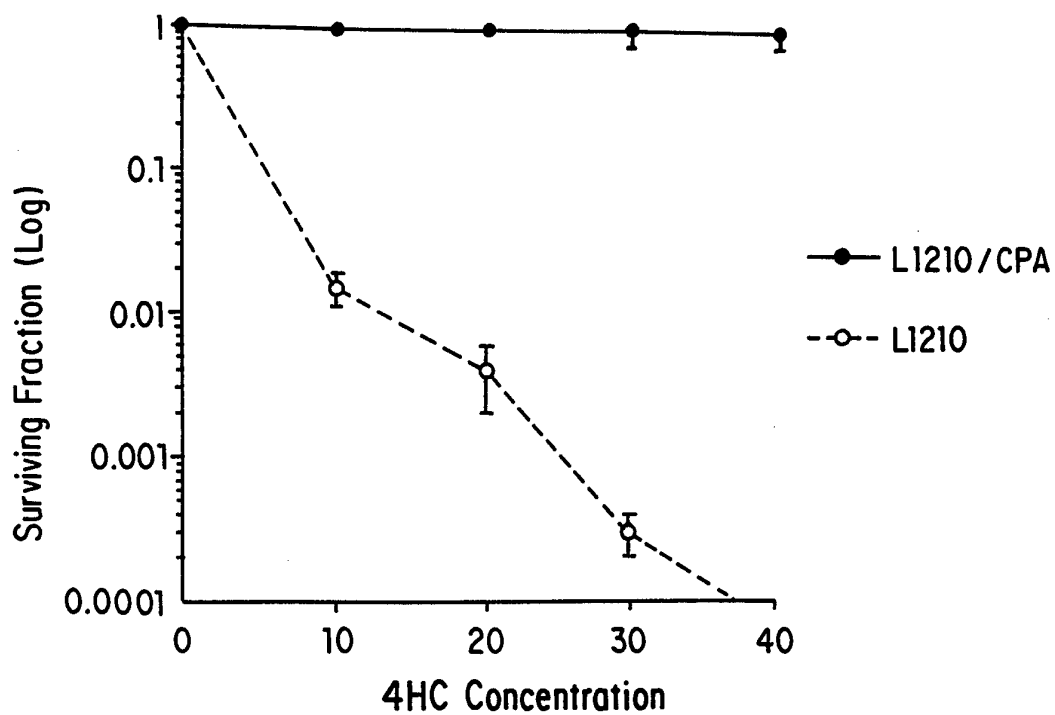


FIG. 4B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/06914

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : 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. : 436/63, 501, 503, 504; 435/7.72, 7.9, 26, 240.2, 240.21; 424/549; 564/441

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, CA, EMBASE, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,061,620 A (TSUKAMOTO ET AL.) 29 October 1991 (29.10.91), see entire patent.	6, 22, 38-42
Y	US 4,714,680 A (CIVIN) 22 December 1987 (22.12.87), see entire patent.	6, 38-42
Y	KASTAN, M.B. et al. Direct Demonstration of Elevated Aldehyde Dehydrogenase in Human Hematopoietic Progenitor Cells. Blood. 15 May 1990, Vol. 75, pages 1947-1950, see entire article.	1-35
Y	HULETT et al. Cell Sorting: Automated Separation of Mammalian Cells as a Function of Intracellular Fluorescence. Science. 07 November 1969, Vol. 166, pages 747-749, see entire article.	5, 6, 14, 22, 32, 33

☒ 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 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

31 JULY 1996

Date of mailing of the international search report

05 SEP 1996

Name and mailing address of the ISA/US
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/06914

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,385,822 A (MELNICOFF ET AL.) 31 January 1995 (31.01.95).	1-35
A, E	US 5,525,461 A (RITTERSHAUS) 11 June 1996 (11.06.96).	1-42
Y	NEBEN et al. Short- and Long-term Repopulation of Lethally Irradiated Mice by Bone Marrow Stem Cells Enriched on the Basis of Light Scatter and Hoechst 33342 Fluorescence. Exp. Hematol. 1991, Vol. 19, pages 958-967, see entire article.	1-6, 14, 19-22, 31, 32, 34-42
Y	SATO et al. Expression and Factor-Dependent Modulation of the Interleukin-3 Receptor Subunits on Human Hematopoietic Cells. Blood. 01 August 1993, Vol. 82, pages 752-761, see entire article.	36-42
Y	US 5,256,560 A (LAWMAN ET AL.) 26 October 1993 (26.10.93), see entire patent.	1-4, 32, 33, 36-42
Y	US 5,087,570 A (WEISSMAN ET AL.) 11 February 1992 (11.02.92), see entire patent.	1-4, 32, 33, 36-42
Y	US 5,132,242 A (CHEUNG) 21 July 1992 (21.07.92), see entire patent.	43-46
Y	BIEDERBICK et al. Monodansylcadaverine (MDC) is a Specific In Vivo Marker for Autophagic Vacuoles. European Journal of Cell Biology. January 1995, Vol. 66, pages 3-14, see entire article.	43-46
Y	PINCUS et al. Dansyl Cadaverine: A Fluorescent Probe and Marker in Cell Membrane Studies. Archives of Biochemistry and Biophysics. 1975, Vol. 169, pages 724-730, see entire patent.	43-46

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/06914

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest



The additional search fees were accompanied by the applicant's protest.



No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/06914

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A61K 35/32; C07C 211/00; G01N 33/48, 33/53, 33/542, 33/566, 33/567; C12Q 1/32; C12N 5/00

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

436/63, 501, 503, 504; 435/7.72, 7.9, 26, 240.2, 240.21; 424/549; 564/441

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-42, drawn to an enriched cellular composition, a method for identifying cells in a cell mixture that express an intracellular marker, a method for preparing a composition enriched in cells that express an intracellular enzyme, a method for identifying cells containing cytosolic aldehyde dehydrogenase (ALDH), a method for preparing a composition enriched in viable cells containing cytosolic aldehyde dehydrogenase, and a method for in vitro propagation of a lineage-differentiated hematopoietic cell line, and a method of treating a subject in need of pluripotent hematopoietic stem cell (HSC) therapy.

Group II, claims 43-46, drawn to a chemical compound.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the chemical compound of Group II is not required to practice the invention of Group I and the methods and composition of Group I are not required to obtain the chemical of Group II. Since the chemical is not related to the methods and the cellular composition of Group I, there is no special technical feature linking the inventions.