P/00/008
Section 29(1)
Regulation 3.1(2)

AUSTRALIA
Patents Act 1990

NOTICE OF ENTITLEMENT

We 1) David SCHEER
2) Bernard M BABIOR

of 1) 51 SAGE HOLLOW ROAD
GUILFORD CT 06437
USA

2) 4295 IBIS STREET
SAN DIEGO CA 92103
USA

being the applicant(s) and nominated person(s) in respect of an application for a patent for an invention entitled STEM CELL AND LYMPHOCYTE STORAGE (Application No. 46519/93), state the following:

1. The nominated person(s) has/have, for the following reasons, gained entitlement from the actual inventor(s):

   THE NOMINATED PERSONS ARE THE ACTUAL INVENTORS.

2. The nominated person(s) has/have, for the following reasons, gained entitlement from the applicant(s) listed in the declaration under Article 8 of the PCT:

   THE APPLICANTS AND NOMINATED PERSONS ARE THE BASIC APPLICANTS.

3. The basic application(s) listed in the declaration under Article 8 of the PCT is/are the first application(s) made in a Convention country in respect of the invention.

DATED: 18 January 1995

David SCHEER
Bernard M BABIOR

GRIFFITH HACK & CO.

Patent Attorney for and
on behalf of the applicant(s)

AU9346519

(12) PATENT ABRIDGMENT (11) Document No. AU-B-46519/93
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 668582

(54) Title
STEM CELL AND LYMPHOCYTE STORAGE

International Patent Classification(s)
(51)[6] **C12N 005/00**
(51)[5] **A01N 001/02**

(21) Application No. : **46519/93** (22) Application Date : **25.06.93**

(87) PCT Publication Number : **WO94/00567**

(30) Priority Data

(31) Number	(32) Date	(33) Country
903858	25.06.92	US UNITED STATES OF AMERICA

(43) Publication Date : **24.01.94**

(44) Publication Date of Accepted Application : **09.05.96**

(71) Applicant(s)
DAVID SCHEER; BERNARD M. BABIOR

(72) Inventor(s)
DAVID SCHEER; BERNARD M. BABIOR

(74) Attorney or Agent
GRIFFITH HACK & CO , GPO Box 1285K, MELBOURNE VIC 3001

(56) Prior Art Documents
US 4,639,373
US 4,923,797

(57) Claim

1. A method of stabilizing cells which are lymphocytes or stem cells obtained from a mammal which comprises providing said cells and suspending said cells in an aqueous mixture comprising gelatin.

OPI DATE 24/01/94 APPLN. ID 46519/93
AOJP DATE 14/04/94 PCT NUMBER PCT/US93/06095

AU9346519

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 11/04, A01N 1/02

A1

(11) International Publication Number: WO 94/00567

(43) International Publication Date: 6 January 1994 (06.01.94)

(21) International Application Number: PCT/US93/06095

(22) International Filing Date: 25 June 1993 (25.06.93)

(30) Priority data:
07/903,858 25 June 1992 (25.06.92) US

(71)(72) Applicants and Inventors: SCHEER, David [US/US]; 51 Sage Hollow Road, Guilford, CT 06437 (US). BABIOR, Bernard, M. [US/US]; 4295 Ibis Street, San Diego, CA 92103 (US).

(74) Agent: FREEMAN, John, W.; Fish & Richardson, 225 Franklin Street, Boston, MA 02110-2804 (US).

(81) Designated States: AU, CA, FI, JP, NO, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: STEM CELL AND LYMPHOCYTE STORAGE

(57) Abstract

Methods of stabilizing lymphocytes and/or stem cells obtained from a mammal by suspending them in an aqueous medium comprising gelatin.

STEM CELL AND LYMPHOCYTE STORAGE

Background of the Invention

This invention relates to storing stem cells and lymphocytes without unacceptable loss of cell function.

Stem cells are undifferentiated cells which are pluripotent -- i.e., they can differentiate into cells which have diverse functional characteristics. They are not fully differentiated, and they retain the ability to duplicate. Of particular interest are lymphohematopoietic stem cells which differentiate into lymphoid and myeloid cells. Lymphohematopoietic stem cells include the following cells, as well as many others: a) cells that are colony-forming cells for granulocytes/monocytes (CFC-GM); b) cells that are colony-forming for erythrocytes (BFU-E); c) colony-forming cells for eosinophiles (CFC-Eo); d) multipotent colony forming cells (CFC-GEMM); e) immature lymphoid precursor cells, including precursors of B-cells and T-cells; and f) pluripotent stem cells. Fig. 1 is a commercially distributed chart depicting the various cells including stem cells involved in hematopoiesis.

Stem cells are essential for transplantation, for example, bone marrow transplantation. Typically bone marrow transplants involve harvesting marrow cells, washing them with a buffer to remove undesired cells and material (e.g., T-cells and/or malignant cells), and suspending in a suitable medium (e.g. RPMI, autologous serum, and DMSO). The resulting material is either immediately infused into the recipient or it is cryopreserved (frozen well below 0°C) for later use. Peripheral blood stem cells are also used for bone marrow transplantation. Stem cells are obtained from peripheral blood by leukapheresis, with similar subsequent processing.

Civin U.S. Patents 4,714,680; 4,965,204; and 5,035,9994 describe a monoclonal antibody specific for an antigen on human pluripotent lymphohematopoietic stem cells, and not for antigens on normal mature human lymphoid and myeloid cells. Tsukamoto et al. U. S. 5,061,620 describe human hematopoietic stem cells, their separation, characterization, and use. Each of those patents is hereby incorporated by reference. These and other rapidly advancing technologies may make bone marrow transplants and other medical procedures available to a far larger patient population than can currently receive this treatment.

The lymphocyte family includes lymphoid stem cells as well as the differentiated cells resulting from them (T-cells, B-cells, and plasma cells). See Fig. 1. In various situations, it is desirable to obtain cell populations enriched in certain lymphocytes and/or depleted in others. For example, it may be desirable to destroy a certain population of malignant or infected lymphocytes. Alternatively, it may be desirable to activate a patient's lymphocytes outside the body (e.g., with IL-2) and then to reinfuse them. In short, various technologies are known for treating and/or engineering stem cells or lymphocytes outside the body, and returning the treated cells to the body. Finally, for patients in remote locations, it may be necessary to ship lymphocytes to remote locations for histocompatibility testing in preparation for a transplant, requiring storage during shipment.

Various technologies are known for segregating or selectively enriching or destroying populations of lymphocytes or stem cells, in addition to the technologies mentioned above. For example, other techniques include the CEPRATE™ stem cell concentrator sold by CellPro, Inc. of Seattle, Wash., panning

techniques, cell sorting (e.g. fluorescent antibody cell sorting (FACS)), and the use of magnetic beads.

There is a particular need for a convenient and effective way to store stem cells and/or lymphocytes after they are obtained (e.g., in the manner described by Tsukamoto et al.) and before they are used.

Summary of the Invention

The invention features methods of stabilizing stem cells or lymphocytes obtained from a mammal (particularly human) by suspending them in an aqueous mixture (or storage composition) comprising gelatin.

In preferred embodiments, the cells are suspended in such a mixture for a period in excess of 24 (most preferably in excess of 36, 48, 60 or even 72) hours, preferably at a temperature between freezing and 25°C. Modified fluid gelatin is preferred, but unmodified gelatin may also be used. Any stem cells or lymphocytes may be stored by this method, but the above-mentioned lymphohematopoietic cells (particularly pluripotent stem cells) are particularly suited for the storage technique. The preferred medium comprises gelatin at a concentration such that the storage composition (including the cells) does not gel below 40°C (e.g. 6-25 percent by weight modified fluid gelatin), and the stem cells are present during storage. Preferably, the mixture comprises a standard cell tissue growth medium. As described below, in preferred applications, the mixture is pharmaceutically acceptable for administration to a human patient. Plasma is optional and the invention may be used with a mixture that has substantially no plasma and may even consist essentially of cell growth medium or buffer, gelatin, and the cells being stored. However, plasma may be advantageous, e.g. optionally, it is possible to practice the invention using a medium that comprises 10-75 percent by weight plasma. The cells

being suspended may be enriched for a desired cell subpopulation, in comparison to the original sample, e.g., of bone marrow or from leukapheresis.

Other features and advantages of the invention will be apparent from the following description of preferred embodiments and from the claims.

Description of the Preferred Embodiment(s)

The following description of preferred embodiments of the invention is intended to illustrate, not to limit, the invention.

I. Stem Cells

Those skilled in the field will understand that there are known methods of obtaining stem cells that can be suitably stored for later use. The stem cells can be obtained either from bone marrow or from peripheral blood. The stem cells can be purified by known techniques, using for example the technique disclosed in the Tsukamoto et al. patet or the Civin patent, each of which is cited above and hereby incorporated by reference. Other such techniques are also cited above. The invention can be used to store mixed stem cell preparations such as unmodified bone marrow or leukapheresis-derived preparations. It may also be used to store preparations derived from unmodified bone marrow or leukapheresis-derived preparations by selective stem cell or other cell enrichment, e.g., using methodology described above, such as the CEPRATE™ system by CellPro, Inc., Seattle Washington, which is an immunoaffinity separation system.

While the above discussion is generally directed to lymphohematopoietic stem cells and particularly to pluripotent stem cells, other types of stem cells can be stored by the method of he invention, including neurostem cells, epithelial stem cells and others. Those skilled

in the art will be able to obtain such cells by known techniques.

II. Lymphocytes

Those skilled in the art are aware of numerous monoclonal antibodies which discriminate between various lymphocytes based on cell surface markers. These antibodies can be used in the various immunologically based cell separation techniques described above to selectively enrich or deplete populations obtained from bone marrow or leukapheresis for specific lymphocytes. Peripheral blood treated in standard ways can be used to recover the mixtures (e.g. buffy coats) from which the lymphocyte subpopulations are purified.

III. Storage mixtures or compositions

The preferred storage mixtures use modified fluid gelatin generally as described by Babior U.S. 4,923,797, which is hereby incorporated by reference. Modified fluid gelatin has been used clinically as a plasma substitute or expander and is widely available under a variety of tradenames, such as PlasmaGel, Haemaccel, Leukogel, Gelofusine, etc. In general, these materials are partly hydrolyzed gelatins which have an average molecular weight from 15,000-40,000 daltons and which form aqueous solutions having a viscosity less than that of a gelatin solution of the same concentration. In some cases they are succinylated, or they are reacted to form e.g. urea linkages, or cross-linked. The commercially available materials usually are in the form of a solution of the modified gelatin in a buffer together with various salts and other ingredients. The term "modified fluid gelatin" as used herein refers to the partly hydrolyzed gelatin component itself whether or not further reacted. In some cases the commercially available solutions can be used, but preferably the partly hydrolyzed gelatin

component is purified by separation from the remaining ingredients of the commercial product.

Preferred mixtures include standard tissue cell culture growth media such as RMPI or Eagle's media. It may also be possible to use conventional buffer solutions that provide isotonicity in the compositions of the present invention;for example, common buffers, e.g., Hank's balanced salt solution. Optionally, it may be possible to add a heterocyclic base to the storage mixture as described by Babior U. S. 4,923,797.

Optionally plasma can be added to the mixture (or it can be retained from the original patient sample). The plasma may be normal human plasma, either autologous or heterologous with the cells being stored, preferably autologous. If plasma is used, the amount of plasma present in the composition can be 10-75 percent by weight based on the total composition exclusive of the cells.

The amount of modified fluid gelatin in the composition may vary considerably, from 4% by weight based on the total compositions, exclusive of the cells being stored, up to the amount which causes the composition to set to a gel at 40°C. The amounts required for optimum results vary depending upon the source of the modified fluid gelatin but can readily be determined in any given case by a simple test.

Optionally, cell-specific growth factors may be added as needed to preserve cell viability, depending on the desired cell type.

The stem cells or lymphocytes can be dispersed in the composition in suitable amounts. During storage of the stem cell or lymphocyte-containing composition, it is preferably maintained at a temperature below 8°C although higher temperatures up to 25°C can be tolerated for relatively short times. For optimum storage life,

storage temperature should be maintained at 4°C or lower, but not below freezing temperate.

After storage the composition containing the stem cells or lymphocytes can be administered after warming without further processing; or if desired the stem cells can be separated from the compositions by washing or centrifuging in order to permit dispersion of the cells in any other desired medium.

It is contemplated that the present invention may be practiced by supplying to blood banks, laboratories or other entities a stock solution suitable for mixing with a suspension of cells to be stored, with directions for mixing the stock solution with the suspension in suitable proportions. Such a stock solution comprises a tissue culture medium or other buffer solution as described above, containing an amount of modified fluid gelatin from about 4-8% by weight up to the amount causing the solution to gel at room temperature, preferably 20 to 40% by weight. Other optional ingredients described above may be included.

An alternate storage media uses gelatin as generally described by Babior U. S. 4,639,373 which is hereby incorporated by reference. In this embodiment, the buffer employed can be any conventional tissue culture medium or non-toxic buffer as described above. For best results, the cells in the gelatin-containing buffer should be stored at low temperature, e.g. below 8°C, although they may be stored at higher temperatures up to about 25°C for at least 12 hours with satisfactory results.

Gelatin from any of the usual commercial sources can be used in the practice of the present invention. The amount of gelatin may vary over a wide range, depending in part on the length of storage desired. There is no critical upper limit on the amount used,

except that it must be low enough so that the composition is a liquid, rather than a gel, at a temperature no higher than 40°C in order to facilitate removal of the gelatin after storage.

If plasma is employed, it can be normal human plasma or autologous plasma. The amount of plasma may vary from 10% by weight of the total composition to as much as 75% by weight.

III. Use of Stored Cells.

After storage, the stem cells can be transfused without further processing except for warming to a temperature no higher than 40°C to liquefy any gel which is present; or the cells can be reconstituted for use simply by washing out the gelatin and plasma (if any) with buffer which can be the same as or different from the buffer used for storage, or by removal of the gelatin-containing buffer by centrifugation followed by resuspending the stem cells in a desired buffer or medium.

What is claimed is:

1. A method of stabilizing cells which are lymphocytes or stem cells obtained from a mammal which comprises providing said cells and suspending said cells in an aqueous mixture comprising gelatin.

2. The method of claim 1 further comprising maintaining said cells in an aqueous mixture comprising gelatin for a period in excess of 24 hours.

3. The method of claim 2 in which said cells are stored in an aqueous mixture comprising gelatin at a temperature between freezing and 25°C for a period in excess of 24 hours.

4. The method of claim 1 in which said mixture comprises modified fluid gelatin.

5. The method of claim 1 in which said mixture comprises a tissue growth medium.

6. The method of claim 1 in which said cells are stem cells.

7. The method of claim 6 in which said cells are pluripotent stem cells.

8. The method of claim 3 in which said mixture comprises plasma.

9. The method of claim 8 in which said mixture comprises 10-75 percent plasma by weight.

10. The method of claim 1 in which said gelatin is present in an amount less than that which causes the mixture (including said cells) to gel at 40° C.

11. The method of claim 1 in which said gelatin is at least 1 percent by weight of the total mixture including said cells.

12. The method of claim 1 in which said mixture is pharmaceutically acceptable for administration to a human patient.

13. The method of claim 1 in which said cells are enriched for a desired cell subpopulation and then suspended in said aqueous mixture.

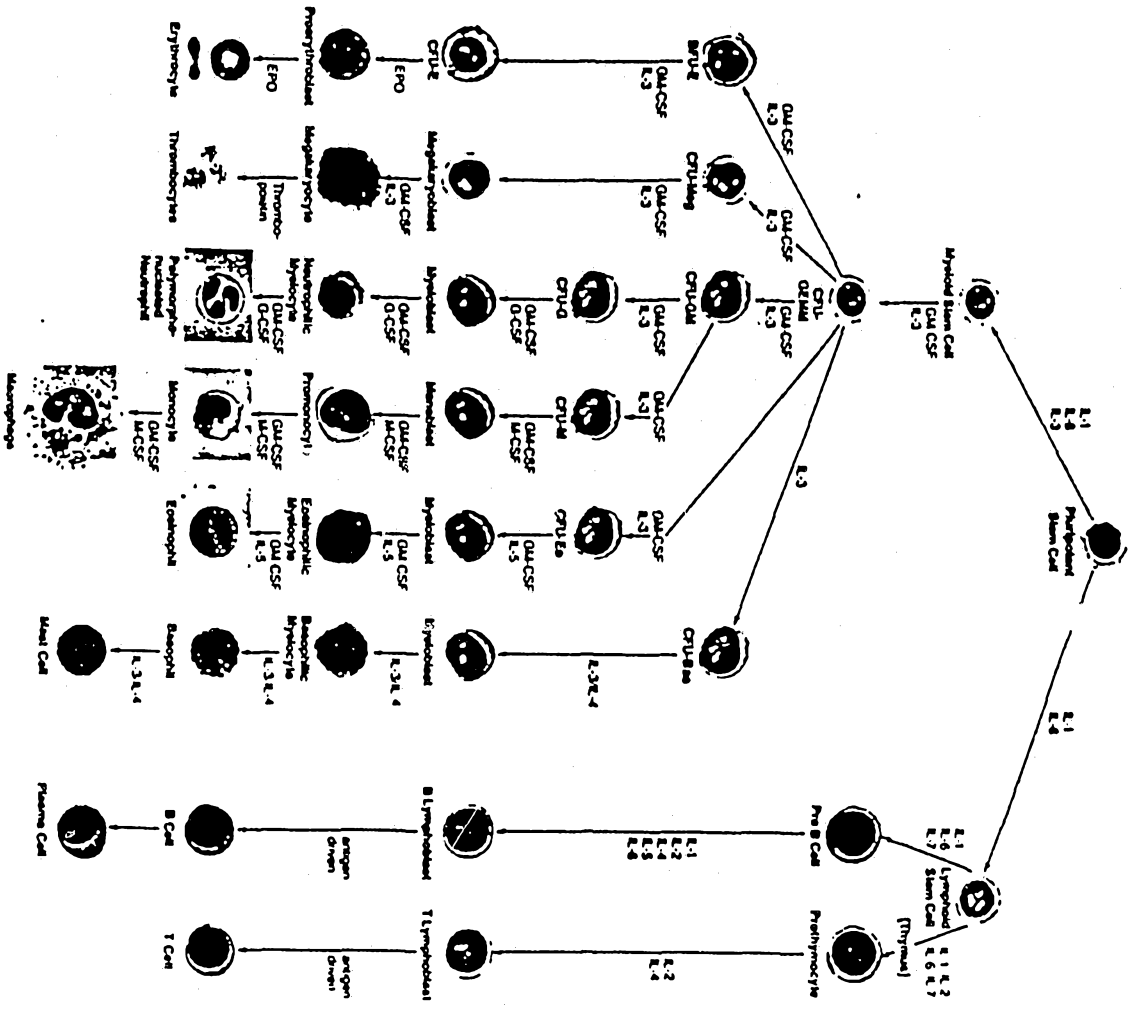
Myeloid Stem Cell
GM-CSF
IL-3
IL-1
IL-6
IL-3
Pluripotent Stem Cell
IL-3
IL-1
IL-4
Lymphoid Stem Cell
IL-1
IL-6
IL-7
IL-1
IL-2
IL-6
IL-7
(Thymus)
BFU-E
CFU-Meg
CFU-GEMM
CFU-GM
CFU-G
CFU-M
CFU-Eo
CFU-Ba
Pro B Cell
Prothymocyte
B Lymphoblast
T Lymphoblast
Proerythroblast
Erythrocyte
EPO
Megakaryoblast
Megakaryocyte
Thrombocytes
Thrombopoietin
Myeloblast
Neutrophilic Myelocyte
Polymorphonuclear Neutrophil
G-CSF
Monoblast
Promonocyte
Monocyte
Macrophage
M-CSF
Eosinophilic Myelocyte
Eosinophil
Basophilic Myelocyte
Basophil
Mast Cell
IL-3,IL-4
Erythroblast
Plasma Cell
B Cell
T Cell
antigen driven
IL-1
IL-2
IL-4
IL-5
IL-6
IL-2
IL-4

Fig. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06095

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 11/04; A01N 1/02
US CL :435/182, 2

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. : 435/182, 2

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)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indicatinn, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,032,508 (Naughton et al) 16 July 1991, see entire document, especially column 2, lines 9-20; column 8, line 57; column 13, lines 52-56; column 14, lines 2-4; column 15, lines 7-16.	1-13
Y	US, A, 4,639,373 (Babior) 27 January 1987, see entire document, especially column 1, lines 6-15 and 64-70; column 2, lines 1-11, 23-34 and 38-41.	1-13
Y	US, A, 4,923,797 (Babior) 08 May 1990, see entire document, especially column 1, lines 4-10 and 40-64.	1-13

☐ 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	Date of mailing of the international search report
04 AUGUST 1993	01 SEP 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer MARIA L. OSOTEO
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

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