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IN VITRO GROWTH OF NEUTROPHIL AND MEGAKARYOCYTE PRECURSORS IN SERUM-FREE MEDIA

Technical Field

The invention relates generally to human hematopoietic cells cultured in serum-free media. More specifically, the invention relates to serum-free cell suspensions enriched for neutrophil and megakaryocyte precursors useful for treating neutropenia and thrombocytopenia.

Background

Cancer treatments such as high-dose chemotherapy and radiation destroy hematopoietic cells in the bone marrow, leaving the patients severely depleted of neutrophils and platelets. After such treatments, patients often spend several weeks in intensive care due to infections and fever resulting from neutropenia. Thrombocytopenia leads to reduced clotting and bleeding disorders requiring platelet transfusions. Lack of neutrophils and platelets is the leading cause of morbidity and mortality following these cancer treatments, and contributes to the high cost of cancer therapy.

Several methods are directed towards restoring the patient's immune system after therapy. Hematopoietic growth factors are administered after therapy to stimulate remaining stem cells to proliferate and differentiate into mature infection fighting cells. Although hematopoietic growth factors can shorten the total period of neutropenia, there remains a critical 10 - 15 day period immediately following therapy when the patient is severely neutropenic and thus infection prone. Even with growth factor stimulation, 10 to 15 days are required for the patient's stem cells to proliferate and progress through the various stages of differentiation leading to mature neutrophils. Megakaryocyte and platelet recovery requires an even longer time, generally greater than 15 days. Thus growth factor treatment leaves a gap during which the

patient is deficient in infection fighting cells and blood clotting ability.

Post-therapy bone marrow transplantation can also ameliorate neutropenia after about 10 - 15 days. Since allogenic bone marrow transplantation is often complicated by Graft versus Host Disease, autologous bone marrow transplantation is preferred whenever practical. Bone marrow is harvested from the patient prior to therapy, frozen, and then thawed and transplanted back into the patient after therapy. Autologous bone marrow transplantation carries the risk that the transplanted bone marrow may harbor tumor cells. In any event, bone marrow does not contain sufficient mature neutrophils or their immediate precursors to restore the patient's immunity during the critical 10 -15 day period after therapy.

A phenomenon known as "mobilization" has also been exploited to harvest greater numbers of stem/progenitor cells from peripheral blood to treat neutropenia. Mobilization occurs as a result of either chemotherapy, or administration of hematopoietic growth factors, or both. It is believed that hematopoietic stem cells in the bone marrow are "mobilized" into the peripheral blood stream as a natural result of the recovery of myelosuppressed bone marrow or in response to relatively large doses of hematopoietic growth factors. Growth factors used for mobilization include interleukin-3 (IL-3), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), stem-cell factor (SCF) and a recombinant fusion protein having the active moieties of both IL-3 and GM-CSF (Brandt, SJ, et al., N Eng J Med 318:169, 1988; Crawford, J, et al., N Eng J Med 325:164, 1991; Neidhart, J, et al., J Clin Oncol 7:1685, 1989). Mobilized peripheral blood stem cells are harvested after chemotherapy or growth factor treatment, and then reinfused into the patient following the next round of high

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dose chemotherapy or radiation. The reinfused stem cells then proliferate and differentiate in vivo, eventually to restore the patient's neutrophil and platelet population.

Combinations of the above approaches have succeeded in reducing the neutropenic period to about 9 days.

In order to obtain cells to treat patients during the early neutropenic period, differentiated hematopoietic cells were generated in vitro in the laboratory of the present inventors (Smith, S.L., et al., Experimental Hematology 21:870-877, 1993). Committed precursors of neutrophils were successfully generated in vitro using fetal bovine and horse serum-containing media. However, the potential for therapeutic use of the cells would be greatly enhanced if the cells were grown without animal sera or animal proteins in the culture medium.

Traditionally, animal serum supplementation was relied upon as a source for protein and growth factors in culture media formulations. Researchers have long sought media formulations free of animal proteins for the growth of therapeutic cells because of the potential for life-threatening immune reactions raised by infusion of foreign proteins. However, animal sera, and in particular fetal bovine sera, contain many unknown growth factors, certain factors being more or less important for each cell type. Even if every factor in fetal bovine serum were identified and chemically synthesized, it would still be a matter of trial and error and much experimentation to discover which factors promote the proliferation and differentiation of each cell type. In spite of the difficulty, researchers have succeeded in formulating serum-free media in which certain hematopoietic cells may be grown.

Serum-free media formulations containing bovine serum albumin and various hematopoietic growth factors were shown to promote the proliferation of murine bone marrow cells (Ponting,

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I.L.O., et al., Growth Factors 4:165-173, 1991; Merchauv, S., et al., Internatl J Cell Cloning 2:356-367, 1984;).

Thirteen different combinations of serum-free media proved disappointing as replacements for serum containing medium for the in vitro culture of human hematopoietic progenitors (Wu, Z-H., et al., Pathology 22:145-148, 1990). The effects of serum-free medium on the growth of leukemic cells were reported (Da, W.M., et al., Brit J Haematology 78:42-47, 1991). The growth of erythropoietic cells in serum-free medium was also reported (Lansdorp, P.M., et al., J Exp Med 175:1501-1509, 1992). All of the above serum-free formulations contained animal protein in the form of bovine serum albumin.

There remains a need for a serum-free, animal protein-free medium formulation which supports the growth and differentiation of human neutrophil and megakaryocyte precursors. The resulting cell suspension would then be suitable for infusion into a patient to restore infection fighting cells and thrombocytes during the critical period immediately following cancer therapy.

Summary of the Invention

The invention provides serum-free, animal protein-free media formulations to be used in conjunction with hematopoietic growth factors for the in vitro growth of human neutrophil and megakaryocyte precursors. The medium is comprised of a base medium, corticosteroid, transferrin, insulin, cholesterol, ethanolamine, and human albumin.

The invention also provides methods for preparing serum-free, animal protein-free suspensions of human hematopoietic precursor cells wherein the cellular component comprises at least about 16% neutrophil precursors and at least about 1% megakaryocyte precursors.

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Serum-free, animal protein-free suspensions of human hematopoietic cells are provided wherein the cellular component comprises at least about 30%, preferably greater than 60% neutrophil precursors. The neutrophil precursors are comprised of blast cells, promyelocytes, neutrophilic myelocytes, and neutrophilic metamyelocytes.

Also provided are serum-free, animal protein-free cell suspensions wherein the cellular component comprises at least about 3%, preferably greater than 8% megakaryocyte precursors.

Also provided are serum-free, animal-protein free cell suspensions wherein the cellular component comprises colony-forming units and cluster-forming units.

Brief Description of the Figures

Figure 1 depicts the differential cell count of cultures grown in serum-free medium (295-1), in serum-containing medium (HLTM), and cultures which were grown for 10 days in 295-1 and then transferred to HLTM for an additional 10 days.

Figure 2 shows blast cells (solid arrows) in a 10 day serum-free culture.

Figure 3 shows promyelocytes (solid arrows) and a neutrophilic metamyelocyte (hollow arrow) in a 10-day serum-free culture.

Figure 4 shows neutrophilic myelocytes (solid arrows) and neutrophilic metamyelocytes (hollow arrows) in a 10 day serum-free culture.

Figure 5 shows the cell phenotypes as determined by flow cytometry.

Figure 6 shows a typical field of colonies (solid arrows) and clusters (hollow arrow).

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Figure 7 shows megakaryocyte precursors labeled by immunocytochemistry (red) in a field of myelocytic cells (blue).

Figure 8 shows forward light scatter of the cells in flow cytometry; (a) control (b) anti-CD41a.

Figure 9 shows a typical megakaryocyte burst developed in a fibrin clot assay.

Figure 10 shows the kinetics of megakaryocyte cell growth in serum-free cultures compared to normal media.

Figure 11 shows the effects of serum-free culture media compared to normal media or megakaryocyte growth from isolated $CD34^+$ cultures.

Figure 12 shows the growth comparisons of megakaryocytes in different cultures.

Figure 13 shows the effects of various cytokine combinations on megakaryocyte growth.

Detailed Description of the Invention

The invention provides serum-free media formulations for the *in vitro* growth of human neutrophil and megakaryocyte precursors. The resulting serum-free, animal protein-free cell suspensions are suitable for infusion into a patient for the treatment of neutropenia and thrombocytopenia. The absence of animal proteins in the cell suspensions is especially advantageous since animal proteins are known to cause immune reactions in humans. Moreover, a high proportion of the cells in the suspensions are at suitably advanced stages of differentiation so that soon after infusion they are expected to differentiate within the patient to form mature neutrophils, megakaryocytes and platelets.

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Essential reagents in the serum-free media formulations are the base medium, corticosteroid, transferrin, insulin, cholesterol, ethanolamine, and human albumin.

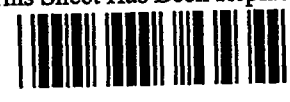
The base medium may be any standard cell culture medium containing inorganic salts, vitamins, amino acids, and at least one energy source such as glucose or pyruvate. Preferably, the base medium contains a bicarbonate buffer and a pH indicator such as phenol red. The pH of the final medium is maintained at 6.8 - 7.4, preferably at about 7.2, by means of a controlled O_2/CO_2 gas atmosphere. The osmolarity of the basal medium is preferably in the range of 260-290 mOsm/Kg.

Hematopoietic growth factors are also necessary. Growth factors are selected from the following: interleukin-3 (IL-3), granulocyte-colony stimulating factor (G-CSF), granulocyte/macrophage-colony stimulating factor (GM-CSF), interleukin-6 (IL-6), interleukin-11 (IL-11), stem cell factor (SCF), interleukin-1 (IL-1), thrombopoietin and gamma interferon. Whole growth factors may be replaced by chimeric or fusion proteins having the active moieties of one or more factors. For instance, in place of IL-3 and GM-CSF, a single fusion protein having the active moieties of both IL-3 and GM-CSF may be used.

The growth factors are produced recombinantly in microorganisms or mammalian single-cell hosts, and have amino acid sequences either identical to or highly homologous with the active moieties of corresponding human growth factors. The factor is highly purified from any host proteins associated with it such that, when added to the herein provided media formulations, no host protein is detectable.

Given the disclosure herein, it will be apparent to one of skill in the art that growth factor combinations and concentrations may be manipulated to yield various results.

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For example, G-CSF and GM-CSF will enhance the production of neutrophils whereas thrombopoietin will enhance the production of megakaryocytes. The serum free media formulations provided herein are expected to further enable the identification of specific growth factor functions due to the absence of confounding factors from serum.

Herein, the term "serum-free" refers to a medium formulation which does not include any form of whole serum, neither animal nor human. Herein, a protein which has been purified from other serum components is considered serum-free.

Human serum albumin (HSA) provides a source of protein in the media. It is generally believed that protein is required to provide a viscosity similar to that of blood so that hematopoietic cells may thrive. Moreover, protein acts as a substrate for proteases which might otherwise digest cell membrane proteins. Albumin is thought to act as a carrier for trace elements and essential fatty acids. HSA is greatly advantageous over protein derived from animals such as bovine serum albumin (BSA) due to the reduced immunogenic potential of HSA. The HSA may be derived from pooled human plasma fractions, or may be recombinantly produced in such hosts as bacteria and yeast, or in vegetable cells such as potato and tomato. Preferably, the HSA used in the present formulations is free of pyrogens and viruses, and is approved by regulatory agencies for infusion into human patients. The HSA may be deionized using resin beads prior to use in the media.

Transferrin and insulin used in these serum-free media formulations may be derived from animal sera, but it is most preferable to use products which are recombinantly synthesized. It is understood that when transferrin and insulin are derived from an animal source, they are purified to remove other animal proteins, and thus are at least 99% pure. Transferrin is used in these formulations at only 25 -

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125 μg/ml, while insulin is used at only 1 - 25 μg/ml. Therefore, any trace of animal protein other than insulin or transferrin in the medium is non-detectable using standard techniques such as HPLC and gel electrophoresis. Herein, the term "essentially free of animal proteins" refers to a medium formulation or a cell suspension in which no animal proteins other than transferrin or insulin are detectable. The term "animal" is understood to exclude microorganisms and humans.

Preferably, the transferrin and insulin are genetically engineered proteins produced by microorganisms such as bacteria and yeast. Most preferably, the amino acid sequences of the recombinant transferrin and insulin are identical to or highly homologous with those of humans. Thus, the most preferable serum-free media formulations herein contain no animal-derived proteins and do not have even a non-detectable presence of animal protein.

The corticosteroid component may be any naturally occurring or synthetic glucocorticoid hormone, preferably hydrocortisone at a concentration of 1 - 10 μM. The corticosteroid may also be dexamethasone, methylprednisone, or other glucocorticoids approved for clinial use.

Cholesterol may be chemically synthesized or purified from human serum. Cholesterol is used in the present media formulations at a concentration of 0.01 - 0.1 mg/ml, most preferably at 0.05 mg/ml.

Surprisingly, it was discovered that ethanolamine added to serum free media in the range of 50 - 200 μM, preferably 100 μM, greatly increased the proliferation and development of neutrophil and megakaryocyte precursors. Ethanolamine, also known as ß-aminoethyl alcohol, is a viscous, hygroscopic liquid which is commonly used as a surfactant in the manufacture of pharmaceuticals. The exact function of

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ethanolamine in the present media formulations is not known, however this does not diminish the importance of the discovery of its beneficial effects, especially within the context of the specific media formulations provided herein.

In the most ideal system, serum-free media are made fresh each day and continuously refreshed in the cultures. However, reducing agents such as α-monothioglycerol and ß-mercaptoethanol, which are thought to diminish free-radical formation, may be added to the serum-free media formulations to enhance stablility during storage times of up to 20 days or longer. Reducing agents may also be helpful when the media formulations are used in static culture and refreshed only after several days of use. Antibiotics such as gentimicin may also be added to the media as a precaution against bacterial infection of the cultures.

Media formulations containing the above reagents, known herein as media "295-1", were found to be optimal for the development of megakaryocyte precursors within a population of neutrophil precursors, as will be described below. However, it was discovered that the addition of other lipids to the above essential reagents could enhance the proliferative potential of neutrophil precursors. Optionally, triglycerides and/or phospholipids are included as additional sources of lipid. A preferable source of lipid is a sterile, non-pyrogenic fat emulsion prepared from soybean oil and egg yolk phospholipids, typified by Intralipid® (Kabi Pharmacia). Such a preparation preferably contains a mixture of neutral triglycerides of predominantly unsaturated fatty acids such as linoleic, oleic, palmitic, linolenic, and stearic acid. Such a preparation may also contain phosphatidylethanolamine and phosphatidylcholine from egg yolk. Another source of lipid is a human plasma fraction precipitated by ethanol and preferably rendered virus-free by pasteurization. For example, a growth-enhancing media supplement, Nutrimax™ (Baxter Hyland), is derived from

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Cohn's fraction IV_4 and contains triglycerides as well as cholesterol. When a cholesterol-containing lipid preparation is used, it may substitute for the cholesterol in the 295-1 formulation above provided the lipid preparation supplies cholesterol at a final concentration of at least about 30 µg/ml.

Using the above serum-free formulations, optimal populations of neutrophil and megakaryocyte precursors are generated in vitro. The cells originate either from bone marrow, from cord blood, or from peripheral blood. Bone marrow samples may be obtained either from normal donors or from patients. Umbilical cord blood is obtained after normal gestations. Peripheral blood is obtained either from normal donors or from cancer patients. In some cases, cancer patients are treated with hematopoietic growth factors to "mobilize" or stimulate their stem cells to move from bone marrow to their peripheral blood stream, thus greatly increasing the number of stem/progenitor cells in their peripheral blood samples.

Herein, the term "stem/progenitor cell" refers to a hematopoietic cell having the CD34+ cell surface antigen (stem cells and colony-forming units). In non-mobilized peripheral blood, the number of CD34+ cells comprises only about 0.1% of total leukocytes. Mobilization brings the number of CD34+ cells up to about 1 - 4% of total leukocytes. In cord blood, CD34+ cells comprise about 0.1 - 1% of total leukocytes. Normal bone marrow typically contains only 1 - 2% CD34+ cells.

White blood cells (leukocytes) are first separated from the samples of bone marrow or cord or peripheral blood by standard methods such as centrifugation through a gradient. A leukocyte population from bone marrow generally contains only about 10 - 15% myeloblasts and promyelocytes (Geigy Scientific Tables, Vol 3, C. Lentner, ed. Ciba-Geigy, Basel, Switzerland, 1984). Mature megakaryocytes in bone marrow, as recognized by

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Wright-Giemsa staining, comprise only about 0.05% of the leukocyte population, while immunostaining specific for megakaryocyte lineage cells labels up to about 0.2%. Since, in a healthy individual, neutrophil and megakaryocyte precursors differentiate fully in the bone marrow, a precursor is only very rarely discovered in normal blood.

After separation, leukocytes may be cultured directly in serum-free medium. Preferably, however, stem/progenitor cells are separated from the leukocyte population by positive selection. Positive selection of stem/progenitor cells may be based on their binding to an antibody specific for CD34+, followed by separation of antibody-bound stem/progenitor cells using paramagnetic beads (Hardwick, RA, et al., J Hematother 1:379-386, 1992; Smith, SL, et al., supra).

Positively selected stem/progenitor cells are placed in culture at densities ranging from 5,000 to 100,000 cells/ml, preferably at 10,000 cells/ml. Any standard tissue culture flasks or bags may be used in either a static or a perfusion culture system (Koller, MR, et al., BIO/TECHNOLOGY 11:358-363; Emerson, SG, et al., PCT WO92/11355). When a static culture system is used, the cells are fed at intervals of 5 to 7 days to replenish nutrients and remove wastes.

Cells are cultured in serum-free medium for 7 - 14 days, more preferably 9 - 12 days, at which time the cell suspension contains a suitable population of neutrophil and megakaryocyte precursors for use in the treatment of neutropenia and thrombocytopenia. When serum-free medium formulation 295-1 is used, the cell population contains at least about 16% neutrophil precursors and at least about 1% megakaryocyte precursors. Preferably, the cell population contains over 30% neutrophil precursors, more preferably over 60% neutrophil precursors. The neutrophil precursor population is composed of blast cells, promyelocytes, neutrophilic myelocytes, and

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neutrophilic metamyelocytes, which are the precursors of mature banded and segmented neutrophils (Marmont, AM., et al., IN "Neutrophils", Atlas of Blood Cells, Function and Pathology,Eds. Zucker-Franklin, D., et al., 2nd Edition, pp. 159-190; Jandl, JH 1987 Blood, Textbook of Hematology, Little, Brown & Co., Boston/Toronto, pp.441-480).

Flow cytometric analysis of the 10-day serum free cultures was performed to determine the cells' phenotypes based on labeling with cell surface antigens. Cells which are positive for the CD15 antigen and negative for the CD11b antigen (CD15+/CD11b-) have been shown by morphological analysis to be predominantly myelocytes and promyelocytes (Smith, SL, et al., supra). Cells which are CD15+/CD11b+ have been shown to be predominantly mature segmented neutrophils. The 10-day serum-free cultures of the present invention were shown to contain 20 - 60% CD15+/CD11b- cells. When the cultures were transferred at day 10 from serum-free to serum containing medium, the phenotypes were seen to progress to predominantly mature forms as identified by CD15+/CD11b+ labeling (Figure 5c).

Fortuitously, it was discovered that cultures grown in serum-free 295-1, without added lipids, contained a high proportion of megakaryocyte precursors. Herein, the term "megakaryocyte precursor" refers to a nucleated cell which expresses the platelet/megakaryocyte specific glycoprotein IIb/IIIa, also known as "CD41a", as identified by immunostaining and/or flow cytometry. The megakaryocyte precursor population is composed mainly of promegakaryoblasts and megakaryoblasts (Long MW, Stem Cells 11:33-44, 1993; Paulus, JM, "Platelet kinetics", in: Williams, WJ, et al., (eds), Hematology, McGraw-Hill, Ner York: 1251-1260, 1990).

Preferably, the megakaryocyte precursor component of the serum-free 9 - 12 day cultures comprises about 1% of the total

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cells, more preferably at least about 3% of the total cells, most preferably greater than 8% of the total cells. Surprisingly, these results in serum-free medium 295-1 were superior to those obtained in the control, serum-containing medium. Culture in serum-free medium yielded 3 - 22 times the number of megakaryocte precursors as compared with control, which suggested that the serum component may have an inhibitory effect on megakaryocyte precursor growth. Moreover, this enrichment of the cell population for megakaryocyte precursors in liquid culture represents a great advance over previous reports of megakaryocyte growth in vitro (Nakef, A., et al., Ser Heamat 8:4, 1975; Metcalf, D., et al., PNAS 72:1744, 1975; Dexter, T.N., et al., J Cell Physiol 91:335, 1977).

This discovery of a cell suspension greatly enriched for megakaryocyte precursors enables the effective treatment of various types of thrombocytopenia in addition to those caused by cancer therapy. Thus, the herein provided cell suspensions may replace platelet infusions in the treatment of thrombocytopenia. Moreover, the co-existence of a high proportion of megakaryocyte precursors with neutrophil precursors renders these serum-free cell suspensions ideal for the co-treatment of thrombocytopenia and neutropenia.

When the above cell population is administered to a patient following ablative therapy, it is expected that the administered cells will further differentiate in vivo to form mature neutrophils and megakaryocytes, which ultimately form platelets. This expectation is based upon the discovery that when this cell population is switched to serum-containing medium for a further 10 days of culture, the population advances to more mature neutrophilic forms (Figure 1). Moreover, when placed in a fibrin-clot assay, the megakaryocyte precursors were observed to form mature megakaryocytes and release platelets. The results from these

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assays suggest that cells from the serum-free cultures can undergo further maturation after they are returned to in vivo conditions.

One of the primary advantages of the 7 - 14 day serum-free cell culture lies in its population of more mature neutrophil and megakaryocyte precursors, as described above. However, another advantage of this cell culture lies in its small population of earlier cell types, i.e. "progenitors", which are capable of a number of successive rounds of proliferation and differentiation. The daughter cells of these progenitors are expected to follow the precursors in maturation to provide a later population of neutrophils and megakaryocytes, thereby extending the duration of effective treatment possible from an infusion of the original cell suspension.

Progenitors within the neutrophilic lineage are identified as colony-forming units (CFU) and cluster-forming units (clFU) The term CFU is herein defined as a single cell which, when plated in a serum-containing methylcellulose culture for 14 days, proliferates to form a closely associated group of 50 or more cells (a "colony"). Under the same conditions, a clFU forms a group of fewer than 50 cells (a "cluster"). Colonies are further defined by the cell type(s) they contain, as identified by Wright-Giemsa staining (Zucker-Franklin, et al., supra). The colonies which are expected to ultimately form mature neutrophils and monocytes are designated "granulocyte/macrophage" or GM. The clusters are composed of early granulocyte precursors and macrophages, with the granulocytic types predominating.

It was discovered that the addition of a lipid to the 295-1 serum-free formulation could increase the percentage of CFU and clFU in the population. As demonstrated in colony-forming assays, this cell population preferably contains about 0.5 to 3.0% CFU and clFU combined. In the above cell population,

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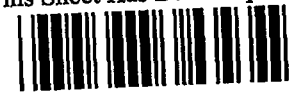


GM-CFU preferably comprise about 5 - 50% of the total CFU/clFU. The cells within the clusters are more differentiated than the cells within the GM colonies. Thus, the clFU within the population are expected to provide a bridge of committed precursors before the GM-CFU have gone through a sufficient number of divisions and stages of differentiation to replenish the precursor population. Preferably, in the above cell population, the clFU comprise about 40 - 60% of the total CFU/clFU.

Compared to neutrophilic CFU and clFU, a colony of cells of the megakaryocyte lineage may contain fewer cells due to their unique form of differentiation. When plated in a fibrin-clot culture for 14 days, a megakaryocyte burst forming unit (MK-BFU) proliferates to form a group of 40 or more megakaryocytes and shows a focal development of many colonies. Thus the MK-BFU is regarded as the early megakaryocyte progenitor. A megakaryocyte colony forming cell (MK-CFC) proliferates to form 2 to 39 megakaryocytes and is regarded as the megakaryocyte progenitor more mature than the MK-BFU. The single megakaryocyte forming unit (S-MK) can only undergo endomitosis (DNA duplication without cell division) and form a single polyploid cell in the colony culture. The S-MK is thus regarded as more mature than the MK-CFC. As demonstrated by specific immunostaining in fibrin clot assays, MK-BFU represent 30 - 57% of the total cells in 5 - 7 day cultures capable of megakaryocyte burst/colony formation. By day 12 in serum-free culture, all the MK colony forming units have progressed to MK-CFC or S-MK. These results in fibrin clot assays confirm that the megakaryocyte precursors grown in serum-free liquid culture for 5 - 12 days are capable of differentiation to mature megakaryocytes.

The serum-free cell suspensions provided herein may be administered to a patient in an amount sufficient to abrogate neutropenia and thrombocytopenia following ablative cancer

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therapy. The enrichment of neutrophil and megakaryocyte precursors in these cell suspensions allows the administration of an effective therapeutic number of desired cells with fewer total cells returned to the patient. Persons skilled in the art will be able to determine the optimal quantity and conditions for administration of the cell suspensions based on their own clinical experience and using guidelines from literature on stem cell infusions (Spitzer, G., et al., Blood 55:317-323; Douay, et al., Exp Hematol 14:358-365, 1986).

The following experimental examples are intended to illustrate the present invention without limiting its scope.

EXAMPLE 1

Serum-free media formulations

Serum free media were formulated as follows. Optimum concentrations were determined by separate titrations based on cell proliferation.

"295-1" Formulation

Isocove's Modified Dulbecco's medium (IMDM, Sigma)
or McCoy's 5A (Sigma)
HEPES buffer, 10-25 mM, optimum 25 mM
hydrocortisone, 1-10 μM, optimum 200 ng/ml
transferrin (human holo) (Sigma), 25-125 μg/ml,
optimum 75 μg/ml
insulin (human recombinant, Novo Nordisk, Princeton, N.J.),
1 -25 μg/ml, optimum 10 μg/ml
selenium, 0.017 - 5 ng/ml, optimum 5 ng/ml
alpha-monothioglycerol, 10 - 160 μM, optimum 216 ng/ml
beta-mercaptoethanol, 20-100 μM, optimum 50 μM
gentamicin, 10 - 50 μg/ml, optimum 50 μg ml
recombinant human hematopoietic growth factors selected from rIL-3 (Amgen, Thousand Oaks, CA), rG-CSF (Amgen), rGM-CSF (Amgen), rSCF (Genzyme, Boston, MA), and PIXY-321 (a recombinant protein composed of the active moieties of IL-3

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and GM-CSF, Immunex, Seattle, WA). Growth factor types and concentrations are stated for each experimental example below.

cholesterol, (Sigma or Miles), 30 - 60 μg/ml, optimum 60 μg/ml

ethanolamine, 50 - 200 μM, optimum 100 μM

human albumin (Baxter Hyland, Duarte, CA), 0.1 - 25 mg/ml, optimum 10 mg/ml. Human albumin was used both as received from supplier and after deionization with resin beads. The deionization procedure was carried out at 4°C by stirring 100 ml albumin (human) 25% solution Buminate (Baxter Hyland Cat no 142-6425) with 0.5-1.0 g of mixed bead resin (AG 501-X8(D) Biorad, Richmond, CA) until the resin beads turned from blue to yellow. The solution was centrifuged at 1000 rpm for 5 minutes to pellet the beads. The albumin solution was decanted, and the stirring procedure with fresh resin beads was repeated. The entire procedure was repeated until the beads no longer changed color (generally, 4 times).

In designated experiments, three different types of lipid preparations were added to formulation 295-1:

1. Lipid derived from a human plasma fraction (Pentex® Ex-Cyte®, Miles Inc., Kankakee, IL; cholesterol, 4.96 mg/ml; triglycerides, 1.67 mg/ml) was used at dilutions from 1:50 - 1:500, optimum 1:100.

2. Lipid derived from ethanol precipitated Cohn's fraction IV_4 from pooled human plasma, pasteurized to kill viruses (Nutrimax™, Baxter Hyland, Duarte, CA), at 0.2 - 5. mg/ml.

3. Lipids derived from soybean oil and egg yolk (Intralipid®, Kabi Pharmacia, Clayton, NC; neutral triglycerides with fatty acid residues comprising 50% linoleic, 26% oleic, 10% palmitic, 9% linolenic, and 3.5% stearic; phospholipids comprising primarily phosphatidylcholine and phosphatidylethanolamine), at 0.03 - 2%, optimum 1%.

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Stock solutions of each lipid were prepared in ethanol at a dilution of 1:10. Optionally, Intralipid® was sonicated in a test tube for 30 minutes.

Control serum-containing medium (HLTM) was composed of the above base media, standard supplements and growth factors, plus 12.5% fetal bovine serum and 12.5% horse serum (Sigma).

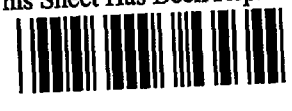
EXAMPLE 2

Preparation of hematopoietic stem/progenitor cells.

Mononuclear cells were obtained from samples of bone marrow, cord blood, or peripheral blood. Normal individuals provided the bone marrow and cord blood. The peripheral blood cells were obtained from cancer patients whose hematopoietic cells had been "mobilized" into their peripheral blood from their bone marrow by the administration of hematopoietic growth factors (Brandt, SJ, et al., supra; Crawford, J., et al., supra; Neidhart, J., et al., supra). The cells in the samples were separated by centrifigation through Histopaque® (Ficoll®Hypaque®, Sigma) and the mononuclear cells from the interface band were collected (Smith, S.L., et al., Exp Hematol 21:870-877, 1993).

The suspensions of mononuclear cells were enriched for stem/progenitor cells by positive selection of CD34+ cells using magnetic beads as follows. The mononuclear cells were first sensitized for 30 minutes on ice with 0.5 μg of anti-CD34 antibody per 10^6 cells [anti-CD34 #9069 prepared by serum-free cell culture (Baxter Hyland, Hayward, CA) and purified by protein G affinity chromatography (Baxter Immuntherapy Division)]. The cells were then washed 3 times by centrifugation in IMDM to remove unbound antibody and mixed with sheep antimouse IgG_1-Fc coated magnetic beads at a bead-to-cell ratio of 1:1. The mixture of beads and cells was then rotated for 30 minutes at 4°C. The bead/cell complexes were then isolated using a magnetic tube holder. After a series of

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washes, CD34+ cells were released from the beads by adding 50 U/ml of Chymodiactin® (Bootes Pharmaceutical, Lincolnshire, IL) in RPMI 1640 (Sigma) and incubating for 15 minutes in a 37°C water bath. The cells released from the beads were then evaluated for CD34+ purity by staining with the FITC-8G12 monoclonal antibody to CD34 (Baxter Immunotherapy Division, Irvine, CA) for 15 minutes on ice and quantitated with a FACSan® flow cytometer (Becton Dickenson, San Jose, CA) as described (Smith, S.L., et al, supra).

EXAMPLE 3

Culture of hematopoietic cells in serum-free media.

Enriched preparations of CD34+ cells were cultured in serum-free media at initial concentrations of $5x10^3$ - $1x10^5$ cells/ml in either polystyrene flasks or plastic bags. Tissue-culture-treated flasks, as well as non-treated flasks of 25, 75 and 150 cm^2 (Corning, Corning, NY) were used. The ethylvinyl acetate (EVA) gas-permeable plastic bags were type PL269 of 250, 500, and 1000 ml capacity (Baxter Fenwal, Deerfield, IL). The 250 ml bags were filled with 20-60 ml, the 500 ml bags were filled with 60-100 ml, and the 1000 ml bags were filled with 100-400 ml of cell suspension. The cultures were placed in a 5% CO_2/5% O_2, 37°C high humidity incubator for the times indicated in the examples below. The cultures were fed at intervals of 5 - 7 days, with dilutions of 1:2 or 1:4.

The concentration of nonadherent cells in the cultures was determined by diluting 0.5 ml from the culture in 9.5 ml 10% cetrimide (Sigma) and counting on a Coulter ZBI (Coulter Electronics, Hialeah, FL).

EXAMPLE 4

Assessment of growth of neutrophil precursors in serum-free medium.

The cell cultures of Example 3 were sampled at indicated days in culture, stained by the Wright-Giemsa method, and

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differentially counted according to morphological characteristics (Marmont, A.M., et al. "Neutrophils", in Atlas of Blood Cells, Eds. Zucker-Franklin, D., et al., 2nd Edition, pp.159-190; Jandl, J.H. (1987) Blood, Textbook of Hematology, Little, Brown & Co., Boston/Toronto, pp.441-480).

Figure 1 depicts the differential cell count of a typical cell culture grown in serum free medium formulation 295-1 (example 1) compared with growth in serum-containing medium ("HLTM"). Hematopoietic growth factors were added to both media as follows: IL-3 at 1000 U/ml, G-CSF at 500 U/ml, GM-CSF at 500 U/ml, and SCF at 20 ng/ml. At day 10 of culture, samples of cells which had been grown in serum-free medium were transferred to serum-containing medium for an additional 10 days.

Examples of the morphology of various cell types are shown in the following figures:

Figure 2. Blast cells (solid arrows).

Figure 3. Promyelocytes (solid arrows) and neutrophilic metamyelocyte (hollow arrow).

Figure 4. Neutrophilic myelocytes (solid arrows) and neutrophilic metamyelocytes (hollow arrows).

Band forms and segmented neutrophils ("Bands/Segs", Fig. 1), the most mature neutrophilic cells, were readily recognized according to standard criteria (Marmont, A.M., et al., supra). The category designated "Other" in Figure 1 includes monocytes, macrophages, and megakaryocyte precursors which are not identifiable by Wright-Giemsa staining. Monocytes and macrophages were identified as described in the Atlas of Blood Cells (supra, Johnston, RB, Jr., The mononuclear phagocyte system, pp.321-377). Megakaryocyte precursors were identified by immunocytochemical staining as described below. Mast cells, which can be identified by toluidine blue staining of

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their heparin granules, represented less than 0.01% of the total cell population.

Differential cell counts varied among experiments due to the wide differences among individual samples obtained from various sources such as normal bone marrow, cord blood, and "mobilized" peripheral blood cells from cancer patients. Typically, however, differential neutrophilic cell counts of cultures grown for 9-12 days in serum-free medium were comparable to cultures from the same samples grown in serum-containing medium. Serum-free cell cultures between 9-12 days in vitro typically contained 10-40% blast cells, 15-30% promyelocytes, 10-25% neutrophilic myelocytes, 10-40% neutrophilic metamyelocytes, and 10-22% "other" (monocytes, macrophages, and megakaryocyte precursors).

When cells were cultured in serum-free medium for 10 days, then transferred to serum-containing medium for an additional 10 days, they differentiated to more mature forms (Fig.1). Their differential neutrophilic cell counts at day 20 were comparable to the cells which had been grown for the entire 20 days in serum-containing medium.

EXAMPLE 5

Neutrophil precursors grown in serum-free medium; phenotype identified by flow cytometry.

At 10 and 20 days of culture, aliquots of $1-2x10^5$ cells were removed from the culture vessels and washed 1 - 2 times in phosphate buffered saline containing 0.05% bovine serum albumin and 0.1% azide (PAB). The cells were then labeled with CD15 (LeuM1) FITC-conjugated and CD11b (Leu15) PE-conjugated monoclonal antibodies (Becton Dickenson) for 10 minutes on ice. Following 1 additional wash in PAB, the cells were suspended in 1 ml PAB and analyzed by flow cytometry for coexpression of CD15 and CD11b.

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At day 10, the CD15+CD11b- phenotype comprised 20 - 60% of the cells in serum-free cultures, which was similar to the day 10 profile of serum-containing cultures (Fig. 5a, lower right quadrant). At day 20, cultures which had been maintained in serum-free media still contained predominantly the CD15+CD11b- phenotype (Fig. 5b). In contrast, at day 20, cultures which had been transferred from serum-free to serum-containing media at day 10 contained 70 - 100% differentiated cells of the CD15+11b+ phenotype, similar to the phenotypic profile of cultures which had been grown in serum-containing media for the entire 20 days (Fig. 5c).

Cells which were CD15+/CD11b- were sorted by FACS cell sorting and identified morphologically as neutrophilic precursors in the promyelocyte and myelocyte stages of differentiation (Smith, SL, et al., supra). Cells which were CD15+/CD11b+ were sorted and identified as the more mature forms, i.e. metamyelocytes, band forms, and segmented neutrophils.

EXAMPLE 6

Colony forming units and cluster forming units in serum free cultures.

Cells were grown in various media formulations using the following growth factors: IL-3 at 300 U/ml, G-CSF at 300 U/ml, GM-CSF at 300 U/ml, and SCF at 20 ng/ml.

At day 0 and at 10 days of culture, colony assays were set up in methyl-cellulose containing IMDM, 30% FBS (Sigma), 7% Leptalb 7 (Armour Pharmaceuticals, Kankakee, IL) and recombinant growth factors at 150 U/ml rIL-3, 200 U/ml rGM-CSF, 150 U/ml rG-CSF, 160 U/ml rIL-6 and 10 U/ml erythropoietin (Amgen, Thousand Oaks, CA). Colony assays were set up in triplicate at 5 to $10x10^3$ cells/ml in 35 ml dishes (Nunc) and scored following 14-day incubation in 5% CO_2/5% O_2 at 37°C. Colonies were defined as groups of greater than 50 cells, and clusters were defined as groups of fewer than 50

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cells (see Figure 6). The colonies were identified macroscopically as either CFU-GM, macrophage (CFU-M), burst-forming unit erythroid (BFU-E) or mixed (CFU-Mix) (myeloid and erythroid) with periodic morphological verification of plucked colonies using Wright-Giemsa staining. Results are shown in Tables 1 - 4 below.

Key for Tables 1 - 4

P.I. = proliferation index (total cells at day 10/total cells at day 0)

GM I.= granulocyte/macrophage-colony forming index (GM colonies formed from 10 day culture/GM colonies formed from day 0 suspension).

Mac I.= macrophage colony forming index

BFUE I.= burst forming unit-erythroid index

Mixed I. = mixed colony forming index

Cluster I.= cluster forming index

PB = peripheral blood sample

B.M. = bone marrow sample

CFU = colony-forming unit; CE = cloning efficiency

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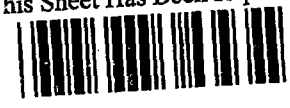


TABLE 1

PB1

	Culture Medium	P.I. Day 10	GM I. Day 10	Mac I. Day 10	BFUE I. Day 10	Mixed I. Day 10	Cluster I. Day 10
Deionized Albumin	Base	11.9	1.5	0.0	7.6	0.0	15.5
	+Intralipid	12.6	3.9	0.0	8.5	0.0	18.9
	+Ethanolamine	9.9	0.7	0.0	0.5	0.0	12.9
	+Intra./Ethan.	10.3	1.4	0.3	0.4	0.0	8.2
Non-Deionized Albumin	Base	13.2	1.4	0.7	7.0	2.0	25.1
	+Intralipid	6.7	0.5	0.0	1.1	0.0	2.0
	+Ethanolamine	7.3	2.0	0.6	8.5	2.2	5.8
	+Intra./Ethan.	10.3	3.3	0.8	16.6	11.8	12.4
Serum Controls	IMDM +25%	14.4	5.8	1.6	14.5	2.2	20.2
	HLTM	14.7	4.9	0.8	6.2	2.2	27.9

PB2

	Culture Medium	P.I. Day 10	GM I. Day 10	Mac I. Day 10	BFUE I. Day 10	Mixed I. Day 10	Cluster I. Day 10
Deionized Albumin	Base	9.9	0.5	0.0	0.4	0.0	2.8
	+Intralipid	33.3	0.5	0.2	0.6	0.1	8.2
	+Ethanolamine	26.2	7.5	0.9	1.7	0.4	19.0
	+Intra./Ethan.	23.7	5.2	2.5	1.1	0.7	53.7
Non-Deionized Albumin	Base	4.2	0.1	0.0	0.1	0.0	0.6
	+Intralipid	23.7	2.4	0.3	1.2	0.3	9.5
	+Ethanolamine	15.1	0.7	0.1	0.6	0.1	6.5
	+Intra./Ethan.	33.3	0.5	0.0	0.2	0.0	1.9
Serum Controls	IMDM +25%	38.2	7.5	1.4	4.7	2.2	18.6
	HLTM	45.3	0.9	0.1	0.5	0.0	9.5

B.M.

	Culture Medium	P.I. Day 10	GM I. Day 10	Mac I. Day 10	BFUE I. Day 10	Mixed I. Day 10	Cluster I. Day 10
Deionized Albumin	Base	7.3	1.0	0.0	0.9	0.0	138.7
	+Intralipid	9.4	0.8	0.1	0.5	0.0	94.0
	+Ethanolamine	8.6	2.2	0.3	0.7	0.0	292.4
	+Intra./Ethan.	10.3	12.1	10.5	2.5	0.0	1565.6
Non-Deionized Albumin	Base	6.5	2.0	0.5	6.0	0.0	221.0
	+Intralipid	7.6	3.3	0.8	8.5	0.1	311.6
	+Ethanolamine	4.8	0.2	0.1	0.8	0.0	91.2
	+Intra./Ethan.	7.6	0.4	0.1	2.1	0.0	76.0
Serum Controls	IMDM +25%	17.5	1.0	0.5	11.5	0.8	455.0
	HLTM	22.6	5.5	1.7	41.3	0.7	949.2

MEAN

	Culture Medium	P.I. Day 10	GM I. Day 10	Mac I. Day 10	BFUE I. Day 10	Mixed I. Day 10	Cluster I. Day 10
Deionized Albumin	Base	9.7	1.0	0.4	2.9	0.0	52.3
	+Intralipid	18.4	1.8	0.4	3.2	0.0	40.4
	+Ethanolamine	14.9	3.5	0.5	1.0	0.1	108.1
	+Intra./Ethan.	14.8	6.2	1.6	1.3	0.2	542.5
Non-Deionized Albumin	Base	6.0	1.2	0.7	4.4	0.7	82.2
	+Intralipid	12.7	2.1	0.7	3.6	0.1	107.7
	+Ethanolamine	9.1	1.0	0.7	3.3	0.8	34.5
	+Intra./Ethan.	17.1	1.4	0.8	8.3	3.9	30.1
Serum Controls	IMDM +25%	23.4	4.8	0.7	10.2	1.7	164.6
	HLTM	27.5	3.8	0.7	16.0	1.0	328.9

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Table 2

Percent CFU and CE for Cells Cultured in 295

P.B

	Culture Medium	%GM Day 10	%Mac Day 10	%BFUE Day 10	%Mix Day 10	%Clusters Day 10	%CE Day 10
Deionized Albumin	Base	11	0	80	0	10	1.3
	+Intralipid	21	0	70	0	9	1.6
	+Ethanolamine	27	0	30	0	43	0.3
	+Intra./Ethan.	48	3	23	0	26	0.3
Non-Deionized Albumin	Base	10	2	71	2	15	1.2
	+Intralipid	21	0	71	0	8	0.4
	+Ethanolamine	12	1	81	2	3	2.4
	+Intra./Ethan.	10	1	79	7	4	3.4
Serum Controls	IMDM +25%	19	2	72	1	6	2.3
	HLTM	28	2	53	2	15	1.3

P.B

	Culture Medium	%GM Day 10	%Mac Day 10	%BFUE Day 10	%Mix Day 10	%Clusters Day 10	%CE Day 10
Deionized Albumin	Base	30	0	46	0	25	1.2
	+Intralipid	17	6	40	1	36	0.7
	+Ethanolamine	51	4	24	2	18	4.2
	+Intra./Ethan.	30	10	13	3	44	5.4
Non-Deionized Albumin	Base	28	9	38	2	23	0.7
	+Intralipid	36	3	38	3	20	2.1
	+Ethanolamine	23	3	43	3	29	1.6
	+Intra./Ethan.	43	0	36	0	21	0.3
Serum Controls	IMDM +25%	33	5	44	7	12	4.4
	HLTM	26	2	30	0	42	0.5

B.M.

	Culture Medium	%GM Day 10	%Mac Day 10	%BFUE Day 10	%Mix Day 10	%Clusters Day 10	%CE Day 10
Deionized Albumin	Base	33	0	21	0	45	0.4
	+Intralipid	38	4	17	0	42	0.2
	+Ethanolamine	38	3	9	0	50	0.7
	+Intra./Ethan.	34	19	5	0	42	3.6
Non-Deionized Albumin	Base	23	4	48	0	25	1.4
	+Intralipid	26	4	45	1	24	1.7
	+Ethanolamine	14	3	31	0	53	0.4
	+Intra./Ethan.	14	3	54	0	29	0.4
Serum Controls	IMDM +25%	7	2	56	4	31	0.8
	HLTM	12	2	63	1	21	2.0

MEAN

	Culture Medium	%GM Day 10	%Mac Day 10	%BFUE Day 10	%Mix Day 10	%Clusters Day 10	%CE Day 10
Deionized Albumin	Base	24	0	49	0	27	1.0
	+Intralipid	25	3	42	0	29	0.9
	+Ethanolamine	39	2	21	1	37	1.7
	+Intra./Ethan.	37	11	14	1	37	3.1
Non-Deionized Albumin	Base	20	5	53	1	21	1.1
	+Intralipid	28	2	51	1	17	1.4
	+Ethanolamine	16	2	51	2	28	1.5
	+Intra./Ethan.	22	1	56	2	18	1.4
Serum Controls	IMDM +25%	20	3	57	4	16	2.5
	HLTM	32	2	49	1	26	1.3

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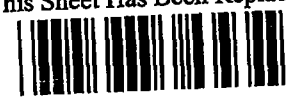


TABLE 3

Culture Medium	P.I.	GM I.	Mac I.	BFUe I.	MixI.	CluI.
295-1 +lipid 1	52	8	5	1	2	522
control +serum	40	8	27	3	24	386
control -serum	20	1	1	1	1	49

(Mean of 4-10 cultures from bone marrow and peripheral blood.)

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Table 4

Colony Forming Units per 10,000 Cells Plated From Cells Cultured In 295

RT2446-PB	GM	Mac	BFUE	Cluster	Total CFU	%GM	%Mac	%Clusters	%CE
295	2	20	30	94	146	1	14	64	1.5
295+ Lipid 3	4	2	10	70	86	5	2	81	0.9
HLTM	4	4	10	120	138	3	3	87	1.4

RT2448-PB	GM	Mac	BFUE	Cluster	Total CFU	%GM	%Mac	%Clusters	%CE
295	14	22	29	88	153	9	14	58	1.5
295+ Lipid 3	5	8	15	34	62	8	13	55	0.6
HLTM	17	83	7	55	162	10	51	34	1.6

UC1107-BM	GM	Mac	BFUE	Cluster	Total CFU	%GM	%Mac	%Clusters	%CE
295	0	0	0	0	0	0	0	0	0
295+ Lipid 1	22	25	19	79	145	15	17	54	1.5
HLTM	6	30	1	84	121	5	25	69	1.2

ALLO-BM	GM	Mac	BFUE	Cluster	Total CFU	%GM	%Mac	%Clusters	%CE
295	4	5	16	32	57	7	9	56	0.6
295+ Lipid 1	6	3	2	30	41	15	7	73	0.4
HLTM	2	14	0	73	89	2	16	82	0.9

RT2613-PB	GM	Mac	BFUE	Cluster	Total CFU	%GM	%Mac	%Clusters	%CE
295	14	2	20	30	66	21	3	45	0.7
295+ Lipid 2	10	0	30	74	114	9	0	65	1.1
HLTM	6	12	8	164	190	3	6	86	1.9

RT2618-PB	GM	Mac	BFUE	Cluster	Total CFU	%GM	%Mac	%Clusters	%CE
295	0	0	24	16	40	0	0	40	0.4
295+ Lipid 2	8	0	42	66	116	7	0	57	1.2
HLTM	10	2	14	34	60	17	3	57	0.6

RP7-BM	GM	Mac	BFUE	Cluster	Total CFU	%GM	%Mac	%Clusters	%CE
295+ Lipid 1	42	66	4	101	213	20	31	47	2.1
295+ Lipid 2	44	33	2	113	192	23	17	59	1.9
HLTM	69	59	4	178	310	22	19	57	3.1

RP8-BM	GM	Mac	BFUE	Cluster	Total CFU	%GM	%Mac	%Clusters	%CE
295+ Lipid 1	30	30	3	91	154	19	19	59	1.5
295+ Lipid 2	55	44	13	136	248	22	18	55	2.5
HLTM	69	40	7	170	286	24	14	59	2.9

%CE=Total CFU/Total Cells Plated
Lipid 1= Excyte
Lipid 2= Nutrimax
Lipid 3= Intralipid

BM= Bone Marrow
PB= Peripheral Blood

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The proliferation index for all cells at day 10 of culture ranged from 10 - 60 fold.

Generally, the number of CFU-GM in day 10 cultures increased 5 - 20 fold over day 0. The number of CFU-Mac in day 10 cultures increased 2 - 10 fold over day 0. Notably, the number of cluster forming units in day 10 serum-free cultures increased 10 -600 fold over day 0.

At day 14 of the methylcellulose assay, GM colonies comprised about 10-50% of total colonies/clusters, Mac colonies comprised about 5-20% of total colonies/clusters, and clusters comprised about 10-60% of total colonies/clusters.

Typically, the percentage of colony-forming units (CFU) plus cluster-forming units (clFU) in 10-day serum-free cultures was relatively low, ranging from about 1 to 5% of the total cells (%CE=%cloning efficiency=total colonies + total clusters (x100)/total cells plated). However, the percentage of clFU within the total CFU/clFU was relatively high, ranging from about 10% to 50% of the total CFU/clFU. The absolute number of cluster-forming units per 10,000 cells ranged from 16 to 136. Cluster-forming units are the individual cells which proliferate directly into more mature forms of myelocytes such as band forms and segmented neutrophils.

EXAMPLE 7

Assessment of megakaryocyte precursors grown in serum-free medium.

Cells were grown in culture as described in Example 4, with serum-free medium formulation 295-1 as described in Example 1. At selected days of culture, cells were cyto-centrifuged onto microscopic slides, fixed with absolute acetone, and incubated with an anti-CD41a monoclonal antibody designated P2 (AMAC, Westbrook, ME). The antibody recognizes platelet/megakaryocyte specific glycoprotein IIb/IIIa.

Glycoprotein IIb/IIIa is present in megakaryocyte precursors, mature megakaryocytes, and platelets, but not in other blood cells. After incubation with the primary antibody, a secondary antibody (biotinylated goat anti-mouse IgG, Kirkegaard & Perry, Gaithersburg, MD) was added and bound to the primary antibody. This was followed by incubation with a streptavidin-peroxidase complex and a peroxidase (AEC) reaction, which produced a red color specifically labeling megakaryocytes, their precursors and their descendents. The cultures were then counterstained with hematoxylin, which produced a blue color on cell nuclei, allowing recognition of negative cells (see Figure 7).

Cells from the same cultures were also analyzed by flow cytometry to detect anti-CD41a labeled cells. Results are shown in Tables 5 - 7 below.

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TABLE 5

Immunocytochemistry and Flow Cytometry Evaluation of MK Cells

Culture	295	Time	CD41a Immu (%)		CD41a Flow (%)		
	+ -	(day)	PL-STK	MK	MK	%mFSC	%hFSC
PB CD34	GF(1)	7	8	12	17.8	83.1	17.4
PB CD34	GF(2)	7	7	18	17.4	82.8	16.7
PB CD34	GF(2)	7	7	19	17.5	81.7	18.9
PB CD34	GF(2)	7	19	25	16.5	81.8	18.2
PB CD34	GF(2)	7	8	20	15.3	83.7	16.4
PB CD34	GF(2)	8	1	7	2.6	84.6	15.4
PB CD34	GF(2)	8	0	6	3.3	89.9	12.1
PB CD34	GF(1)	8	0	4	3.0	90.0	10.0
		Mean	6.3	13.9	11.7	84.7	15.6
		SEM	2.2	2.7	2.6	1.2	1.1
PB CD34	GF(1)	10	0	23	20.0	77.0	23.5
PB CD34	GF(2)	10	0	19	20.7	73.4	26.6
PB CD34	GF(2)	10	0	29	19.8	73.2	26.8
PB CD34	GF(2)	10	0	33	21.3	76.1	23.9
PB CD34	GF(2)	10	0	23	19.6	71.9	28.6
PB CD34	GF(2)	10	0	22	20.7	80.2	20.3
PB CD34	GF(1)	10	0	6	6.7	77.6	22.4
PB CD34	GF(2)	10	0	7	5.2	90.4	10.5
PB CD34	GF(1)	10	1	8	4.5	86.7	13.3
		Mean	0.1	18.9	15.3	78.5*	21.8#
		SEM	0.1	3.3	2.6	2.1	2.1

Key: Immu, immunocytochemistry; flow, flow cytometry; PL-STK, platelet stuck, CD41a-negative cells; MK, megakaryocytic, CD41a-positive cells; %mFSC, % medium forward scatter, smaller cells in the MK population; %hFSC, % high forward scatter, larger cells in the MK population. * significantly lower than mFSC at 7-8 days. # significantly higher than hFSC at 7-8 days. Growth factors: (1) SCF, IL-3, and IL-6; (2) SCF, IL-3, IL-6, and IL-11.

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TABLE 6

Evaluation of Megakaryocytes in BM Samples

Sample	Wright's	Immunocytochemistry		Flow Cytometry		
	MK Morph	CD41a*	CD42b*	CD41a	CD42b	CD61
Fresh Samples:						
BM MNC1	0.05	0.4 (32)	ND	23.3	16.7	22.1
BM MNC2	0.01	0.5 (39)	ND	27.3	5.5	9.5
BM MNC3	0.01	0.2 (45)	0.5 (0)	9.5	4.2	6.5
BM $CD34^+$	0.00#	9.0 (35)	ND	40.0	ND	ND
Cultured Samples (day 10-14):						
BM MNC	2.7	14.7 (0)	4 (0)	15.6	3.7	ND
BM Macroph	0	0 (0)	0 (0)	0.02	0	0

Frequencies (%) of indicated cells are presented in the table. Abbreviations: BM Macroph, bone marrow macrophages (98% of the cells in this culture were morphologically macrophages); ND, not done.

* Numbers in the parenthesis represented frequencies (%) of platelet-stuck, $CD41a^-$ or $CD42b^-$ cells.

\# Morphologically $CD34^+$ cells were lymphocyte- or blast cell-like.

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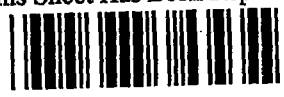


TABLE 7

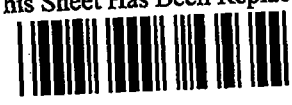
DISTRIBUTION OF MEGAKARYOCYTE IN DIFFERENT CULTURES

Sample	Conditions	Time (Day)	CD41a$^+$ (%)	Cell (10^5/ml)
BM MNC	HLTM+(1)*	7	2 (0)#	7.4
	HLTM+(1)	10	4 (0)	9.4
	Plasma+(1)	12	14.7 (2)	13.0
RT 555	295+(2)*	12	10 (2)	3.2
(3/23/93)	HLTM+(2)	12	3 (1)	5.8
UC 166	295+(2)	14	11 (9)	17.3
(3/17/93)	HLTM+(2)	14	0.5 (1)	8.4
GB (2/1/93)	HLTM+(2)	13	0 (0)	---

* Cytokine combination (1): SCF, IL-3 & IL-6. Cytokine combination (2): SCF, IL-3, GM-CSF & G-CSF.

\# Numbers in ( ) represent platelet-stuck cells (false positive).

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Platelets stained positive with anti-CD41a, CD41b (GP Ib) and CD61 (GP IIIa) were often observed to adhere to other cells not of megakaryocyte lineage. It was found that flow cytometry, as shown in Table 6, was subject to the artifact of platelet-adherence by counting false positive cells. Therefore, CD41a immunocytochemistry using the same monoclonal antibody was utilized to confirm the true megakaryocyte lineage cells. As shown in Table 5, most of the adherent platelets ("PL-STK") disappeared after 7-10 days in culture, and comparable results were obtained from immunocytochemistry and flow cytometry.

Using flow cytometry, the expression of CD41a antigen and the cell size were simultaneously analyzed. Figure 8 shows the flow cytometric dot-plot of a culture sample which was grown in the serum-free medium for 10 days. The X axis represents the forward light scatter (FSC) which measures the cell size. Those cells which plotted further to the right are higher in FSC (hFSC) and larger in cell size. The Y axis represent the green fluorescence intensity which measures the FITC conjugated antibody staining. The isotype control staining of the sample using FITC conjugated anti-mouse IgG is presented in Figure 8a, showing no non-specific antibody binding to the cells. The same cell sample incubated with FITC conjugated anti-CD41a antibody is presented in Figure 8b, showing distinctively CD41a positive megakaryocytes in the culture. The cells plotted in the upper right quadrant are considered large megkaryocytes and their cell size is larger than that of the majority of the cell population in the sample. Those cells plotted in the upper left quadrant are considered small megakaryocytes. It has been reported that large megakaryocytes are more mature than small megakaryocytes (Tomer, A, et al., Blood 70:1635-1742, 1987). As shown in Table 5, large megakaryocytes (hFSC) comprised about 16% of the total labelled cell population in day 7 - 8 cultures. After 10 days of culture, the frequency of large

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megakaryocytes had progressed to about 22%, demonstrating a statistically significant increase in megaryocyte maturation.

As shown in Table 7, when grown in serum-free medium (295), hemopoietic cells from the same donor yield more megakaryocytes (3 - 22 fold) than when grown in the serum-containing medium (HLTM). The results strongly suggest that the serum-free culture medium provides a more favorable microenvironment for megakaryocyte growth than does the serum-containing medium.

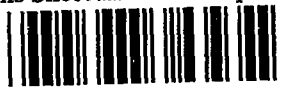
EXAMPLE 8

Megakaryocyte colony formation analyzed by fibrin clot assay.

At selected days in culture, cells were plated in a serum-free fibrin-clot colony culture (MK-CFC assay) and evaluated for their ability to form megakaryocyte colonies. Cells (2 x 10^5/ml) were suspended in a semisolid fibrin-clot culture medium containing 1% BSA (Sigma), 0.02 % fibrinogen (KABI, Sweden) and 0.02 U/ml thrombin (Sigma) in IMDM (Zauli, G, et al., Exp Hematol 20:850-854, 1992). Cytokines including SCF, IL-3, IL-6, and/or IL-11 were also utilized as indicated. A 0.5 ml sample of the cell/medium suspension was placed on an ordinary microscope slide. The slide was placed in a humidified 150 mm Petri dish and cultured at 37°C, 5% CO_2 for 14 days. During this time, megakaryocyte precursors in the original culture proliferated to form megakaryocyte bursts and colonies, or differentiated into more mature single megakaryocytes. The fibrin-clot cultures were then fixed and immunostained as described in Example 7.

The above described MK-CFC assay offers several improvements and advantages over previously described assays. Firstly, the Petri dish has been replaced by the microscope slide as a substrate for the megakaryocyte colony cultures, which offers these advantages:

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(1) The slide enables the use of absolute acetone, the best fixative to preserve the protein antigen for the immunocytochemical detection. Traditionally, the colony cultures were grown in plastic Petri dishes, which would dissolve in acetone.

(2) The slide facilitates the immunostaining by decreasing the amount of antibody used for each culture and diminishing the artifact caused by antibody dry out during staining.

(3) The slide permits clear examination of the in situ colonies by using high power microscopic objectives.

The traditional evaluation methods included morphological recognition wherein megakaryocyte colonies were described as clusters of big, highly refractile cells under the inverted microscope without any staining (Sonoda, Y., et al., Blood 81:624-630, 1993) and immunfluorescent microscopy where megakaryocyte colonies were distinguished from other colonies as bright green vs. dull green (background) (Bruno, E., et al., Blood 73:671-677, 1989; Zauli, G., et al., Exp. Hematol. 20:850-854, 1992). These methods yielded ambiguous results because it was difficult to distinguish between true megakaryocyte colonies and non-megakaryocyte lineage colonies, which also grow in fibrin clots.

The present method provides more accurate counting of megakaryocyte colonies because they are identified by a specific red label, within blue-labelled non-megakaryocytes, under the light microscope (Figure 9). Results are summarized in Table 8 below.

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Table 8

EFFECTS OF CULTURE MEDIA AND CYTOKINES ON MEGAKARYOCYTE LINEAGE DEVELOPMENT (Serum-Free Fibrin-Clot Assay)

Sample	Pre-Cul Time (days)	Pre-Cul Cond & Cytokine*	MK Lineage Cells per 10^5 Cells		
			BFU-MK	CFC-MK	S-MK
Exp 1	5	HLTM+(1)	0	1.9	13.5
	5	295+(1)	0	0	2.65
	5	295+(2)	40	30	335
	12	HLTM+(1)	0	25.9	98.2
	12	295+(1)	0	32.2	652.4
	12	295+(2)	0	66	641.5
Exp 2	7	295+(1)	0	19.6±9.7	134.4±71.9
	7	295+(2)	43.7±9.4	104.3±14.2	15.0±5.8
	7	295+(3)	7	36.5	7.5

PB $CD34^+$ cells were grown in the pre-cultured conditions for the indicated times and then plated into fibrin-clot cultures for a 14-day of further growing.

Abbrevs: Cul, culture; Cond, conditions; MK, megakaryocyte; BFU-MK, megakaryocyte burst-forming cell, the colony containing 40-100 megakaryocytes; CFC-MK, megakaryocyte colony-forming cell, the colony containing 2-39 megakaryocytes; S-MK, single megakaryocyte found in the colony culture.

* Cytokine combinations: (1) stem cell factor, IL-3 & IL-6; (2) stem cell factor, IL-3 & GM-CSF; (3) stem cell factor, IL-3, GM-CSF & IL-11.

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As shown in Table 8, the early megakaryocyte progenitors (BFU-MK) were detectable only in the serum-free (295) but not the serum-containing (HLTM) cultures during days 5 - 7. On day 12, serum-free cultures contained more megakaryocyte colony forming cells (MK-CFC) and single megakaryocytes (S-MK) than serum-containing cultures. The addition of GM-CSF into the serum-free culture appeared to facilitate the growth of megakaryocytes.

EXAMPLE 9

DIFFERENTIATION OF MEGAKARYOCYTES IN SERUM-FREE MEDIUM

This example further assesses the growth and differentiation of $CD34^+$ cell cultures into megakaryocytes in serum-free media compared to serum containing media. The effects of different combinations of growth factors on megakaryocyte differentiation was also determined.

The isolation and culture of $CD34^+$ hematopoietic cells from bone marrow (BM) and peripheral blood (PB) was performed by methods similar to those described in Examples 1 through 3. Briefly, apheresis products of PB cells and plasma were simultaneously collected using a CS3000™ blood cell separator (Fenwal Division, Baxter Healthcare, Deerfield, IL) from cancer patients 4-15 days after G-CSF and/or chemotherapy mobilization. Cell samples were collected in citrate dextrose formula A (ACDS) and diluted 1:1 with Iscove's modified Dulbecco's medium (IMDM) (Gibco, Grand Island, NY) containing 2% fetal bovine serum (FBS) (Sigma, St. Louis, MO). Low-density MNC were obtained after density centrifugation over Histopaque™ ficoll-hypaque (1.077 g/ml) for 20 min at 300g. The plasma was spun for 10 min at 200g to remove platelets, passed through a 0.45 μm filter and stored at -20°C. Human aplastic serum was obtained from thrombocytopenic patients after BM transplantation as described by Debili et al. (*Blood* 80:3022 (1992)). Aplastic serum and plasma (AS) that demonstrated megakaryocyte stimulatory activities in pre-

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screened studies were utilized in these experiments at a final concentration of about 10%.

CD34 positive cells were selected as previously described in Example 2 with slight modifications. Briefly, adherent cells were depleted by mixing the MNC with uncoated polystyrene Dynal paramagnetic beads (Fenwal Division, Baxter Healthcare, Deerfield, IL). Non-adherent cells were incubated with the anti-CD34 monoclonal antibody (mAB) 9C5 (Immunotherapy Division, Baxter Healthcare, Santa Ana, CA) and then mixed with magnetic beads coated with sheep anti-mouse IgG_1 (Fenwal Division, Baxter Healthcare, Deerfield, IL) at the ratio of 0.5 - 1 bead to 1 cell. Bead-cell rosettes were collected after the positive selection using a Dynal MPC-1 Magnet (Immunotherapy Division, Baxter Healthcare, Santa Ana, CA) or Isolex™ 50 magnetic cell separator (Immunotherapy Division, Baxter Healthcare, Santa Ana, CA). Cells were released from beads by overnight incubation with 200 U/ml IL-3 at 37°C, 5% CO_2. For quantitation aliquots of selected cells were stained with another mAB against CD34, fluorescein (FITC) conjugated 8G12 (Immunotherapy Division, Baxter Healthcare, Santa Ana, CA). Flow cytometry demonstrated that about 80-97% of the selected cells were CD34 positive.

BM mononuclear cell (MNC) cultures were initiated at 10^5 cells/ml while CD34 cell cultures were initiated at 10^4 cells/ml. Cultures were grown in the human long term culture medium (HLTM), the serum-free medium (Immunotherapy Division, Baxter Healthcare) or the human serum or plasma-supplemented medium. Immunocytochemistry and flow cytometry was performed as described in Example 7.

The kinetics of megakaryocyte growth in culture was determined in serum-free and HLTM cultures supplemented with SCF, IL-3 and IL-6. Immunocytochemistry showed that, prior to culture on day 0, CD41a$^+$ cells represented 0.33% (ranged 0.01 - 0.95%,

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... and 5.3% (ranged 2 - 9 %, n = 5) of purified BM $CD34^-$ or PB $CD34^+$ cells (Figure 10, day 0). No obvious cell proliferation was observed during the first several days after plating. In order to maintain cultures for later evaluation, cells were not harvested until day 5. Cultures were grown for a period of 10 - 30 days. Some stromal foci were occasionally formed in HLTM BM CD34 cell or MNC cultures. Serum-free cultures had a few scattered, macrophage like adherent cells but no stromal foci.

On day 5, all CD34 cell cultures increased 4 - 5 fold in cellularity, with viable cells greater than 95%. The differential cell count showed that day 5 serum-free $CD34^+$ cell cultures contained 66.2% blast cells (ranged 40 - 98%) and 37% granulocytic lineage cells (ranged 9 - 53%). At day 10 -12, serum-free CD34 cell cultures contained 21.3 ± 6.5% blast cells (ranged 9 - 47%), 70.3 ± 6.7% granulocytic cells (ranged 42 - 88%), 8.2 ± 1.8% macrophages (ranged 2 - 14%) and occasionally erythroid cells (5%). HLTM CD34 cell cultures generally contained less blast cells (9.2 ± 3.2%, ranged 1 - 22%) and more granulocytic cells (78.2 ± 5.3%, ranged 65 - 96%). Cell viability and proliferation were slightly higher in HLTM cultures than in serum-free culture. Morphologically recognizable mature megakaryocytes were found in HLTM cultures but not in serum-free cultures. As compared to mature megakaryocytes present in BM, culture-derived, morphologically recognizable megakaryocyte had a smaller cell size and less lobulated nuclei (2 - 3 lobes).

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TABLE 9
megakaryocyte GROWTH IN CD34$^+$ CELL CULTURES

CULTURE	CD41a$^+$ (%)	VIABLE (%)	TOTAL CELL INCREASE (fold increase)	megakaryocyte fold increase**
Serum-Free	16.2 ±4.3*	75.9 ±1.0	22.0 ±6.0	51.0 ±3.8*
HLTM	3.1 ±1.8	83.4 ±2.1	29.1 ±9.2	14.1 ±2.2

PB CD34$^+$ cell cultures were grown in HLTM (n=6) or serum-free media (n=7) supplemented with SCF, IL-3 & IL-6 for 10-14 days prior to examination.

** estimated fold expansion of megakaryocyte, where the initial cell populations was estimated to contain 5.3 ±1.8% CD41a$^+$ megakaryocyte.

* Significantly higher than that in HLTM.

Distinctive CD41a$^+$ cells were observed in day 10-14 in serum-free CD34 cultures (Fig. 9). Under serum-free conditions, the percent of CD41a$^+$ megakaryocyte progenitors was substantially higher than that observed under serum containing conditions. Similarly, the fold expansion of these cells as compared to the starting cell population was 51 fold in serum-free media versus 14.1 fold in serum containing media (Table 9). These cells showed cytoplasmic CD41a immunoreactivity, containing a single, non-lobulated nucleus and non-granular cytoplasm. Their size was similar or slightly bigger than the surrounding granulocytes. Without the antibody staining, they could not be recognized by light microscopy. These CD41a$^-$ megakaryocyte lineage cells increased around day 5-7 (1.4 ± 3.1%) peaked at day 10-12 (16.2 ± 4.3%) and decreased around day 15-17 (10.5 ± 0.5%) in serum-free CD34 cell cultures (Figure 10). HLTM cultures, however, showed no increase of megakaryocyte frequency throughout the entire culture period (Figure 10); but absolute megakaryocyte number increased and peaked also at day 10-12, mainly due to the high cellularity and the moderate megakaryocyte frequency at that time. Morphologically recognizable megakaryocyte accounted for about 1/3 of total

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CD41a+ cells in day 10-12 HLTM cultures. Others were morphologically unrecognizable megakaryocyte similar to those found in serum-free cultures. Megakaryocytes disappeared from HLTM cultures around day 15. HLTM cultures progressed to day 30 did not regain megakaryocytes. Megakaryocyte derived from day 10-12 serum-free or HLTM cultures were brightly stained with CD41a and easily recognized by immunocytochemistry and flow cytometry.

Paired PB CD34+ cell cultures were established to compare the ability of different culture media to support megakaryocyte growth. PB CD34+ cells from individual donors were grown in serum-free and HLTM cultures. Cultures were supplemented with the identical cytokine combination and refed with the same regimen. Results from 6 experiments are presented in Figure 11. Experiments 1-3 were supplemented with SCF, IL-3, GM-CSF & G-CSF; while experiments 4-6 were supplemented with SCF, IL-3 & IL-6. Cell viability in paired cultures was similar. Cell count was generally lower in serum-free cultures than in HLTM cultures with the exception of experiment 2 (Figure 11). Megakaryocyte frequency in serum-free cultures was 7.6 ± 3.1 fold higher (ranged 3-22) than their paired HLTM cultures. The absolute megakaryocyte number in serum-free cultures was 17.4 ± 7.1 fold higher (ranged 2-45) than their comparative HLTM cultures (Figure 11, megakaryocyte cellularity).

In other independent experiments, in vitro total megakaryocyte expansion was calculated by dividing the estimated cultured megakaryocyte output by the initial megakaryocyte population, and standardized by the cultured cell viability. Megakaryocyte fold expansion was 51-fold in serum-free medium and 14-fold in HLTM cultures (Table 9). Therefore, the serum-free media supported better megakaryocyte growth than the HLTM medium, probably by facilitating megakaryocyte differentiation from CD34+ cells and Mk proliferation.

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Culture medium support of megakaryocyte growth from different cell groups including BM MNC, BM $CD34^+$ and PB $CD34^+$ cells were also investigated (Figure 12). SCF, IL-3 & IL-6 were added to all cultures. As a positive control, IMDM BM MNC cultures were further supplemented with AS. HLTM cultures constantly yielded 2-3% megakaryocyte, no matter if they were derived from purified $CD34^+$ cells or un-purified BM MNC. Serum-free cultures, except for BM MNC, yielded a higher percentage of megakaryocyte than HLTM cultures. In addition, serum-free PB CD34 cell cultures grew more megakaryocyte (16.1 ± 4.3%) and expanded more (22.0 ± 6.0 fold) than serum-free BM CD34 cell cultures (9.3 ± 1.6% megakaryocyte, 8.9 ± 1.3 fold expansion) (Figure 12). Cell viability in both cultures was similar (80.0 ± 6.0% vs. 86.3 ± 1.3%). Therefore, the serum-free medium appeared to support megakaryocyte growth better in PB CD34 than in BM CD34 cell cultures, or there may have been more megakaryocyte precursors to begin with. On the other hand, the serum-free medium compared unfavorably with HLTM BM MNC cultures (Figure 12). In addition, the cell count increased in HLTM BM MNC (4-10 fold) but not in serum-free BM MNC cultures. The growth of megakaryocytes from BM MNC appeared to depend on the presence of stromal foci, which were formed in IMDM (containing human AS) and HLTM (containing animal serum) but not in serum-free cultures. The addition of AS to the serum-free medium on day 0 rendered the medium supportive of megakaryocyte growth from BM MNC in both HLTM and IMDM media (Figure 12).

To determine the effects of cytokines, paired PB $CD34^+$ cell cultures were set up and assayed for their ability to support megakaryocyte growth. Results from a typical experiment are presented in Figure 13. In HLTM cultures, the only effective cytokine combination was SCF + IL-3 + IL-6. In serum-free cultures, SCF + IL-6 or SCF + GM-CSF combinations supported baseline megakaryocyte growth. The addition of IL-3 to either of these combinations increased megakaryocyte proliferation

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(Figure 13). GM-CSF stimulated more cell proliferation than IL-6 when combined with SCF & IL-3, without changing the megakaryocyte frequency. Serum-free cultures supplemented with GM-CSF + SCF + IL-3 thus contained higher total cell count and megakaryocyte number than cultures supplemented with IL-6 + SCF + IL-3 (Figure 13, megakaryocyte cellularity). The same was true when comparing serum-free cultures supplemented with SCF, IL-3, GM-CSF & G-CSF (Experiment 1-3 in Figure 11) with those supplemented with SCF, IL-3 & IL-6 (Experiment 4-6 in Figure 11). The addition of 200 U/ml IL-11 to the SCF + IL-3 + GM-CSF combination did not stimulate further megakaryocyte proliferation (Figure 13). To exclude the possibility of improper dosage, IL-11 was titrated at concentrations between 50 and 800 U/ml with the SCF + IL-3 + IL-6 combination in PB $CD34^+$ cell cultures. Although high dose IL-11 (400 U\ml) might enhance cell proliferation, no obvious synergistic effects of IL-11 with this cytokine combination on megakaryocyte growth were observed.

As morphologically recognizable megakaryocyte were found in serum-containing but not in serum-free cultures, serum effects on megakaryocyte growth and morphology differentiation were evaluated. Human AS was added to cultures at different times. On day 0, paired BM CD34 cell cultures (n=3) were supplemented with AS alone, cytokines (SCF, IL-3 & IL-6) alone, or AS + cytokines. At day 10-12, AS-alone cultures showed a high megakaryocyte frequency (around 15%) but virtually no cell expansion (0.5 - 1 fold); while cytokines-alone and cytokines + AS cultures had a lower megakaryocyte frequency (around 8%) and a high cell expansion (around 20 fold and 38 fold, respectively). Morphologically recognizable megakaryocyte accounted for 1/3-1/2 and 1/7-1/3 of total $CD41a^-$ cells in AS-alone cultures and AS + cytokine cultures, respectively. Virtually no cells with megakaryocyte morphology were found in cytokine-alone cultures. However, when these cytokine-alone cultures were added with AS at day 10-12, morphologically

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recognizable megakaryocyte emerged 3-4 days later and accounted for 1/5-1/3 of total CD41a$^+$ cells. Megakaryocytes in control cultures (without AS addition) remained morphologically unrecognizable. BM MNC cultures initially depleted of megakaryocyte morphology and stimulated with As + cytokines yielded morphologically recognizable megakaryocytes (1/5-1/3 of total CD41a$^+$ cells) after 10-12 days of culture. These results indicated that serum can be used to facilitate megakaryocyte morphology differentiation whereas serum-free conditions tend to maintain an immature phenotype.

The above results demonstrate that the serum-free medium is better than the serum-containing HLTM medium in support of megakaryocyte growth from purified CD34$^+$ hemopoietic progenitors. PB CD34$^+$ cells showed a higher megakaryocyte potential than BM CD34$^+$ cells when they were grown in the serum-free medium. The majority of megakaryocyte derived from serum-free cultures expressed the GP IIb/IIIa antigen, and were morphologically unrecognizable as mature megakaryocytes. They could undergo further differentiation and acquired megakaryocyte morphology upon further stimulation by aplastic serum.

EXAMPLE 10

SERUM-FREE SUSPENSIONS OF HUMAN HEMATOPOIETIC CELLS

This example shows the preparation and growth of serum-free suspensions of human hematopoietic cells in media other than the 295-1 media described previously.

Apheresis blood products from breast cancer patients pre-treated with chemotherapy and G-CSF were obtained from the University of Chicago. The CD34$^+$ cells were selected from the apheresis blood products with the Isolex™ 50 (Baxter Healthcare, Corp., Fenwal Division) or the Isolex™ 300 (Baxter Healthcare, Corp., Immunotherapy Division, Irvine, CA) Magnetic Cell Separator. These systems are designed to select

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$CD34^+$ cells from apheresis blood using an anti-CD34 monoclonal antibody (9C5) and retrieval using Dynal IgG_1FC, ST magnetic beads. The 9C5 anti-CD34 monoclonal antibody was added to the apheresis blood product at 0.5 ug (stock vial at 1 ug/ul) per $1x10^4$ cells in the Isolex™ 50 or Isolex™ 300 column. The cells were then rotated for 30 minutes on the Isolex™ rotator. The cells were washed twice, in order to remove the unbound 9C5 monoclonal antibody, in RPMI 1640 containing 1% human serum albumin (HSA). the cells were then diluted in the column to $1-2x10^7$ cells/ml with RPMI 1640 containing 10 mg/ml (1%) HSA and 1 mg/ml (0.1%) Gammagard® (Baxter Healthcare Corp., Hyland Division, Glendale, CA). Dynal IgG_1FC, ST magnetic beads (Dynal, Oslo, Norway) were added to the cell suspension at 0.5 bead to cell ratio and rotated for 30 minutes on the Isolex™ rotator. The CD34 cell:bead complexes were then removed using a magnet.

The $CD34^+$ cell:bead complexes were resuspended to $3x10^5$ cells/ml in X-VIVO 10 (BioWhittaker, Inc., Walkersville, MD) containing 1% HSA and PIXY 321 at 100 ng/ml (Complete Media). PIXY is a fusion protein containing the active domains of both IL-3 and GM-CSF. Depending on the number of cell:bead complexes obtained, the suspension was then placed in a gas-permeable culture bag (Baxter, Roundlake, IL) and incubated for 48 hours in a cell culture incubator set at 5% carbon dioxide and 37°C. The starting culture medium was 20-50 mls in volume. These containers were referred to as the primary culture containers.

Following a 48 hour culture release period the released cells were retrieved from the culture by removal of the magnetic beads using the MaxSep™ Cell Separator (Baxter Healthcare, Corp., Irvine, CA) (Day 9 of culture). The released cells were then transferred into a secondary culture container and cultured as described in Example 3. At day 5, the cultures were analyzed for cell number and cell viability using a

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hemacytometer and trypan blue viability staining. The cell concentration was then readjusted to $1x10^5$ cells/ml with Complete Media and cultured for an additional 5 days. Flow cytometry, colony assays and morphology evaluations were performed as described in Examples 5-7.

Table 10 below summarizes the results of 5 experiments for cell proliferation at day 5 and 10 in which the viable proliferation index (P.I.) was calculated by dividing the total number of cells at day 5 or 10 by the total number of cells at day 0 and then multiplying by the % viable.

TABLE 10
VIABLE PROLIFERATION INDEX AT DAY 5 AND DAY 10 OF CULTURE

CULTURE I.D.	DAY 5	DAY 10
A.F. (11/22/93)	2	7
L.D. (11/22/93)	2	14
C1514 (12/11/93)	4	14
C1515 (12/11/93)	8	40
V.T. (12/31/93)	2	25
MEAN ±S.D. (n=5)	4 ±2	20 ±13

The increases in cell number, total CFU, clusters, CFU-GM, $CD15^-$ cells, and early granulocytes at day 10 of culture were also determined. The increases in total CFU, clusters, and CFU-GM were calculated from the change in the numbers present at day 0 to the final numbers at day 10 relative to the P.I. (Table 11). The increase in $CD15^+$ cells and early granulocytes at day 10 of culture takes into account that 80 ± 17% (mean purity) of the cells were $CD34^-$ and defined as blasts at day 0.

These results show that the serum-free X-VIVO 10 media supplemented with only 1% HSA and the IL-3 and GM-CSF growth

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factors were able to support the proliferation of neutrophil progenitors and maintain good cell viability (89 ± 3%) in a 10-day liquid culture (Table 11). As stated above the cells used in these experiments had a mean CD34 purity of 80 ± 20% when selected with the magnetic cell separator (Table 12). The percentage of $CD15^+$ cells, CFUs and cell morphology are presented in Tables 12-14. The distribution of early granulocytes as observed by cell morphology were not significantly different as shown in Table 14. However, the total number of early granulocytes was significantly different when the number of cells counted per 100 cells was multiplied by the P.I.

TABLE 11
PROLIFERATION INDEX AT DAY 5 AND 10,
PERCENT OF VIABLE CELLS AT DAY 0, 5, AND 10

	DAY 0	DAY 5			DAY 10		
CULTURE	% VIABLE	P.I.	% VIABLE	VIABLE P.I.	P.I.	% VIABLE	VIABLE P.I.
MEAN (n=5)	90	4.0	89	3.6	22.9	89	20.0
STDEV	8	2.6	4	2.4	15.5	3	13.0

TABLE 12
CD34 PURITY AT DAY 0, CD15 AND CD11B
PHENOTYPE AT DAY 10 OF CULTURE

CULTURE	%CD34 DAY 0	$\%CD15^-$ $\%CD11b^-$	$\%CD15^-$ $\%CD11b^+$	$\%CD15^+$ $\%CD11b^+$	$\%CD15^+$ $\%CD11b^-$	TOTAL $\%CD15^+$
MEAN (n=5)	80	36.05	22.38	15.52	26.05	41.57
STDEV	21	8.11	6.39	1.78	6.32	6.23

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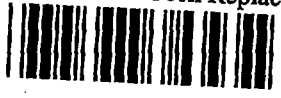


TABLE 13
COLONY FORMING UNITS (CFU) PER 10,000 CELLS
AT DAY 0 AND DAY 10 OF CULTURE

	DAY	CFU-GM	CFU-Mac	BFUE	CFU-MIX	TOTAL CFU	CLUSTERS
CULTURE	0	652	122	318	82	1174	41
MEAN (n=5)	10	51	3	108	3	165	28
CULTURE	0	334	124	337	105	806	46
STDEV	10	19	3	75	4	5	23

TABLE 14
CELL MORPHOLOGY PER 100 CELLS AT DAY 10 OF CULTURE

		EARLY GRANULOCYTES			
CULTURE	BLASTS	PROMYELO-CYTES	MYELO-CYTES	MYTAMYELO-CYTES	MONOCYTES
MEAN (n=5)	20	38	26	1	14
STDEV	15	15	15	1	5

In a separate experiment, the growth and differentiation of $CD34^+$ cells into megakaryocytes in X-VIVO 10 supplemented with various combinations of IL-3, SCF and IL-6 was assessed. Briefly, $CD34^+$ cells were cultured in X-VIVO 10 media containing 1% HSA, 300 U/ml IL-3 and 20 ng/ml SCF with or without the addition of 40 ng/ml IL-6. At day 11 of culture, cell counts were performed and analyzed for the percentage of $CD41a^+$ cells by immunocytochemistry. The results from 5 independent experiments are shown below in Tables 15-17. The data indicate that 1) $CD41a^+$ cells can be generated in this serum-free media under these conditions, and 2) that there was no significant difference seen on the proliferation index of $CD34^+$ cells, percentage of $CD41a^+$ cells and the total number of megakaryocytes in the presence or absence of IL-6. Thus,

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the human hematopoietic cell compositions described herein can be cultured in serum-free media containing, for example, just IL-3 and SCF.

TABLE 15
PROLIFERATION OF DAY 12 CULTURED $CD34^+$ CELLS

SAMPLE	NO IL6	R&D IL6 40 ng/ml
MEAN	31.00	35.10
S.D.	13.30	15.50

TABLE 16
TWO PERCENT CD41a POSITIVE CELLS IN DAY 12 CULTURED $CD34^+$ CELLS

SAMPLE	NO IL6	R&D IL6 40 ng/ml
MEAN	27.0	21.6
S.D.	21.20	10.50

TABLE 17
TOTAL NUMBER OF $CD41a^+$ CELLS IN DAY 12 CULTURED $CD34^+$ CELLS $x10^6$

SAMPLE	NO IL6	R&D IL6 40 ng/ml
MEAN	6.25	5.90
S.D.	3.68	1.63

EXAMPLE 11

COMPARISONS OF SERUM-FREE SUSPENSIONS OF HUMAN HEMATOPOIETIC CELLS

This example compares the proliferation of human hematopoietic stem/progenitor cells in various serum-free media and characterizes the precursor cells produced from the different cultures.

The isolation and culture of $CD34^+$ hematopoietic cells from peripheral blood (PB) was performed by methods similar to

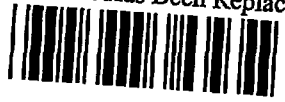
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those described in Example 9. Apheresis products from five patients who received G-CSF during recovery from cyclophosphamide chemotherapy were used for the comparison of proliferation and precursor production. Briefly, following isolation of $CD34^+$ cells, cultures were seeded on day 0 with the selected cells at a density of $1\text{-}5x10^4$ cells/ml in either X-vivo 10, X-vivo 15 or X-vivo 20 (BioWhittaker). The media was supplemented with 300 U/ml IL-3, 40ng/ml IL-6 and 20 ng/ml SCF and the cultures were incubated at 37°C, 5% CO_2, 5% O_2 and high humidity for 8-12 days. The percentage of $CD34^+$ cells was determined as described in Example 9. Similarly, procedures for flow cytometry, cell morphology determination and immunochemistry were performed as described in Example 9.

To determine whether either of the three medias resulted in differential isolation of $CD34^+$ cells, the percentage of $CD34^+$ cells was determined by flow cytometry at day 0 of culture. The results showed no difference in purity with $CD34^+$ cells comprising between 92% and 99% of the cultures in each of the medias. Similarly, no difference was seen in the cell viability or morphology following release in either the X-vivo 10, X-vivo 15 or X-vivo 20 medias.

Expansion of the $CD34^+$ cells was determined at the end of the culture period by calculation of the proliferation index (total number of cells at day 10/number of cells at day 0). Results determined from all five cultures showed no significant difference between the serum-free medias with the mean proliferation indexes being 95.8±22.0, 112.0±7.4 and 170.2±39.5 for X-vivo 10, 15 and 20, respectively. However, in three of the five cultures, the proliferation index of cells grown in X-vivo 20 was significantly higher then those observed using the either of the other two medias ($p=0.047$, paired t test). These results indicate that all three serum-free medias support the growth of human hematopoietic cells.

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In cultures that are highly proliferatory, X-vivo 20 can out perform either X-vivo 10 or 15 for cell expansion.

The percentage of early neutrophil precursors ($CD15^+/CD11b^-$) was also determined in each of the serum-free medias. The results obtained again showed no significant difference in any of the medias. Specifically, the mean percentage of neutrophil precursors for each of the five cultures was 20.76±5.98, 17.20±6.63 and 17.63±6.20 for X-vivo 10, X-vivo 15 and X-vivo 20, respectively. Conversely, the percentage of more mature neutrophils was less then 5% in all cultures in the 10 day time period.

Megakaryocyte percentages was also determined in the above cultures at the end of the culture period. The results showed that X-vivo 10 had a significantly higher percentage of $CD41a^+$ cells as determined by flow cytometry compared to X-vivo 15 and 20 ($p=0.0247$ and $p=0.0211$ paired t test, respectively). When assessed by immunochemistry, the data parallelled the results obtained by flow cytometry. The results are shown below in Table 18.

TABLE 18
PERCENTAGE OF MEGAKARYOCYTES ($CD41a^+$) FROM $CD34^+$ CELLS CULTURED IN SERUM-FREE MEDIA

SAMPLE	X-VIVO 10		X-VIVO 15		X-VIVO 20	
	FACS	ICC	FACS	ICC	FACS	ICC
MEAN	9.39	10.25	6.00	6.60	6.37	8.50
± S.D.	3.75	1.70	3.47	3.43	3.43	5.19

FACS: percentage $CD41a^+$ cells determined by flow cytometry
ICC: percentage $CD41a^+$ cells determined by immunochemistry

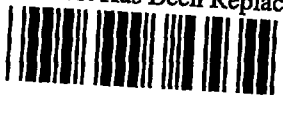
The total number of neutrophil precursors and megakaryocyte cells expanded in each of the three serum-free medias was also

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assessed. The cell expansion for each lineage was determined by multiplying the initial number of $CD34^+$ cells (10^6) by the proliferation index at day 10 and by the percentage of cells within the particular lineage. For both lineages, the results indicate that there was no significant difference between each of the medias. For example, the fold expansion of neutrophil precursors was 20.9±4.7, 17.20±2.98 and 17.63±2.77 in X-vivo 10, 15 and 20, respectively. Fold expansion for megakaryocytes was determined to be 9.77±3.1, 6.6±1.9 and 12.2±4.7 in X-vivo 10, 15 and 20, respectively.

Collectively, the above results demonstrate the feasibility of <u>in vitro</u> expansion of $CD34^+$ cells into neutrophil precursors and megakaryocytes in serum-free media to obtain serum-free suspensions of human hematopoietic cells.

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What is claimed is:

1. A serum free medium to be used in conjunction with hematopoietic growth factors for the <u>in vitro</u> growth of human neutrophil and megakaryocyte precursors, comprising

a base medium, corticosteroid, transferrin, insulin, cholesterol, ethanolamine, and human albumin.

2. The serum free medium of claim 1 wherein said human albumin is deionized.

3. The serum free medium of claim 1 wherein said corticosteroid is hydrocortisone.

4. The serum free medium of claim 1 further comprising at least one lipid selected from the group consisting of triglycerides and phospholipids.

5. The serum free medium of claim 4 wherein said lipid is a triglyceride having fatty acid side chains selected from the group consisting of linoleic acid, oleic acid, palmitic acid, linolenic acid, and stearic acid.

6. The serum free medium of claim 4 wherein said lipid is a phospholipid selected from the group consisting of phosphatidylcholine and phosphatidylethanolamine.

7. The serum free medium of claim 3 further comprising selenium, alpha-monothioglycerol, beta-mercaptoethanol, and HEPES buffer.

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8. The serum free medium of claim 7, further comprising the following concentrations:

hydrocortisone, 1-10 μM
cholesterol, 0.01 - 0.1 mg/ml
triglycerides, .0034 - .034 mg/ml
transferrin, 25 to 125 μg/ml
insulin, 1 to 25 μg/ml
alpha-monothioglycerol, 10 - 160 μM
beta-mercaptoethanol, 20 - 100 μM
ethanolamine, 50 to 200 μM
human albumin, 0.1 to 25 mg/ml
HEPES buffer, 10 to 25 mM

9. The serum free medium of claim 8 comprising the following concentrations:

hydrocortisone, 200 ng/ml
cholesterol, 0.05 mg/ml
triglycerides, 0.017 mg/ml
transferrin, 75 μg/ml
insulin, 10 μg/ml
alpha-monothioglycerol, 216 ng/ml
beta-mercaptoethanol, 5 x 10^{-5} M
ethanolamine, 100 μM
human albumin, 10 mg/ml
HEPES buffer, 25 mM

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10. A method for preparing a serum-free suspension of human hematopoietic precursor cells wherein the cellular component comprises at least about 16% neutrophil precursors and at least about 1% megakaryocyte precursors said method comprising the steps of;

(a) obtaining hematopoietic stem/progenitor cells from bone marrow, peripheral blood, or cord blood,

(b) culturing said stem/progenitor cells for about 7 to 14 days in a serum free culture medium containing hematopoietic growth factors, said medium comprising,

a base medium, corticosteroid, transferrin, insulin, cholesterol, ethanolamine, and human albumin.

11. The method of claim 10 wherein said human albumin is deionized.

12. The method of claim 10 wherein said corticosteroid is hydrocortisone.

13. The method of claim 10 further comprising at least one lipid selected from the group consisting of triglycerides and phospholipids.

14. The method of claim 13 wherein said lipid is a triglyceride having fatty acid side chains selected from the group consisting of linoleic acid, oleic acid, palmitic acid, linolenic acid, and stearic acid.

15. The method of claim 13 wherein said lipid is a phospholipid selected from the group consisting of phosphatidylcholine and phosphatidylethanolamine.

16. The method of claim 12 further comprising selenium, alpha-monothioglycerol, beta-mercaptoethanol, and HEPES buffer.

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17. A cell suspension prepared by the methods of any one of claims 10 - 16.

18. A serum-free suspension of human hematopoietic cells, wherein the cellular component comprises at least about 16% neutrophil precursors and at least about 1% megakaryocyte precursors, said cell suspension being essentially free of animal proteins.

19. The cell suspension of claim 18 wherein the cellular component comprises at least about 30% neutrophil precursors.

20. The cell suspension of claim 19 wherein the cellular component comprises at least about 60% neutrophil precursors.

21. The cell suspension of claim 18 wherein said neutrophil precursors are selected from the group consisting of blast cells, promyelocytes, neutrophilic myelocytes, and neutrophilic metamyelocytes.

22. The cell suspension of claim 18 wherein the cellular component comprises at least about 3% megakaryocyte precursors.

23. The cell suspension of claim 22 wherein the cellular component comprises at least about 8% megakaryocyte precursors.

24. The cell suspension of claim 18 further comprising about 3 to 25% monocytes, and less than about 0.01% mast cells.

25. The cell suspension of claim 18 further comprising colony-forming units (CFU) and cluster-forming units (clFU).

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26. The cell suspension of claim 25 wherein at least about 40% of the total CFU/clFU are granulocyte-macrophage colony forming units, and at least about 40% of the total CFU/clFU are granulocyte cluster forming units.

27. A serum-free suspension of human hematopoietic cells wherein at least about 20% of the cells are CD15+/CD11b-, said cell suspension being essentially free of animal proteins.

28. A method for determining the number of megakaryocyte precursors in a suspension of hematopoietic cells, comprising

(a) combining a sample of said cell suspension with fibrinogen and thrombin to form a mixture,

(b) placing said mixture on a microscope slide, said mixture forming a fibrin clot containing said hematopoietic cells,

(c) culturing said cells in said fibrin clot for 14 days, said megakaryocyte precursor cells proliferating to form megakaryocytes,

(d) fixing said cells within said fibrin clot,

(e) contacting said fixed cells with a primary antibody specific for platelet glycoprotein IIb/IIIa, said primary antibody thereby specifically binding to megakaryocytes,

(f) labeling said megakaryocytes by means of a secondary antibody and immunoperoxidase color-forming reaction, said megakaryocytes thereby acquiring a specific color,

(g) assessing the number of megakaryocytes labeled with said specific color within the total cell population, and

(h) extrapolating the number of megakaryocyte precursor cells present in the original suspension of hematopoietic cells.

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29. A method for preparing a differentiated suspension of human megakaryocytes wherein the cellular component comprises morphologically recognizable megakaryocytes of the CD41a$^-$ cell population comprising the steps of:

(a) obtaining hematopoietic stem/progenitor cells from bone marrow, peripheral blood, or cord blood,

(b) culturing said stem/progenitor cells in a serum free culture medium containing at least one hematopoietic growth factor, and

(c) subsequently supplementing the cultures with serum.

30. The method of claim 29 wherein said serum is aplastic serum.

31. The method of claim 29, wherein said hematopoietic growth factor is selected from the group consisting of IL-3, G-CSF, GM-CSF, SCF, thrombopoietin, or a recombinant protein containing one or more active moieties of a hematopoietic growth factor.

32. A cell suspension prepared by the method of claim 29.

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