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(54) Title: YOLK SAC STEM CELLS

(57) Abstract

The present invention is directed to yolk sac stem cells. In particular, it relates to the characterization, culturing, and uses of yolk sac stem cells for hematopoietic reconstitution and therapy. Yolk sac stem cells isolated from the early embryonic yolk sac prior to blood island formation exhibit a homogeneous morphology and primitive cell surface phenotype without the expression of mature leukocyte markers and major histocompatibility complex encoded antigens. The cells can be cultured and expanded long-term without alteration of their pluripotency. Therefore, yolk sac stem cells may have a wide range of applications including but not limited to the reconstitution of a destroyed or deficient human hematopoietic system, and the construction of large and small animal models for the production of human blood cells, human antibodies, and testing of human diseases, immune function, vaccines, drugs and immunotherapy.

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YOLK SAC STEM CELLS

1. INTRODUCTION

The present invention is directed to yolk sac stem cells. In particular, it relates to the characterization, culturing, and uses of yolk sac stem cells for hematopoietic reconstitution and therapy. Yolk sac stem cells isolated from the early embryonic yolk sac prior to blood island formation exhibit a homogeneous morphology and a primitive cell surface phenotype without the expression of mature leukocyte markers and major histocompatibility complex encoded antigens. The cells can be cultured and expanded long-term without alteration of their pluripotency. Therefore, yolk sac stem cells may have a wide range of applications including but not limited to the reconstitution of a destroyed or deficient human hematopoietic system, and the construction of large and small animal models for the production of human blood cells, human antibodies, and testing of human diseases, immune function, vaccines, drugs and immunotherapy.

2. BACKGROUND OF THE INVENTION

A multipotential stem cell population is capable of giving rise to blood cells of diverse morphology and function (Golde, 1991, Scientific American, December:86). Since blood cell formation is first detectable in the embryonic yolk sac early in embryogenesis, it has been hypothesized that pluripotent hematopoietic stem cells may be present within the yolk sac, but the characteristics of such cells are still poorly understood and such cells have not heretofore been identified (Moore and Metcalf, 1970, 18:279). During fetal development, the stem

cells migrate to the fetal liver where they reside temporarily, and eventually move to give rise to the bone marrow which is the permanent site of blood cell formation in the adult. Studies on the development of blood cells have led to the identification of a variety of important growth and differentiation factors that regulate hematopoiesis. Further, tissue typing technology has ushered in dramatic advances in the use of hematopoietic stem cells as a form of therapy in patients with deficient or abnormal hematopoiesis.

2.1. HEMATOPOIETIC STEM CELLS

A pluripotent stem cell is believed to be capable of self-renewal and differentiation into blood cells of various lineages including lymphocytes, granulocytes, macrophages/monocytes, erythrocytes and megakaryocytes (Ikuta et al., 1992, Ann. Rev. Immunol. 10:759). The mechanism by which a stem cell commits to a specific cell lineage has not been fully elucidated. The mechanisms involved in stem cell replication without differentiation are also unknown. However, it is clear that such events must, in part, be influenced by a variety of growth and differentiation factors that specifically regulate hematopoiesis. Other factors which are not yet identified may also be involved (Metcalf, 1989, Nature 339:27). The commonly known hematopoietic factors include erythropoietin (EPO), granulocyte/macrophage colony stimulating factor (G/M-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating (M-CSF), interleukin 1-12 (IL-1 to IL-12), and stem cell factor (SCF).

An understanding of hematopoiesis is critical to the therapy of hematopoietic disorders.

Neoplastic transformation, immunodeficiency, genetic abnormalities, and even viral infections all can affect blood cells of different lineages and at different stages of development. For example, basic knowledge of blood cell development has contributed to the success of bone marrow transplantation in the treatment of certain forms of hematopoietic malignancies and anemias.

Conventional therapy utilizes whole bone marrow harvested from the iliac crest but this approach has certain limitations. Bone marrow stem cells are present at extremely low concentrations, and they may not be at the earliest stage of differentiation. An impediment in bone marrow transplantation is the need for matching the major histocompatibility complex (MHC) between donors and recipients through HLA tissue typing techniques. Matching at major loci within the MHC class I and class II genes is critical to the prevention of rejection responses by the recipient against the engrafted cells, and more importantly, donor cells may also mediate an immunological reaction to the host tissues referred to as graft versus host disease. In order to facilitate graft acceptance by the host, immunosuppressive agents often have been employed, which render the patients susceptible to a wide range of opportunistic infections.

Hollands examined the in vivo potential of embryonic cells, and found that day 7 embryonic mouse cells could colonize the hematopoietic system of normal non-irradiated allogeneic mice (Hollands, 1988, British J. Haematol. 69:437). However, it was not clear which embryonic cell population actually contributed to this result, as total embryonic cells were used for in vivo transfer. In a study on the

effects of in utero cell transfer, day 9 yolk sac cells were injected into syngeneic fetuses which differed from the donor cells only at the β-globin locus (Toles et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:7456). The donor cells were shown to induce hematopoiesis. Both of these in vivo studies utilized freshly isolated cells from mouse embryos, and there was no suggestion that long-term cultured and expanded embryonic yolk sac cells could retain their pluripotency. Such methods involved diffusion chambers embedded within the species of origin of the yolk sac tissue (Symann et al., Exp. Hemat. 6:749, 1978) or methods that led to in vitro malignant transformation of the yolk sac cells. For example, long-term yolk sac cell lines were established from day 10-13 mouse embryos, and they were shown to give rise to tumor cells at high frequency (Globerson et al., 1987, Differentiation 36:185). Therefore, the potential of tumor formation renders such long-term cultured cells undesirable for use in reconstitution therapy.

2.2. MAJOR HISTOCOMPATIBILITY COMPLEX

The MHC is a highly polymorphic complex of genes (Bach and Sachs, 1987, New Eng. J. Med. 317:489). It was first discovered by its close association with the phenomenon of transplantation rejection of tissue grafts. Subsequent studies conclusively demonstrated that antigens encoded by MHC class I genes are the major targets of transplantation rejection responses. Such antigens are expressed by all somatic cells.

MHC class II genes encode molecules on a limited array of cells, most of which are related to

the hematopoietic system. They can also elicit reactions by allogeneic immune cells.

Studies on the expression of MHC antigens by embryonic yolk sac cells yielded inconsistent results. Billington and Jenkinson (Transplantation 18:286, 1974), working with cells of the yolk sac of 10-14 day mouse embryos, found that these cells expressed both H-2 and non-H2 (murine major and minor histocompatibility) antigens. The work of Patthey & Edidin (Transplantation 15:211, 1973), cited by Billington and Jenkinson, reported that H-2 antigens first appeared on day 7 embryos which could provoke a strong immune reaction, but the latter suggested that these antigens did not make an appearance in utero until day 9 or later. See THE EARLY DEVELOPMENT OF MAMMALS 219 (Balls and Wild, eds., Cambridge U.:1975). Heyner reported that H-2 antigens were detectable in day 7 mouse embryos (Heyner, 1973, Transplantation 16:675). Further, mouse yolk sac cells obtained at day 9 of gestation were shown to be capable of generating a graft-versus-host response in vitro (Hofman and Globerson, 1973, Eur. J. Immunol. 3:179). However, Parr et al. demonstrated that H-2 antigens were absent on the apical or the laterobasal membrane of the mouse yolk sac endoderm even at day 20 of pregnancy (Parr et al., 1980, J. Exp. Med. 152:945). Thus, no consensus has been established in regard to the antigenicity of yolk sac cells.

3. SUMMARY OF THE INVENTION

The present invention relates to yolk sac stem cells, a method of isolating and culturing yolk sac stem cells, and a method of using the cultured yolk sac cells for reconstituting an allogeneic or xenogeneic hematopoietic system.

The invention is based, in part, on Applicants' discovery that the murine yolk sac, isolated from mouse embryos prior to visible blood island formation, contains a homogeneous population of cells that are $CD34^+$, $Thy\text{-}1^-$, MHC class I^- and class II^-. Such cells can be expanded in number by long-term in vitro culture with minimal differentiation, and can give rise to mature blood cells of diverse lineages when subsequently treated with the appropriate hematopoietic growth and differentiation factors. Further, the long-term cultured cells also can mature into functionally competent blood cells in vivo, capable of mediating antigen-specific immune responses, repopulating lympho-hematopoietic organs, and prolonging survival of animals with a destroyed hematopoietic system. The yolk sac cells of the invention can be successfully transplanted into allogeneic fetuses in utero and into non-immunosuppressed xenogeneic hosts and since these cells do not induce graft-versus-host and host-versus-graft reactions, transplantation will result in tissue chimerism.

The invention is described by way of examples in which murine yolk sac cells are isolated, and their cell surface phenotype is characterized. The homogeneous population of yolk sac cells is expanded in long-term culture, and shown to retain pluripotency in vitro and in vivo. A wide variety of uses for the yolk sac cells are encompassed by the invention described herein.

4. BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. A schematic drawing of the appearance of mouse embryos around day 7 and day 8.5 of gestation.

FIG. 2. Murine yolk sac cells from a day 7 embryo are more homogeneous in appearance than cells from a day 8.5 embryo by flow cytometry analysis.

FIG. 3. Murine yolk sac cells from a day 7 embryo express CD34 but not Thy-1, MHC class I and class II antigens.

FIG. 4. Cultured yolk sac cells can differentiate into mature blood cells in vitro, including (4A) monocytes, (4B) megakaryocytes, (4C) erythrocytes, and (4D) lymphocytes.

FIG. 5. Yolk sac cells recovered from recipient mouse spleens following in vivo transfer demonstrate the expression of mature leukocyte antigens by donor cells.

FIG. 6. Hemagglutination of red blood cells coated with antigens (FIG. 6A, lipopolysaccharide, and FIG. 6B, human serum albumin) by sera of immunodeficient mice treated with yolk sac cells, demonstrating restoration of immune function by yolk sac cells in vivo.

FIG. 7. Cultured yolk sac cells repopulate the spleens of chemically-ablated mice and give rise to colony-forming units in vivo; (7A) A comparison between a chemo-ablated mouse spleen and a fully repopulated spleen; (7B) A repopulated spleen at day 7 post-yolk sac treatment; (7C) A populated spleen at day 14 post yolk-sac treatment.

FIG. 8. In utero injection of yolk sac cells into allogenic mice leads to tissue chimerism in new born mice.

FIG. 9. Survival and differentiation of long-term cultured murine yolk sac cells in a sheep and a goat which had received multiple high doses of yolk sac cells.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to yolk sac stem cells, to methods of isolating and culturing the yolk sac stem cells, and to methods of using the yolk sac stem cells.

Although the specific procedures and methods described herein are exemplified using murine yolk sac cells, they are merely illustrative for the practice of the invention. Analogous procedures and techniques are equally applicable to all mammalian species, including human subjects. Therefore, human yolk sac stem cells may be isolated from the embryonic yolk sac prior to blood island formation. The cells having the phenotype of $CD34^+$, $Thy\text{-}1^-$, and MHC class I^- and II^- may be cultured under the same conditions described herein, infra.

Mammalian development may be divided into three distinct stages: the zygote, from fertilization to cleavage; the embryo, from cleavage to the formation of all somites; and the fetus, from the formation of the last somite until birth. This invention takes advantage of the unique properties of embryonic yolk sac cells after their course of development is determined, but before they have lost either immuno-incompetency or the ability to proliferate rapidly.

It is known that when completely undifferentiated cells of the blastula or morula are transplanted into a developed animal, they produce tumors. These totipotent, tumorigenic cells are of no value for in vivo reconstitution therapy. However, in accordance with the invention, it is advantageous to transplant cells which have reached a stage of specialization at which they have become committed to a particular sequence of development, or lineage. Such cells may be used alone or to deliver genetic material, or its expression products, into a particular tissue of the body, including blood cells. The cells can be transplanted into a host before or after transformation with an exogenous gene of interest, and allowed to develop into the target tissue.

While it is necessary to use cells which have matured to the point of losing totipotency, fully mature cells will be rejected by a histoincompatible host. Consequently, it is desirable to use cells which have just lost totipotency, but still retain pluripotency for a particular tissue type. Such cells also may retain the ability to colonize, thus facilitating their delivery to the target tissue.

Stem cells of the embryonic yolk sac offer particular advantages for hematopoietic reconstitution. Unlike the cells of the embryo, the cells of the yolk sac develop into only a small number of different tissues. Among those tissues is the hematopoietic system, which includes the red and white blood cells, and the tissue of the veins, arteries and capillaries. Thus, by day 8 in the development of the mouse embryo, mesodermal cells in the yolk sac begin to form blood islands. The cells of the blood islands differentiate, the peripheral cells becoming the

endothelium of the future blood vessels, and the central cells becoming first mesenchymal cells and then the red and white blood cells. The blood islands establish communications to form a circulatory network, which is extended into the embryo proper.

The yolk sac cells of the subject invention do not express MHC antigens, and can mature in allogeneic and xenogeneic hosts, demonstrating their ability to escape immune rejection. By contrast, research with bone marrow cells has depended on the use of immunocompromised hosts. The culture methods described herein maintain the yolk sac in their undifferentiated state, and are applicable to mass culture of yolk sac cells, providing donor cells for large numbers of recipients.

5.1. ISOLATION OF YOLK SAC CELLS

The embryonic yolk sac is the first identifiable site of blood cell formation in ontogeny. The yolk sac cells travel to the fetal liver during embryogenesis and eventually migrate to the bone marrow where they reside and differentiate into mature blood cells throughout the entire adult life.

The embryonic development of the mammalian yolk sac is rapid and occurs within a narrow time frame. The murine yolk sac is fully formed by day 7 of gestation, and the formation of blood is detectable in the mesenchyme of the body stalk and in neighboring areas of the yolk sac. Shortly thereafter, masses of mesenchymal cells round up and become aggregated to form blood islands. By day 8.5, extensive blood island formation in the murine yolk sac is readily visible microscopically. At this stage, embryonic development has reached a level where fetal liver is formed and yolk sac cells begin to migrate to the

fetal liver. Upon the departure of the yolk sac stem cells, the yolk sac begins to atrophy. Similar events also occur in embryonic development of other species, but the timing of developmental events varies between different species. In humans, the yolk sac is formed by day 10 of gestation, and blood island formation occurs shortly thereafter. Thus, human yolk sac cells isolated at day 10 may be comparable to the murine cells at day 7.

Since the yolk sac is where blood cell formation is first established in development and the yolk sac cells eventually reach the bone marrow to become the bone marrow hematopoietic cells, it is reasoned that the yolk sac represents the earliest site for the generation of primordial hematopoietic cell precursors. The cells have committed to the hematopoietic differentiative pathway so that they are no longer totipotent. However, the yolk sac cells are still pluripotent, since they have not yet committed to a particular blood cell lineage as seen by their ability to make cells of lymphoid, myeloid, and erythroid lineages. Hence, yolk sac cells may be the ideal cell population for use in reconstitution therapy including, but not limited to, bone marrow transplantation. In addition, the primitive nature of these cells, as evidenced by the absence of cell surface expression of various mature markers and MHC transplantation rejection antigens, may render these cells uniquely capable of being used as a universal donor cell population in allogeneic and even xenogeneic hosts.

The isolation of the embryonic yolk sac may be achieved using a variety of surgical methods. Traditionally, the yolk sac of a mouse embryo is disaggregated by the use of enzymatic digestion and

mechanical separation upon surgical removal. A gentler method of detaching the cells from the yolk sac membrane and separating them from each other is described in Section 6.2.1. in which a yolk sac is immersed in an EDTA solution which causes the cells to segregate and form a single cell suspension. This method minimizes cell lysis due to physical force and cell surface protein alteration due to enzymatic treatment.

Since the establishment of blood islands in the yolk sac marks the beginning of cellular differentiation and blood cell formation, it is preferable that yolk sac cells be isolated prior to extensive blood island formation. Large numbers of highly homogeneous yolk sac cells of day 7 murine embryos (or similar stage human yolk sac cells), can be isolated using the method described herein, and cells obtained at this stage should in principle contain the least committed and least differentiated pluripotent stem cells suitable for long-term in vitro culture, for use in immediate in vivo therapy or as carriers of specific exogenous genes for use in gene therapy.

For long-term maintenance of the yolk sac cells, the cells are grown in medium containing a relatively high concentration of serum supplement, between 15-20%. Various cytokines may be added to suppress differentiation of the stem cells, including but not limited to, leukemia inhibitory factor (LIF) or stem cell factor/the c-kit ligand (SCF) or SCF in combination with other cytokines such as IL-3. Such factors accelerate the multiplication of cultured cells, while inhibiting cellular differentiation in vitro. The examples presented in Section 6, infra, were all performed using yolk sac cells grown in the

presence of 10-100 U/ml of LIF. However, higher LIF concentrations may be used to achieve stronger suppression of differentiation. The growth of cells using SCF could produce similar results.

Alternatively, a number of other known hematopoietic factors such as IL-3, CSF's and EPO also may be used in combination depending on the need to select for a particular cell type. For example, the combined use of IL-3 and EPO may assist in driving cultured yolk sac cells towards the erythroid pathway. The maintenance of cells at the appropriate temperature, CO_2 concentration, humidity level and the frequency of changing the culture media are within the ordinary skill of the art.

5.2. CHARACTERIZATION OF YOLK SAC CELLS

As shown by the examples described herein, yolk sac cells obtained from mouse embryos prior to blood island formation are more homogeneous in appearance than cells obtained at a later stage. Freshly isolated yolk sac cells from day 7 and day 8.5 murine embryos were compared by light scattering using flow cytometry analysis, see Section 6.2.1., infra. It is apparent that yolk sac cells of day 7 mouse embryos are extremely uniform with respect to both cell size and cell shape. By day 8.5, distinct populations of cells are clearly visible, suggesting that the earlier stage yolk sac cells may be clonally derived and the difference of 1 day in development may be critical to the nature of the yolk sac cells.

Another indication of the primitive nature of the early yolk sac cells is their cell surface phenotype in regard to the expression of various lineage-specific blood cell markers. This form of analysis may be most conveniently carried out by the

use of a panel of marker-specific monoclonal antibodies. When the day 7 yolk sac cells were reacted with antibodies, the results showed that they lacked expression of all mature blood cell markers. In addition, such cells did not express MHC-encoded products which are the major targets of transplantation rejection responses. Thus, yolk sac stem cells can be characterized as $CD34^+$, $Thy\text{-}1^-$, MHC class I^- and MHC class II^-. Similarly, human yolk sac stem cells obtained from day 10 embryos should display an identical cell surface phenotype.

The CD34 and Thy-1 markers previously have been demonstrated to be associated with bone marrow hematopoietic stem cells (Spangrude et al., 1988, Science 241:58). While CD34 expression declines as stem cells differentiate and mature, the presence of Thy-1 is retained and its density increased in certain mature blood cells, particularly T-lymphocytes. The finding that yolk sac stem cells are positive for CD34 expression is consistent with these cells being stem cells. However, the absence of Thy-1 expression suggests that yolk sac cells may represent an earlier cell population than the bone marrow stem cells which express low levels of Thy-1 in the bone marrow microenvironment. In fact, when yolk sac cells are cultured in vitro, a small percentage of the cells escape the effect of LIF and begin to express Thy-1, further suggesting that Thy-1 expression is a later event of stem cell development.

MHC-encoded class I and class II molecules are involved in immune regulation between T, B, and antigen presenting cells. These highly polymorphic molecules also serve as targets in major transplantation rejection responses between genetically mismatched individuals. Therefore, HLA

tissue typing is currently a routine clinical procedure in ensuring graft acceptance in human transplant patients by matching the donors and recipients at the major MHC genetic loci. The absence of MHC antigens on the yolk sac cell surface strongly suggests the possibility of using such cells as universal donors in hematopoietic reconstitution therapy, alleviating the need of tissue typing and the restrictive use of only MHC-matched tissues as donor cells. The development of adoptively transferred yolk sac cells in the environment of the host may lead to specific tolerance between the host and donor cells for each other, causing a diminution of the potential for inducing graft-versus-host and host-versus-graft reactions.

The above-described yolk sac phenotype is seen with the vast majority of cells isolated from day 7 murine embryos. Therefore, early isolation of yolk sac cells provides for a highly homogeneous and enriched population of stem cells. This is in contradistinction to the purification procedure needed for murine bone marrow hematopoietic stem cells which are of $CD34^+$ and $Thy\text{-}1^+$ phenotype. Such cells must be isolated and enriched by a series of selection steps, as they constitute only less than 0.1% of the total cells in the bone marrow (Spangrude et al., 1991, Blood 78:1395). On the other hand, yolk sac stem cells can be obtained in an essentially homogeneous state without requiring additional purification, and such cells retain their phenotype and functional activity during long-term in vitro growth.

5.3. FUNCTIONAL ACTIVITIES OF YOLK SAC CELLS

The pluripotency of yolk sac stem cells to differentiate and mature into functionally competent

blood cells of various hematopoietic lineages was tested by a number of in vitro and in vivo methods described herein. The presence of a pluripotent population in long-term cultured yolk sac cells was first demonstrated as follows. After 10 passages of in vitro growth, yolk sac cells were washed from LIF and exposed to a combination of cytokines including IL-3, CSF's, and EPO at previously determined optimal concentrations for an additional three weeks in culture. At the end of the period, the stimulated yolk sac cells were prepared as blood smears and stained with hematoxylin. The result of this analysis reveals the appearance of blood cells that can be identified as erythrocytes, granulocytes, megakaryocytes, and lymphocytes.

A similar study also was carried out in vivo by recovering donor cells four weeks after in vivo injection into allogeneic SCID mice. The yolk sac cells used in this study had been expanded in culture for over 40 passages. Double-staining of the spleen, bone marrow, and thymus cells of the SCID mice was performed using antibodies specific for the donor cell haplotype of H-2^d and antibodies against mature blood cell markers such as B220 for B cells, CD3 and Thy-1 for T cells, and Mac-1 for macrophages. The results of this in vivo study confirm the in vitro study that long-term cultured yolk sac cells are capable of giving rise to mature T cells, B cells and macrophages/monocytes.

In addition to morphologic evidence of blood cell maturation from yolk sac cells, the adoptively transferred yolk sac cells were tested for functional activities in the form of specific antibody production. One month after receiving an infusion of yolk sac cells, the mice were immunized with either

lipopolysaccharide (LPS) or human serum albumin (HSA). Sera of mice were diluted serially, reacted with the two antigens, and compared with normal mouse sera as controls. LPS is a T cell-independent antigen which activates polyclonal B cells directly. The high titer of LPS specific antibodies in the sera of yolk sac cell-bearing beige nude xid mice after LPS immunization indicates the presence of functionally competent antibody producing cells, i.e., B lymphocytes and plasma cells. Additionally, HSA, which is a T cell dependent antigen, elicited a weaker yet detectable specific antibody production in mice. Since the anti-HSA antibody response requires T cell help which, in turn, is first activated by antigen-presenting cells such as macrophages, this result provides evidence for the presence of mature and functional T cells, B cells, and macrophages which co-operate and interact in the generation of antibodies. As a corollary, this also suggests that other T cell and macrophage-mediated functions such as cytotoxicity, lymphokine and cytokine secretion, phagocytosis, antigen processing and presentation may all develop from the transferred yolk sac stem cells.

The in vivo transfer of yolk sac cells also repopulated the spleens of mice whose hematopoietic system had been previously destroyed by chemical ablation or lethal doses of irradiation. This resembles situations in which a patient's lymphohematopoietic system is deficient due to a genetic disorder or an acquired viral infection, or a patient's system is intentionally destroyed by chemotherapy or radiotherapy in order to eradicate tumor cells in the bone marrow. The administration of yolk sac cells induced colony forming units-spleen (CFU-S) in lethally irradiated or chemo-ablated mice

whose spleens, otherwise, frequently exhibited a necrotic appearance. On the other hand, expansion of the yolk sac cells over a period of time in vivo supported repopulation and restoration of spleens completely normal in appearance. Further, the yolk sac cell-treated mice experienced a prolongation of survival time when compared with the untreated control group. Therefore, long-term cultured yolk sac cells may be useful in a variety of settings in which bone marrow reconstitution can be applied as an effective means of therapy.

Transplantation of murine yolk sac cells into allogeneic fetuses in utero and xenogeneic new born animals did not induce graft rejection reactions. The yolk sac cells persisted in vivo and established hematopoietic chimerism in the spleen, liver, and peripheral blood of the host. Thus, yolk sac cells may be useful as universal donor cells in various mammalian species, including humans.

5.4. USES OF YOLK SAC STEM CELLS

The absence of MHC antigen expression by yolk sac stem cells provides for a source of donor cells for in vivo transplantation and reconstitution therapy. The cells may be used immediately after isolation from the yolk sac or after long-term expansion in vitro, in order to procure larger numbers for more effective doses. Introduction of exogenous genes into the yolk sac cells may be achieved by conventional methods during in vitro culture and/or in vivo gene therapy. Long-term cultured cells may be used as a mixed population or progenitors can be pre-selected based on the primitive phenotype of $CD34^+$, Thy-1, MHC class I^- and class II^-, or by limiting dilution cloning, prior to in vivo use.

5.4.1. HUMAN YOLK SAC CELLS IN MICE

Human yolk sac cells may be obtained, grown in vitro and transferred into immunodeficient or immunocompromised mice. Such mice contain a human hematopoietic system and may be used for the study of human blood cell development in vivo, the identification of novel hematopoietic growth and differentiation factors, and testing for cytotoxic and/or inhibitory compounds that affect various stages of blood cell formation as well as anti-cancer drugs. Such a chimeric mouse referred to as HumatoMouse™ herein would be superior to the conventional SCID/Hu mouse model in which mice are reconstituted with human bone marrow stem cells because HumatoMouse™ would permit studies in the delineation of the earliest events in hematopoiesis. Furthermore, yolk sac cells may be implanted in utero into normal mouse fetuses for engraftment of human blood cells in a normal mouse environment. Such yolk sac cells may be transfected with a drug-resistance gene so as to allow subsequent selective ablation of only the host cells using the corresponding drug.

It has been observed that SCID mice are not totally immunodeficient and that a small amount of restoration of immune function is correlated with the age of the mice. SCID mice possess detectable natural killer cell and macrophage activities. A small percentage of mice even re-acquire T and B cell function as they mature. Thus, conventional SCID mice may not be the most appropriate hosts for the construction of the HumatoMouse™ as their immune function may interfere with the analysis of the donor yolk sac cells. The steel mice possess a mutation at the steel locus which encodes SCF, a ligand for the proto-oncogene c-kit cell surface receptor. Mouse

fetuses that are homozygous for this mutation live only to about day 15 of gestation before they are aborted due to the absence of a hematopoietic system and blood cell formation. Hence, human yolk sac cells may be injected into the developing homozygous fetuses in utero prior to abortion, e.g., at day 8, to reconstitute their hematopoietic function. The resulting neonates should have a fully humanized system with no contribution by the host as they would not normally have lived to birth.

Studies described herein demonstrate that cultured yolk sac cells can develop into mature blood cells in vivo, suggesting that the cells secrete the necessary growth and differentiation factors for supporting their own development. A further improvement of the Humatomouse™ model includes the introduction of human growth and differentiation factor genes in the mice. In the event that certain of the critical cytokines for human blood cell formation are species-specific, such as SCF, and mouse molecules do not act effectively to promote growth and differentiation of human cells, transgenic SCID or steel mice may be constructed to result in endogenous production of human cytokines of interest such as IL-3, CSF's, and SCF. Alternatively, human yolk sac cells may be transfected with murine receptor genes. The subsequent transfer of human yolk sac cells to these mice should give rise to a more complete and efficient human hematopoietic system in mice.

5.4.2. TRANSPLANTATION USING YOLK SAC CELLS

The repeated transfer of high doses of long-term cultured mouse yolk sac cells into a foreign species, i.e. sheep, has shown that the cells persist in vivo, differentiate into mature lymphocytes, and do

not mediate graft versus host disease. Although the mature donor mouse cells eventually express MHC antigens in vivo, the donor cells are present in high quantities in the peripheral blood of the xenogeneic host. The absence of graft rejection (host versus graft) and graft versus host reactions may be attributed to the primitive nature of the yolk sac cells, particularly the lack of MHC antigen expression, allowing the cells and the host immune system to "learn" each other as self prior to MHC expression and thus, induce a state of specific tolerance.

Xenogeneic transplants of solid organs have been carried out in humans in situations where there is a shortage of HLA-matched organs. With respect to xenogeneic transplant of primitive hematopoietic stem cells, yolk sac cells may be used to reconstitute the hematopoietic system of any mammalian species, for example, in a human patient with HIV infection. Since non-human T cells cannot be infected by human HIV, this approach may serve as a means of limiting HIV infection in humans. Yolk sac cells may also be transfected with genes which are designed to disrupt HIV gene sequences involved in HIV replication prior to in vivo administration. Such exogenously introduced genes may encode anti-sense RNA or ribozyme molecules that specifically interfere with HIV replication. Further, the induction of tolerance by the transfer of xenogeneic yolk sac cells may allow subsequent transplantation of solid organs, including but not limited to heart, liver and kidney from donor animals sharing the same genetic makeup of the yolk sac donors. This raises the possibility of using MHC-mismatched yolk sac cells not only for reconstitution purposes, but also as first step

tolerogens for inducing specific tolerance in a recipient for subsequent organ transplants.

In addition, this form of yolk sac cell transplantation may be applied in situations where a genetic defect has been detected in a fetus. Human or other mammalian yolk sac cells carrying a normal wild type gene or an exogenously introduced gene may be injected into the developing fetus in a routine procedure similar to that of amniocentesis in utero. The genetic disorders for which this approach may be applicable include, but are not limited to, sickle cell anemia, thalassemia, and adenosine deaminase deficiency. Alternatively, yolk sac cells may be used in settings where a pregnant mother is diagnosed to carry HIV, and reconstitution of the fetus with yolk sac cells may prevent viral infection of the fetus.

The ability of yolk sac cells to grow in xenogeneic animals with no irradiation or chemical treatment allows for large scale production of human hematopoietic cells and their secreted factors in vivo. Human yolk sac cells may be injected in a large farm animal, the blood collected, and large quantities of human proteins or cells such as red blood cells, lymphocytes, granulocytes, platelets, monoclonal antibodies and cytokines purified for clinical use.

5.5. BONE MARROW REPLACEMENT THERAPY IN HUMANS

A protocol for the replacement of bone marrow cells in human patients requiring bone marrow transplantation may be devised using cultured human or xenogeneic yolk sac cells. Yolk sac cells obtained from human yolk sac at day 10 of gestation may be isolated using the procedures described herein, expanded in culture, and cryogenically preserved as donor cells for the transplant.

Ablation of recipient patient bone marrow cells may not be required, but if it is used, it can be accomplished by standard total body irradiation (Kim, et al., Radiology, 122:523, 1977) or by chemotherapy with a variety of commonly used compounds including, but not limited to Busulfan (Tutschka, et al., Blood, 70:1382-1388, 1987), following the conventional methods. Yolk sac cells can be introduced into the recipient, using similar methods for bone marrow cells. Prior to in vivo transfer, yolk sac cells may be transformed with a drug-resistance gene, such as the methotrexate resistance gene. This allows the subsequent use of high doses of the corresponding chemotherapeutic drug to eradicate the less resistant host cells in a patient, without damage to the transferred yolk sac cells. Post-operative care would be the same as with transplantation using bone marrow cells from a donor.

High doses of yolk sac cells obtained from allogeneic or xenogeneic sources may be continuously infused into a bone marrow transplant recipient in the absence of prior chemotherapy or radiotherapy. This presents a novel approach to bone marrow transplantation without immunosuppressing the recipient.

5.6. IDENTIFICATION OF NEW MARKERS ON YOLK SAC CELLS

Murine yolk sac cells express CD34 but none of the other known leukocyte markers. It is possible that yolk sac cells express other early markers which have not yet been identified. If so, previous failure in identifying these unique molecules might be due to their decreased expression in more mature cells or even stem cells after migration to other sites out of the yolk sac. Therefore, yolk sac cells may be used

to generate antibodies against their cell surface antigens in order to identify and characterize such unknown markers.

Also within the scope of the invention is the production of polyclonal and monoclonal antibodies which recognize novel antigenic markers expressed by yolk sac cells. Various procedures known in the art may be used for the production of antibodies to yolk sac cells. For the production of antibodies, various host animals can be immunized by injection with viable yolk sac cells, fixed cells or membrane preparations, including, but not limited to, those of rabbits, hamsters, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

Monoclonal antibodies to novel antigens on yolk sac cells may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Kohler and Milstein (1975, Nature 256, 495-497), the more recent human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72; Cote et al., 1983, Proc. Natl. Acad. Sci. 80:2026-2030) and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Techniques developed for the production of "chimeric

antibodies" by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule can be used (e.g., Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature 314:452-454). In addition, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies.

Syngeneic, allogeneic, and xenogeneic hosts may be immunized with yolk sac cells which can be prepared in viable form, or in fixed form, or as extracted membrane fragments. Monoclonal antibodies can be screened differentially by selective binding to yolk sac cells, but not to mature macrophages, granulocytes, T, and B cells.

Antibody fragments which contain the binding site of the molecule may be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments.

6. EXAMPLE: GENERATION OF YOLK SAC STEM CELLS FOR IN VIVO HEMATOPOIETIC RECONSTITUTION

6.1. MATERIALS AND METHODS

6.1.1. ANIMALS

BALB/c, C57BL/6, beige nude X-linked immunodeficient (BNX), and C3H/SCID mice were purchased from Jackson Laboratories (Bar Harbor, ME)

and kept in the animal facility of Edison Animal Biotechnology Center.

6.1.2. ISOLATION OF THE YOLK SAC

On day 7 of gestation (day of plug was counted as day 0, female mice were sacrificed by cervical dislocation, and uteri containing embryos were placed in petri dishes with Dulbecco's Phosphate Buffered Saline (PBS) plus penicillin and streptomycin antibiotics (final concentration :1000 units potassium penicillin G and 1000 μg streptomycin sulfate/ml).

Under a laminar air-flow bench ,each uterine segment containing an embryo was aseptically removed by dissection with the aid of a dissenting microscope. Each embryo surrounded by decidua capsularis was transferred to another petri dish containing PBS plus penicillin-streptomycin. The decidua capsularis was opened with watchmaker's forceps and each embryo transferred into an individual petri dish where yolk sac tissue was excised from the amnion, placenta, embryo, and Reichert's membrane in 0.02% EDTA in PBS at 4°C for 15-30 minutes. The yolk sac cells in single cell suspension were then washed in PBS before culturing.

6.1.3. CULTURE CONDITIONS

Disaggregated yolk sac cells were grown in alpha medium (Sigma) supplemented with 18% heat-inactivated fetal calf serum, 0.2 Mm β-mercaptoethanol, 50 μg/ml of gentamicin and 10% LIF conditioned medium (medium of a LIF-producing cell line, Cho LIFD at 100-1000 u/ml). Cells were grown without feeder layers on collagen or gelatin coated dishes and incubated at 37°C in 5% CO_2 in air. Media were changed every other day.

6.1.4. FLOW CYTOMETRY ANALYSIS

10^6 yolk sac cells were washed twice in cold PBS containing 0.1 BSA and sodium azide. The cell pellets were suspended in the same buffer containing the test antibodies at 4°C for 30 minutes. Cells were then washed in cold PBS twice and analyzed by flow cytometry. Antibodies specific for Thy-1, Ly-1, Ly-2, Mac-1, MHC class I and class II were purchased from Boehringer Mannheim. Anti-M1/70, anti-$H2^d$ and anti-$H2^b$ antibodies were purchased from Pharmingen (San Diego, CA). Anti-CD34 was used as hybridoma supernatant.

6.1.5. INDUCTION OF YOLK SAC DIFFERENTIATION

BALB/c yolk sac cells were grown to approximately 50% confluency in medium containing LIF. The cells were harvested, washed and medium containing growth factors was added. Growth factors used were: LIF (100-1000 U/ml), SCF (50 U/ml), EPO (1-25 U/ml), IL-2 (10-200 U/ml), and IL-3 (10-200 U/ml) in various combinations. The medium was changed every 2 days until confluency was reached, at which time the yolk sac cells were passed 1:4 into new gelatinized 35 mm culture dishes. At day 5, and 21, cells were prepared for blood staining. Day 0, 5, and 21 cells were analyzed by flow cytometry for the appearance of differentiated blood cells.

6.1.6. HEMAGGLUTINATION ASSAY

Lipopolysaccharide (LPS) conjugated to trinitrophenol (TNP) and human serum albumin (HSA) conjugated to TNP were injected at 20 μg/mouse intraperitoneally into BNX mice and SCID mice, respectively, both of which had previously received 10^6 murine yolk sac cells intraperitoneally a month earlier. A second injection of the antigens was

performed one week later, animals were bled after seven days and sera assayed for the presence of specific antibodies.

A two-fold serial dilution of the mouse sera was made in microtiter plates. Sheep red blood cells (SRBC) coated with dinitrophenol (DNP) were added to each well. The plates were incubated at room temperature for one hour. The results of the assay were assessed visually. A diffused pattern of SRBC indicated a positive TNP-specific antibody response. Negative wells had a small, tight pellet of SRBC.

6.2. RESULTS

6.2.1. ISOLATION OF MURINE YOLK SAC CELLS

In the mouse, the yolk sac is fully formed by day 7 and blood island formation usually appears by day 8.5 of gestation. Therefore, in order to isolate homogeneous and undifferentiated yolk sac cells, mouse embryos were surgically removed prior to visible blood island formation, preferably at day 7 of gestation. The yolk sac region of the embryos was separated by excision, and the external surface of the yolk sac was immersed in cold EDTA which caused the detachment of the yolk sac cells from the membrane into a single cell suspension (FIG. 1). When the physical appearance of yolk sac cells obtained from day 7 and day 8.5 embryos was compared by flow cytometry analysis, freshly isolated day 7 cells clearly displayed a much more uniform cell shape and cell size than the day 8.5 cells, suggesting that yolk sac cells were a homogeneous population at day 7 but by day 8.5, differentiative activities had already occurred to generate a mixed population of cells in the yolk sac (FIG. 2). Therefore, day 7 yolk sac cells were used

for all in vitro and in vivo studies described herein, infra.

6.2.2. CELL SURFACE PHENOTYPE OF YOLK SAC CELLS

The freshly isolated yolk sac cells from day 7 mouse embryos were immediately examined for their cell surface expression of a number of known leukocyte markers by reactivity with monoclonal antibodies. Such uncultured yolk sac cells express CD34 but not Thy-1, MHC class I and class II antigens (FIG. 3). The expression of CD34 by yolk sac cells is consistent with them being primitive stem cells as CD34 is currently the earliest detectable marker on bone marrow hematopoietic stem cells. The absence of MHC antigen expression at this stage is significant in that the likelihood of rejection of these cells by a genetically disparate host upon in vivo transfer is greatly reduced. Further, the lack of Thy-1 expression indicates that the yolk sac cells of the invention represent an earlier cell population in ontogeny than the Thy-1$^+$ hematopoietic stem cells found in bone marrow, thus should contain a pluripotent population that is less committed to any specific cell lineages.

6.2.3. LONG-TERM MAINTENANCE OF YOLK SAC CELLS

The yolk sac cells isolated from day 7 embryos were established in culture in the presence of leukemia inhibitory factor (LIF) at 10-100 U/ml without a feeder layer. The cells expanded in number, having a doubling time of about 18 hours. Such cultured cells have been grown in vitro for over 41 passages covering a period of time over nine months in continuous culture. Alternatively, yolk sac cells

could also be grown in stem cell factor with similar results.

Although LIF is capable of suppressing differentiation of the yolk sac cells over an extended period of in vitro growth, the effect of LIF is incomplete because a small fraction of the cultured cells began to express certain differentiation markers including Thy-1 and MHC-encoded molecules. However, the majority of the long-term cultured cells retained their original cell surface phenotype. Further, such cells continued to be pluripotent as evidenced by their ability to give rise to mature blood cells in vitro and in vivo, infra. The cells with the original phenotype in long-term cultures may be obtained by cell sorting or by repeated limiting dilution cloning.

6.2.4. DIFFERENTIATION OF YOLK SAC CELLS IN VITRO

After one month of in vitro culture in the presence of LIF, the yolk sac cells were tested for their ability to differentiate into mature blood cells of all lineages in response to various known hematopoietic growth factors including IL-3, IL-2, and EPO. When cultured in IL-3 and EPO, the appearance of red blood cells was readily detectable in the yolk sac cultures. In response to CSF's and IL-3, the yolk sac cells matured into megakaryocytes and granulocytes. Fig. 4 is a blood stain of a yolk sac culture grown in the presence of a combination of cytokines and the appearance of various blood cell lineages can be identified. In addition, the expression of various leukocyte surface markers by these cells became detectable, including CD34, CD45, LFA, MAC-1, Ly-1 and Ly-2.

6.2.5. DIFFERENTIATION OF YOLK SAC CELLS IN VIVO

A long-term culture of yolk sac cells of BALB/c origin was injected into allogeneic C3H/SCID mice after 22 passages in vitro. Four weeks later, spleens and livers of the treated animals were analyzed for the presence of donor cells by monoclonal antibodies.

The donor cells were identified by antibodies specific for the donor H-2^d haplotype. Double-staining experiments utilizing two antibodies further demonstrated that certain subpopulations of the donor cells expressed CD3, Thy-1, B220 and M1/70 (FIG. 5). Therefore, these results indicated that the long-term cultured mouse yolk sac cells were capable of differentiating naturally in vivo into T cells, B cells and macrophages.

6.2.6. GENERATION OF IMMUNOCOMPETENT CELLS BY YOLK SAC CELLS IN VIVO

In order to examine whether the yolk sac cells could give rise to functionally mature blood cells, the long-term cultured yolk sac line was transferred in vivo into allogeneic SCID or BNX mice, and tested for specific antibody production. When the BNX mice received yolk sac cells and subsequently were immunized with LPS a month later, specific antibody titers were detected in the sera (FIG. 6). As LPS is a polyclonal B cell activating agent, this result shows the presence of functionally active antibody-producing B cells. Additionally, when SCID mice were injected with yolk sac cells followed by HSA immunization, which is a T cell dependent antigen, an antibody response was again detectable, suggesting that long-term cultured yolk sac cells could differentiate to become immuno-competent T and B cells in vivo.

6.2.7. YOLK SAC CELLS REPOPULATE CHEMO-ABLATED MOUSE SPLEENS

Certain classes of chemotherapeutic drugs are effective, and have been used, as ablative agents for bone-marrow in bone-marrow transplantation procedures (Floersheim and Ruszkiewicz, 1969, Nature 222:854). One of the most effective agents used to replace whole body irradiation in bone-marrow transplantation procedures is the drug Busulfan (Tutschka et al., 1987, Blood 70:1382). Through careful titration of the dose of Busulfan and the use of inbred lines of mice (C57BL/6) of a defined age and weight (3-4 weeks of age), doses of Busulfan have been determined which fully ablate the bone-marrow of these mice but do not directly kill them. These doses of Busulfan result in the eventual death of the treated mice between 11 and 14 days if they do not receive transplanted bone-marrow. This dose is 65 mg of Busulfan/g of body weight administered in a single dose by I.P. injection. When C57BL/6 mice were treated with this dose of Busulfan and then received an I.P. injection of 10^6 syngeneic cultured yolk-sac cells 24 hrs. following Busulfan treatment, the transplant recipients revealed spleen repopulation at day 7 and 14 post Busulfan treatment (FIG. 7). On day 7, spleen colony formation within the recipient was observed, indicative of the initial stages of splenic repopulation by the transplant. Additionally, comparison at day 12 post treatment, of the spleens of control Busulfan treated mice not receiving yolk-sac transplants and those animals receiving transplants showed a marked difference in splenic viability. While the spleens of control animals were dark, almost black in color, and appeared necrotic, the spleens of transplant recipients displayed a red/pink color and appeared normal and healthy. Further, the survival

time of the yolk sac cell-treated mice was extended to between 18 and 20 days.

6.2.8. IN UTERO ADMINISTRATION OF YOLK SAC CELLS RESULTS IN TISSUE CHIMERISM

A long-term cultured yolk sac cell line was tested for its ability to survive in an allogeneic host. 10,000-50,000 BALB/c yolk sac cells after 13-20 passages in vitro were injected in utero in day 8 embryos of C57BL/6 mice. At birth, the spleens and livers of the neonates were harvested and analyzed for the presence of donor cells.

Since the donor cells were of the H-2^d haplotype, a monoclonal antibody specific for H-2^d antigens was used to identify the donor cells by flow cytometry analysis. FIG. 8 presents the results from two neonates examined and it clearly shows that donor cells were present in both the liver and spleen of the recipient mice in substantial numbers. Therefore, in utero administration of yolk sac cells into MHC-mismatched mice resulted in tissue chimerism, and survival and homing of the cells to the lymphohematopoietic organs. Tissue chimerism was retained when the mouse tissues were examined even one month after birth.

6.2.9. XENOGENEIC TRANSPLANTATION OF YOLK SAC CELLS RESULTS IN LONG-TERM PERSISTENCE OF CELLS IN VIVO

In order to test the feasibility of using yolk sac cells in xenogeneic transplantation and reconstitution, long-term cultured BALB/c yolk sac cells were injected into a newborn Hampshire sheep and a Nubian goat. The sheep received $40x10^6$ murine yolk sac cells intravenously at day 3 after birth and the

goat received the same cell dose at day 7 after birth. Four days later, both animals received a second dose of $200x10^6$ cells. After four additional days, a final injection of $60x10^6$ cells was given, the peripheral blood mononuclear cells were harvested for antibody staining and flow cytometry analysis about one and a half month later.

FIG. 9 demonstrates that a substantial number of blood cells obtained from the sheep were reactive with anti-H-2^d antibody. While there were lower numbers of donors in the peripheral blood of the goat, donor cells were nonetheless detectable. In addition, cells expressing the murine T cell marker Ly-1 were also present from both animals. However, neither animal had cells that were positive for the murine macrophage marker Mac-1, consistent with the fact that macrophages are not normally present in the peripheral blood.

The results of this experiment are revealing in a number of ways. It illustrates the possibility of xenogeneic reconstitution using murine yolk sac cells. Neither animal was pre-treated with irradiation or cytotoxic drug. The high cell doses and the repetitive injections did not induce graft rejection. Both animals also appeared normal and healthy, having no indication of graft versus host reaction. The consistent finding of a high number of donor cells recoverable from the sheep than the goat may be a result of the goat being of an older age before receiving the first cell injection. The younger age of the sheep when it was given the first cell dose might have resulted in a more efficient induction of tolerance. However, there was still acceptance of the donor cells in the goat in the absence of any prior immunosuppressive treatment. If

induction of tolerance is the mechanism underlying this observation, this further suggests that the tolerized hosts may also accept other solid organs including the heart, liver and kidney from xenogeneic donors sharing the same haplotype of the original donors. Finally, the expression of a T cell marker indicates normal differentiation and maturation in vivo, and the absence of macrophages in the peripheral blood suggests the appropriate homing of the right cell lineages in the host upon intravenous administration of yolk sac cells.

7. EXAMPLE: IN VIVO TRANSFORMATION OF YOLK SAC CELLS

In this example, both untransformed yolk sac cells and a retroviral vector containing the exogenous gene of interest are injected into the target animal. The exogenous gene used is the growth hormone gene (bGH).

Yolk sac cells harvested from day 8 C57/SJL mouse embryos are cultured on an STO feeder layer system until approximately 20 x 10^6 cells per culture flask (150 cm) were generated. Cells were passed to new flasks when the cell density became greater than 80%. All experiments were performed with cells at passage 10 or greater.

Newborn mice were injected I.P. with yolk sac cells (2-4 x 10^6) at 3 to 5 days of age. Two months (positive results have been observed with infections as early as two weeks following yolk sac injection) after I.P. injection of yolk sac cells, animals received 1x10^6 viral particles of a replication deficient retroviral vector produced from the Moloney murine Leukemia Virus based Mulligan ψ2 packaging cell line after transformation with the plasmid pLJPCKbGH by I.V. injection into the tail vein.

Of 145 animals treated by this procedure, 112 were positive for bGH in the serum by ELISA assay. The following is a breakdown of the positive bGH levels of these test animals:

20-59 ng/ml	= 56 mice
60-100 ng/ml	= 20 mice
100-200 ng/ml	= 14 mice
200-500 ng/ml	= 13 mice
> 500 ng/ml	= 9 mice

An equal number of control animals were injected with the retroviral vector but not with the yolk sac cells. None of these were positive for bGH.

It is believed that either the retroviral vector specifically can transfect the injected yolk sac cells, and/or the yolk sac cells secrete factors having an effect on the nearby cells, rendering them susceptible to transfection.

WHAT IS CLAIMED IS:

1. A cellular composition comprising a substantially homogeneous population of yolk sac stem cells displaying a phenotype of $CD34^+$, $Thy\text{-}1^-$, MHC class I^- and MHC class II^- which are capable of differentiating into mature blood cells in vivo.

2. The composition of Claim 1 wherein the yolk sac stem cells are isolated from a yolk sac prior to blood island formation.

3. The composition of Claim 2 wherein the yolk sac stem cells are isolated from a mouse yolk sac at day 7 of gestation.

4. The composition of Claim 2 wherein the yolk sac stem cells are isolated from a human yolk sac at day 10 of gestation.

5. A method of generating a cellular composition of yolk sac stem cells comprising isolating a substantially homogeneous population of yolk sac stem cells displaying a phenotype of $CD34^+$, $Thy\text{-}1^-$, MHC class I^- and MHC class II^-.

6. A method of expanding a cellular composition of yolk sac stem cells comprising culturing, in the presence of an agent which suppresses cellular differentiation, a substantially homogeneous population of yolk sac stem cells displaying a phenotype of $CD34^+$, $Thy\text{-}1^-$, MHC class I^- and MHC class II^-.

7. The method of Claim 6 wherein the agent is leukemia inhibiting factor.

8. The method of Claim 6 wherein the agent is stem cell factor.

9. A method of hematopoietic reconstitution comprising administering the yolk sac stem cells of Claim 1 to an animal.

10. The method of Claim 9 wherein the cellular composition is administered intravenously.

11. The method of Claim 9 wherein the cellular composition is administered in utero.

12. The method of Claim 9 wherein the animal is a mouse.

13. The method of Claim 9 wherein the animal is a sheep.

14. The method of Claim 9 wherein the animal is a goat.

15. The method of Claim 9 wherein the animal is a human.

16. The method of Claim 15 wherein the human is infected with the human immunodeficiency virus.

17. A non-human animal having a hematopoietic system reconstituted with yolk sac stem cells.

18. The animal of Claim 17 wherein the animal is a mouse.

19. The animal of Claim 17 wherein the animal is a sheep.

20. The animal of Claim 17 wherein the animal is a goat.

21. A method of producing blood cells in vitro, comprising culturing, in the presence of a growth factor, a substantially homogeneous population of yolk sac stem cells displaying a phenotype of $CD34^+$, $Thy\text{-}1^-$, MHC class I^- and MHC class II^- which are capable of differentiating into mature blood cells in vivo.

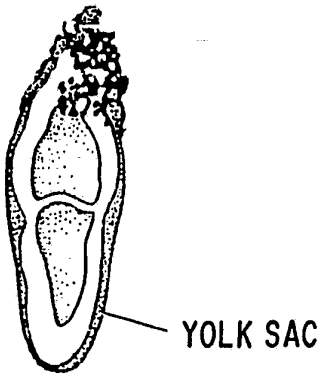
22. The method of Claim 21 wherein the growth factor is EPO, IL-2, IL-3, G-CSF, M-CSF, GM-CSF, or a combination thereof.

23. A method of producing blood cells in an animal comprising:

(a) injecting into an animal a substantially homogeneous population of yolk sac stem cells displaying a phenotype of $CD34^+$, $Thy\text{-}1^-$, MHC class I^- and MHC class II^- which are capable of differentiating into mature blood cells in vivo; and

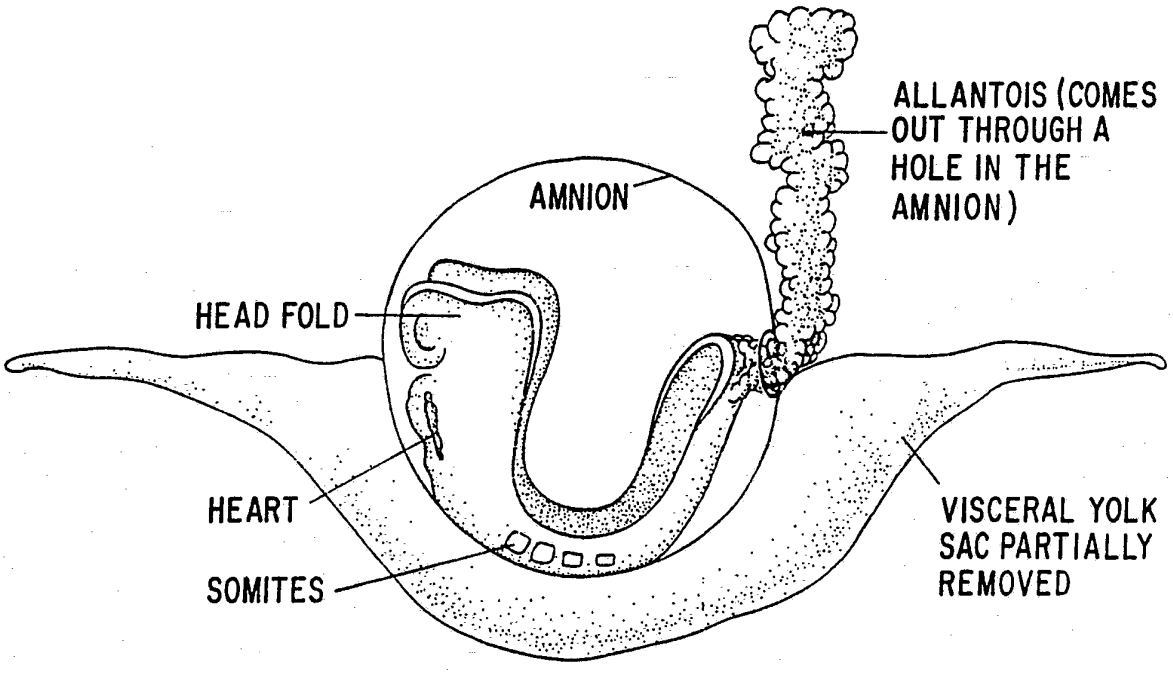
(b) collecting blood cells from the animal.

24. A method of tolerizing an animal comprising administering a substantially homogeneous population of allogeneic or xenogeneic yolk sac stem cells displaying a phenotype of $CD34^+$, $Thy\text{-}1^-$, MHC class I^- and MHC class II^-.



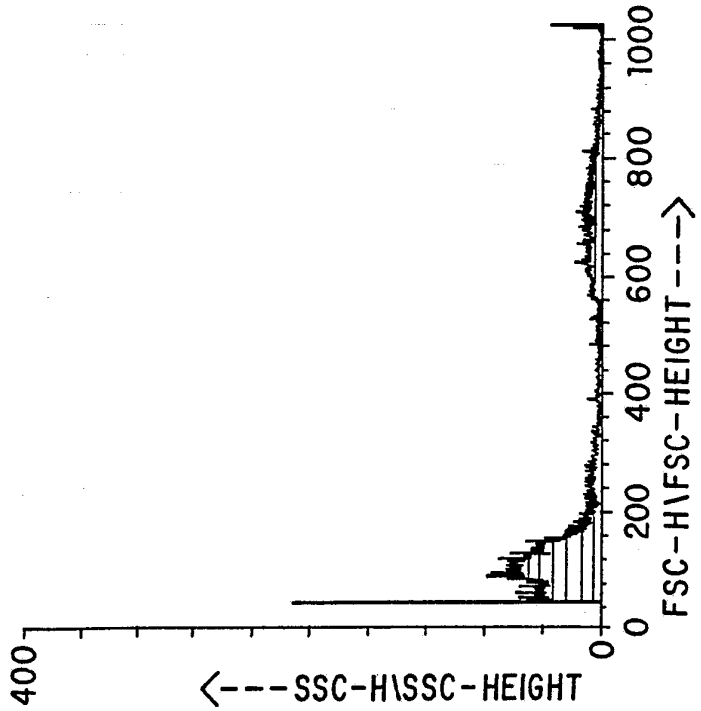
DAY 7

FIG. 1A



DAY 8.5

FIG. 1B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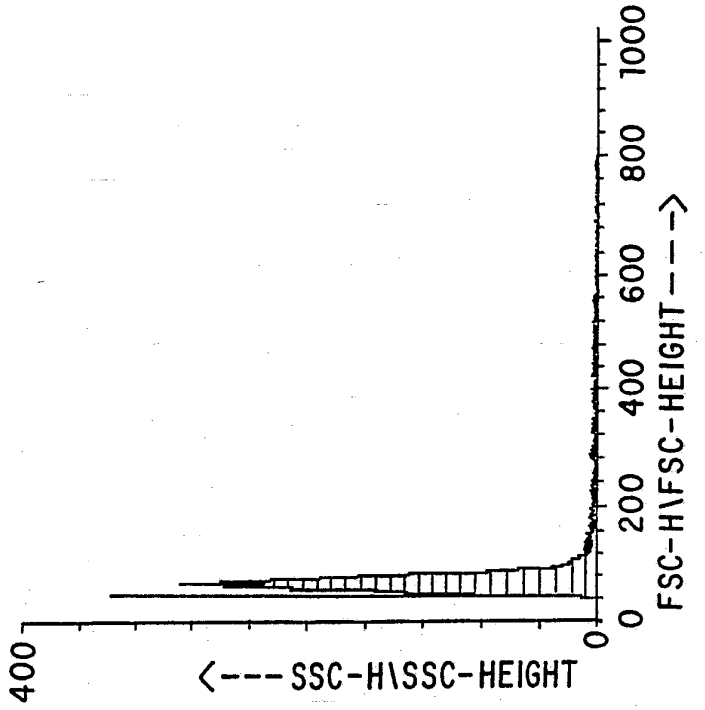
0
200
400
600
800
1000
FSC-H\FSC-HEIGHT--->
FIG. 2B


400
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<---SSC-H\SSC-HEIGHT
0
200
400
600
800
1000
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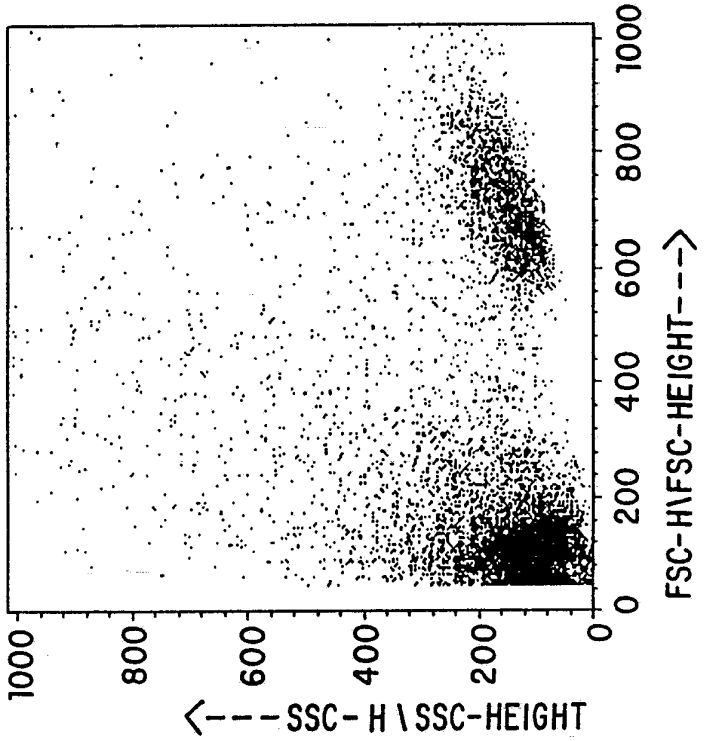
FIG. 2A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600
400
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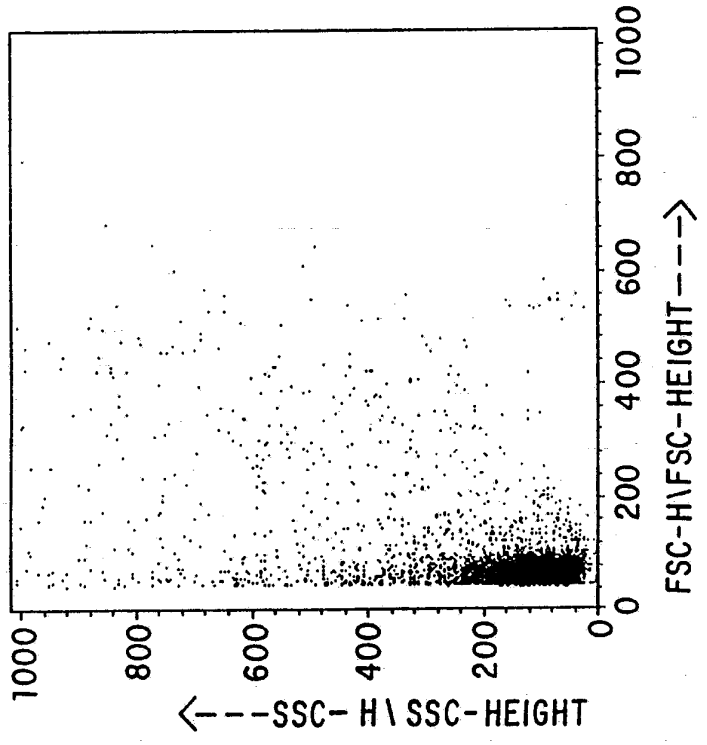
800
1000
FSC-H\FSC-HEIGHT-->

FIG. 2D


1000
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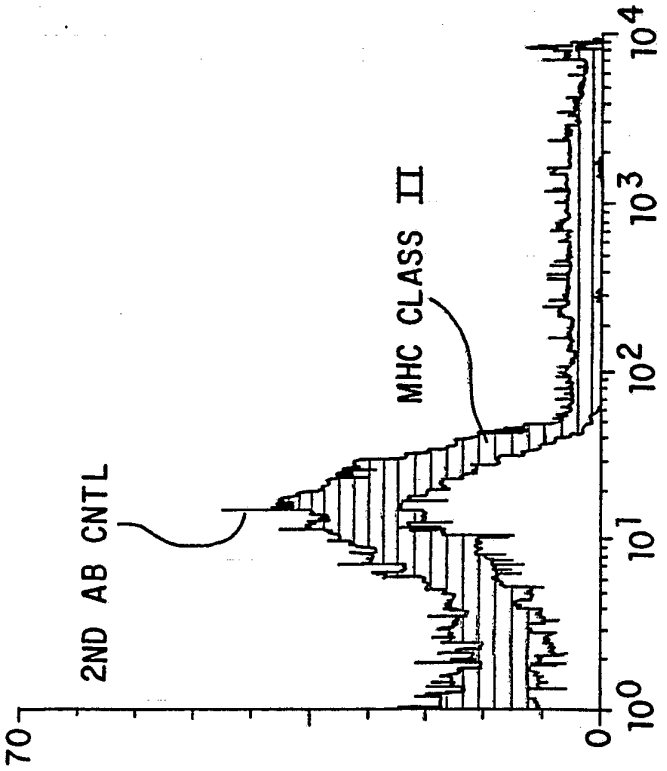
FSC-H\FSC-HEIGHT-->

FIG. 2C


2ND AB CNTL
MHC CLASS II
70
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10^0
10^1
10^2
10^3
10^4

FIG. 3B

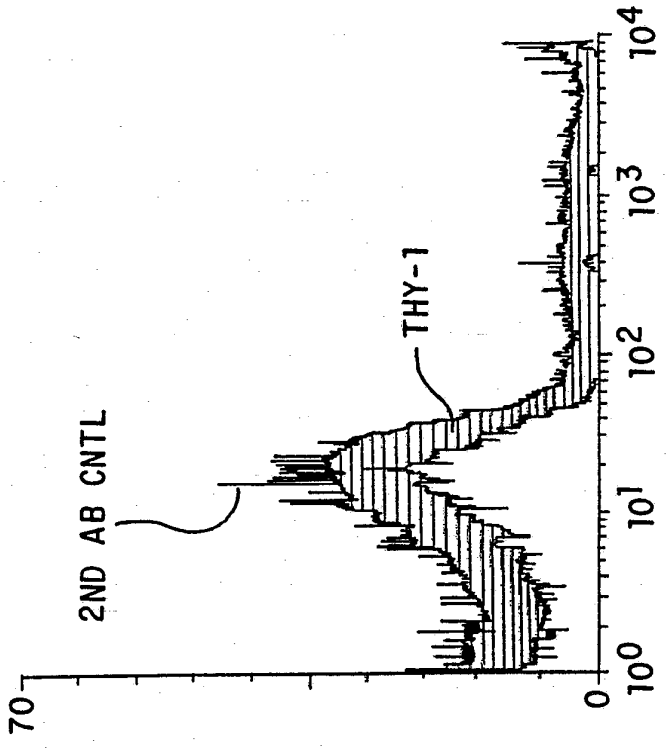
2ND AB CNTL
THY-1
70
0
10^0
10^1
10^2
10^3
10^4

FIG. 3A

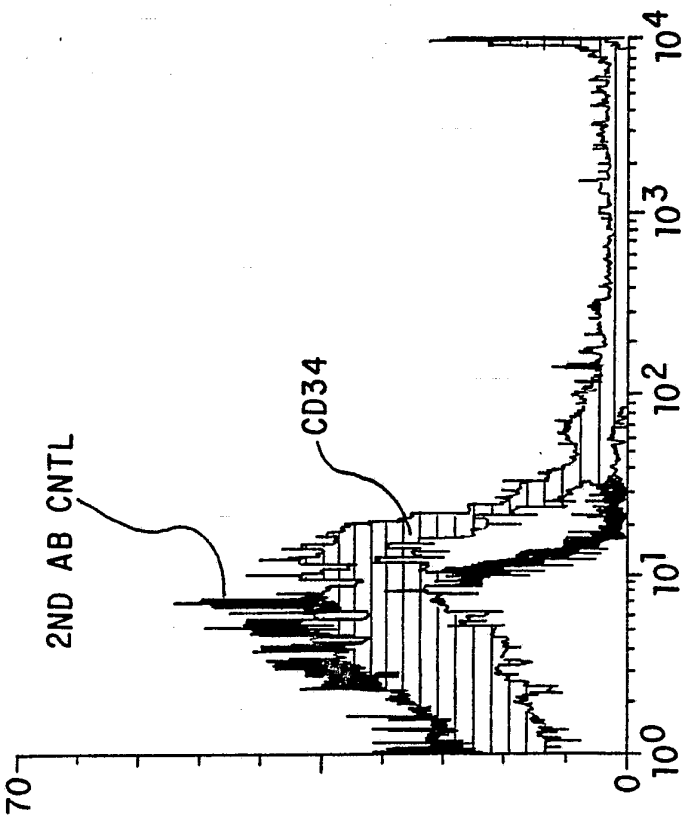
2ND AB CNTL
CD34
70
0
10^0
10^1
10^2
10^3
10^4

FIG. 3D

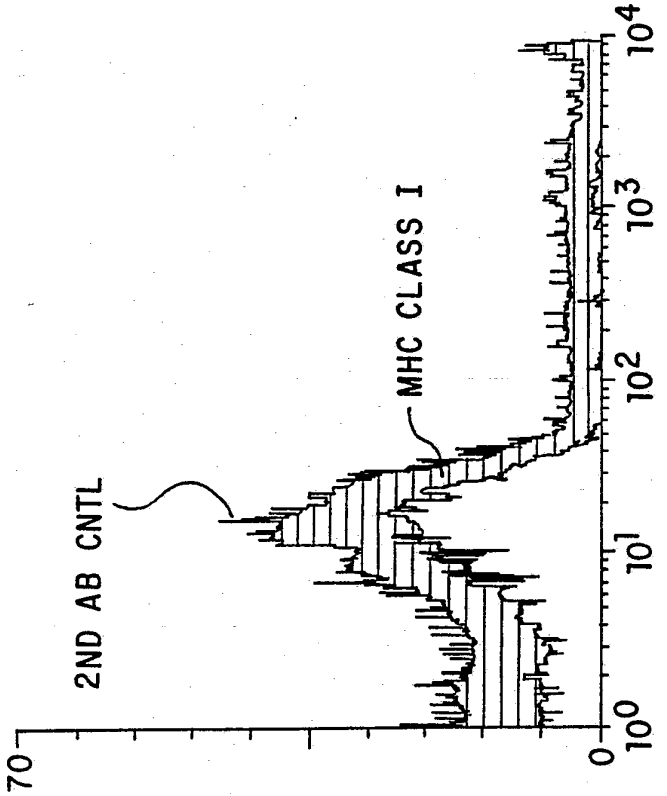
2ND AB CNTL
MHC CLASS I
70
0
10^0
10^1
10^2
10^3
10^4

FIG. 3C

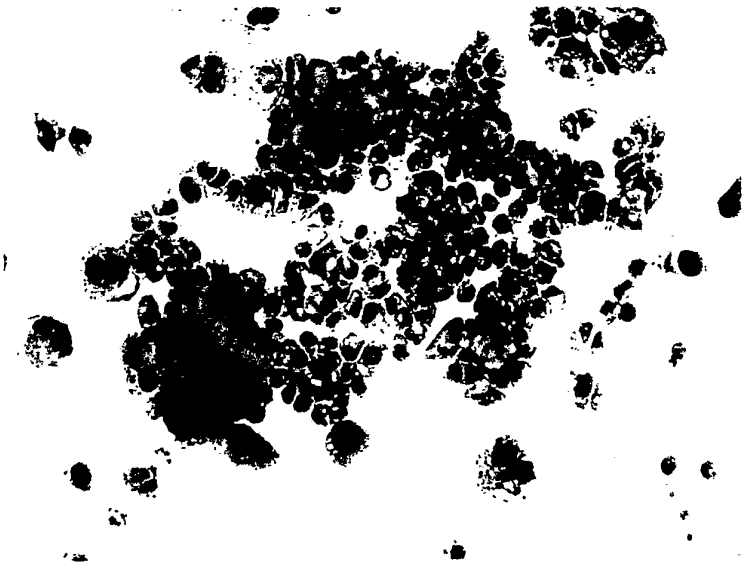

FIG. 4A

FIG. 4B

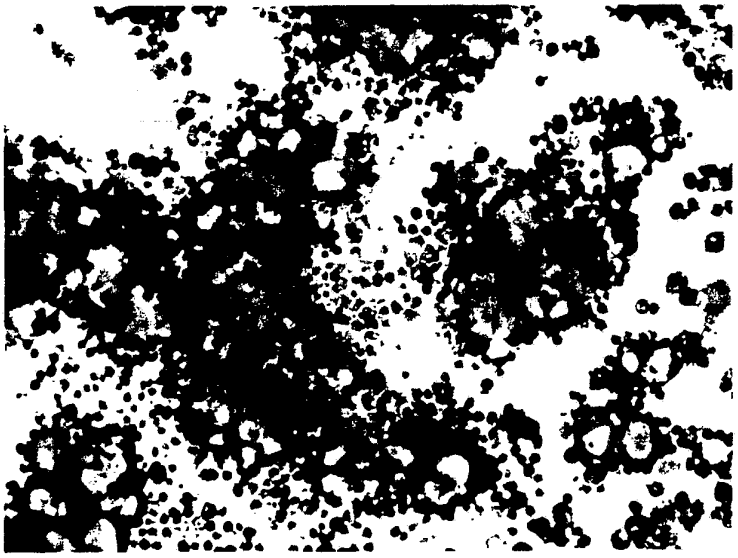

FIG. 4C

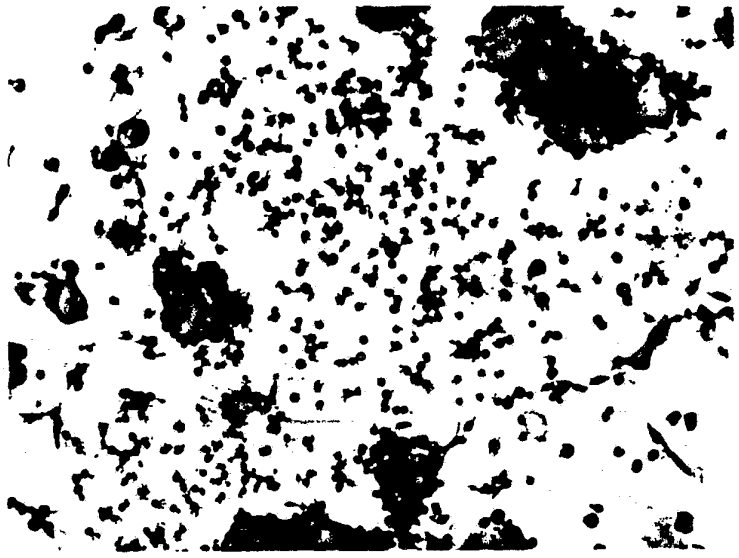

FIG. 4D

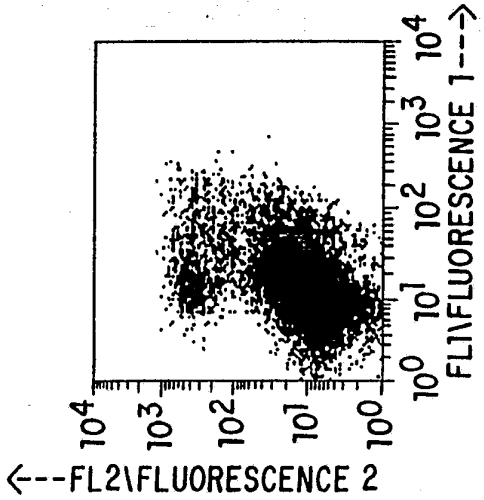

FIG. 5C

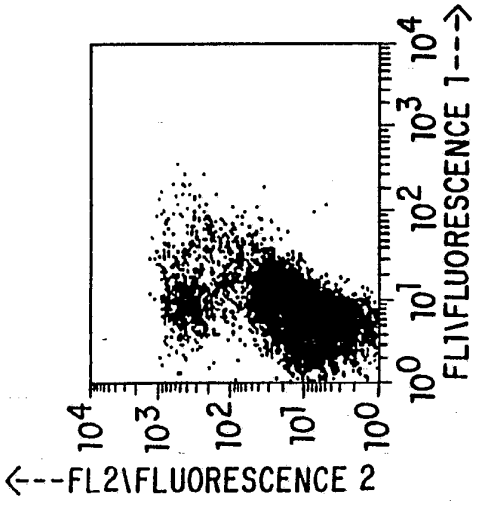

FIG. 5B

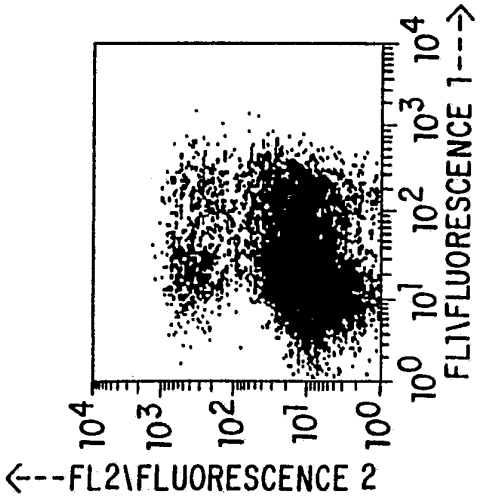

FIG. 5D

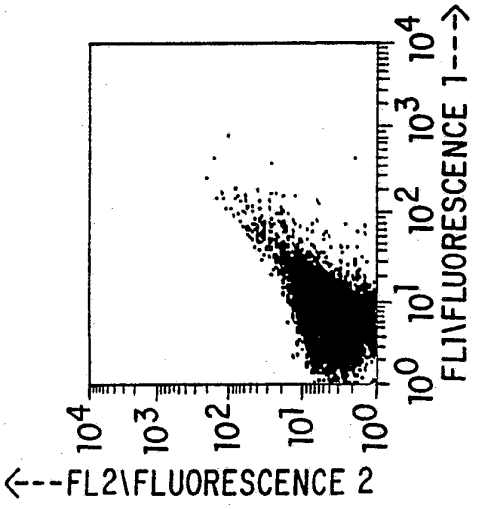

FIG. 5A

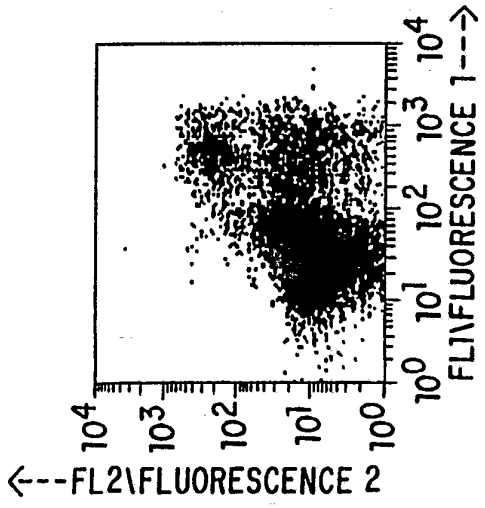

FIG. 5G

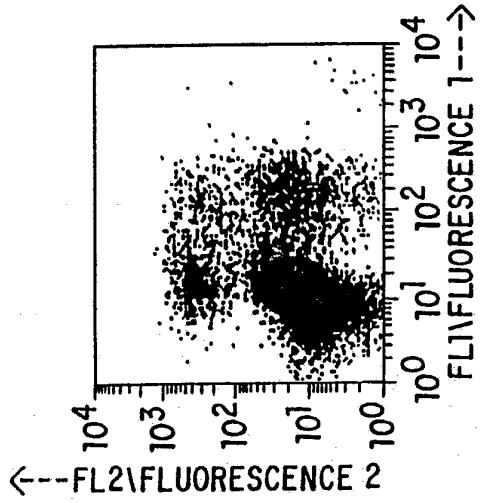

FIG. 5F

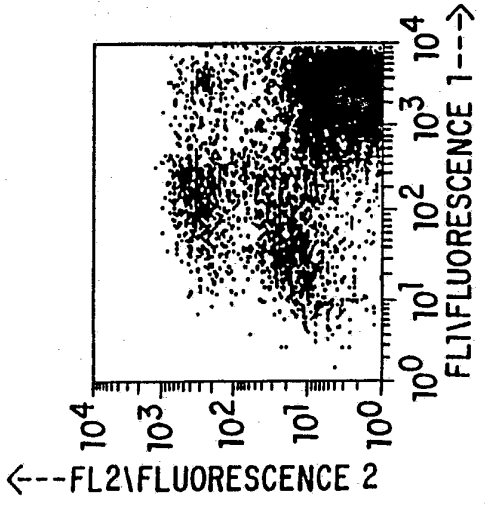

FIG. 5E

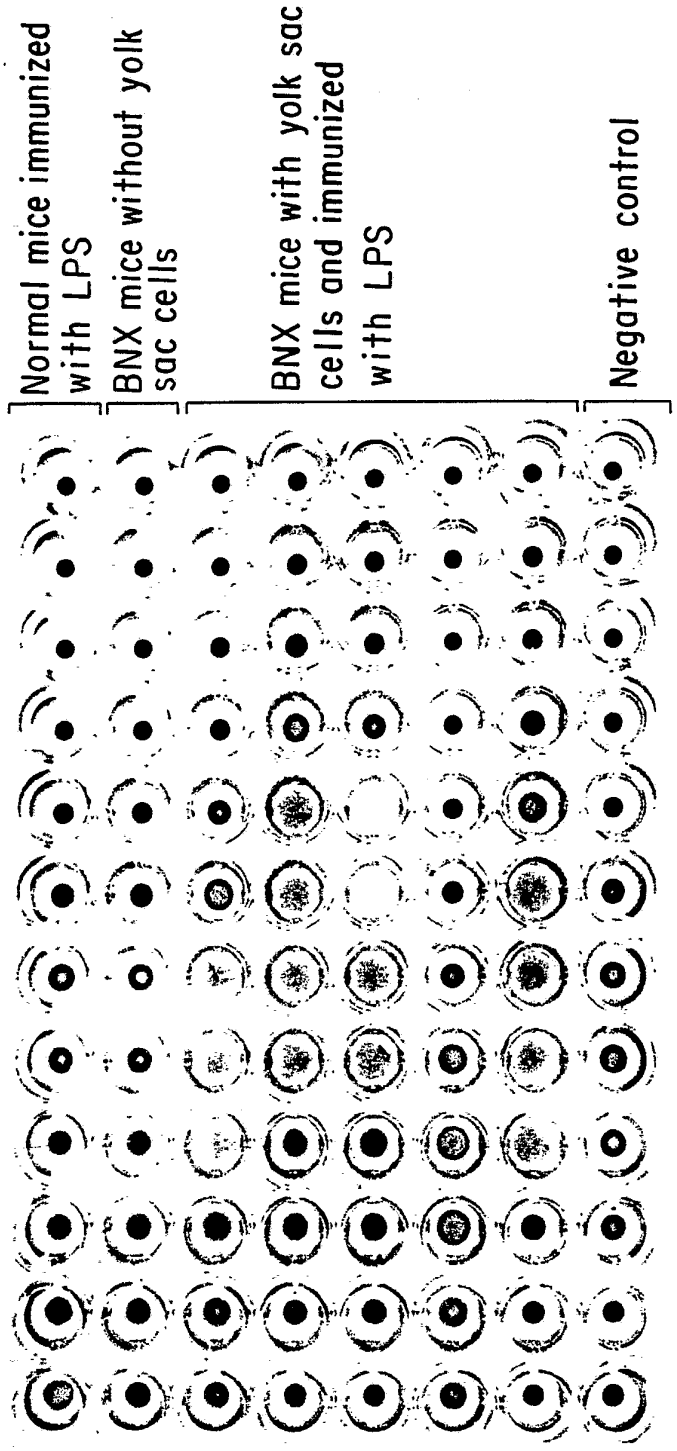
Normal mice immunized with LPS
BNX mice without yolk sac cells
BNX mice with yolk sac cells and immunized with LPS
Negative control

FIG. 6A

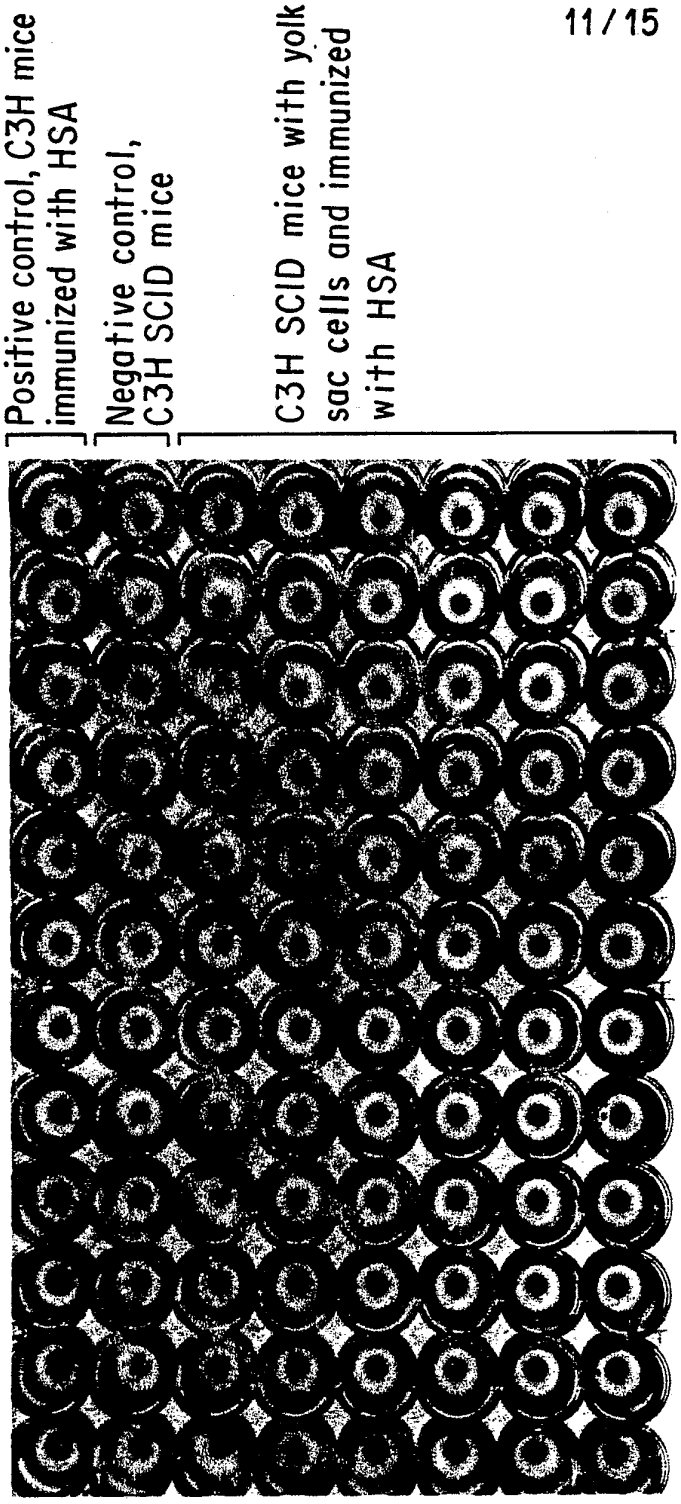
Positive control, C3H mice immunized with HSA
Negative control, C3H SCID mice
C3H SCID mice with yolk sac cells and immunized with HSA

FIG. 6B

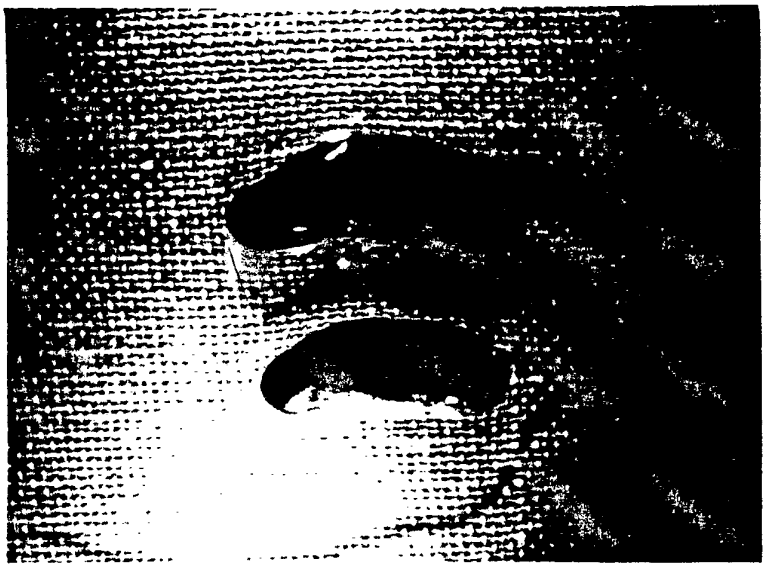

FIG. 7A

FIG. 7B

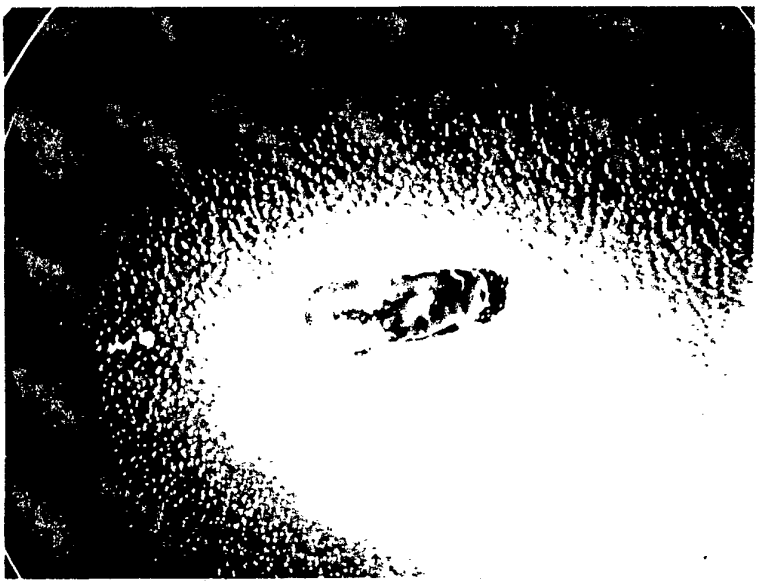

FIG. 7C

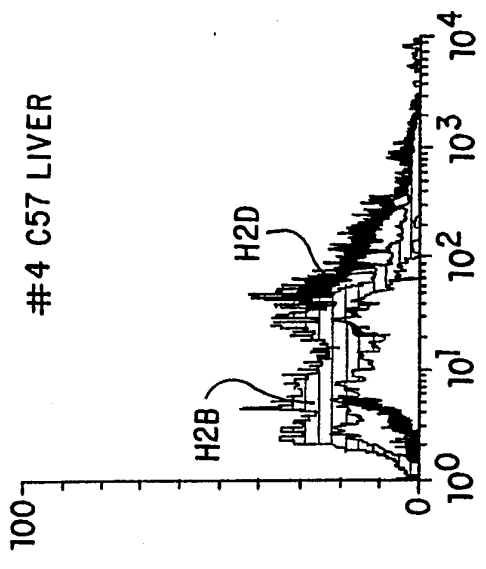

FIG. 8C

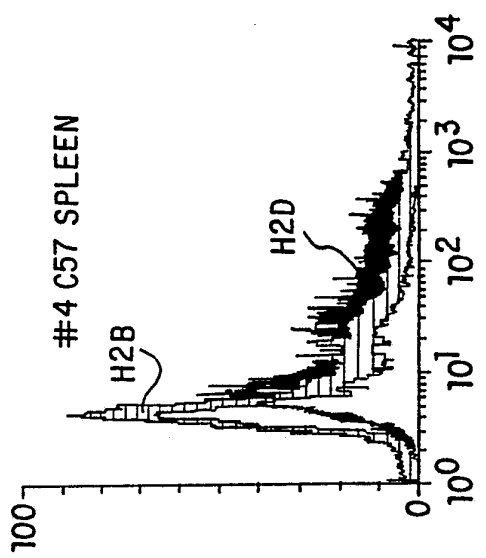

FIG. 8B

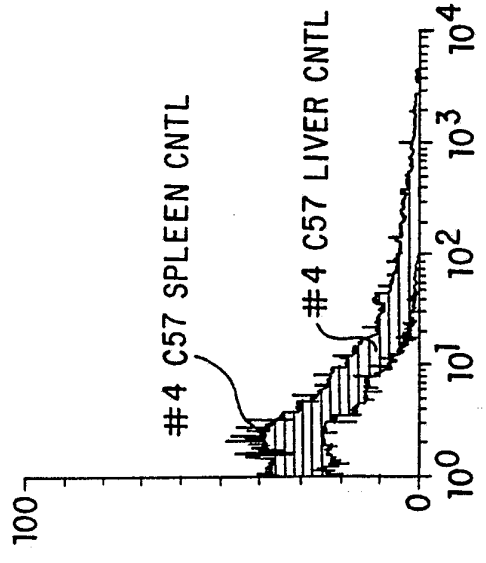

FIG. 8A

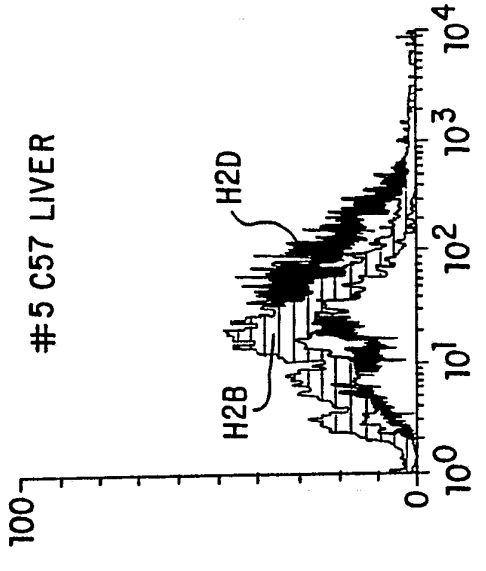

FIG. 8F

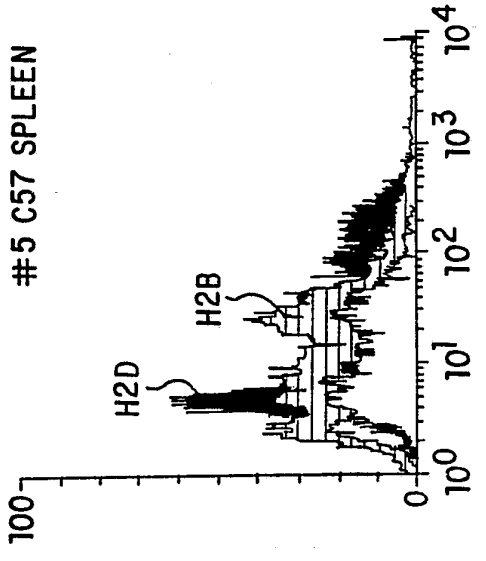

FIG. 8E

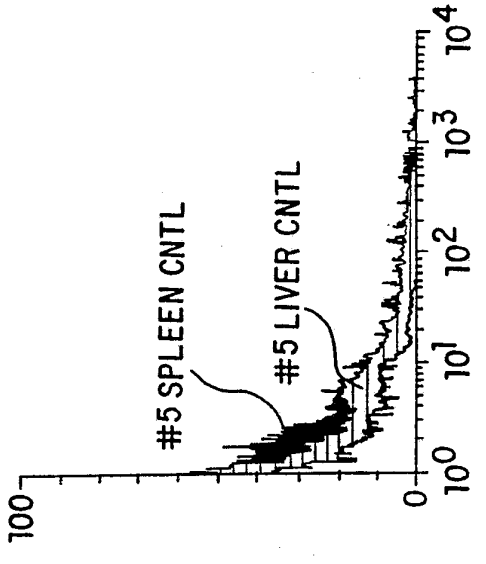

FIG. 8D

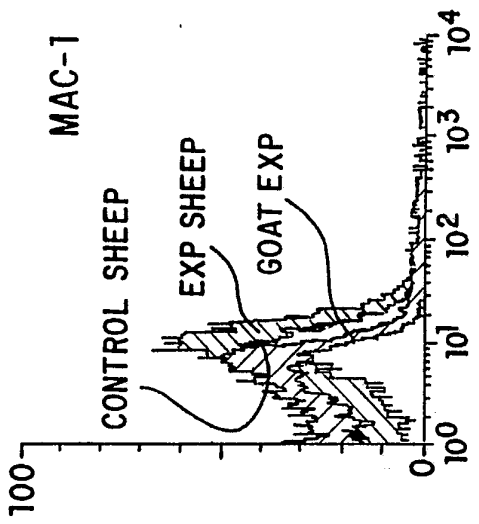

FIG. 9A

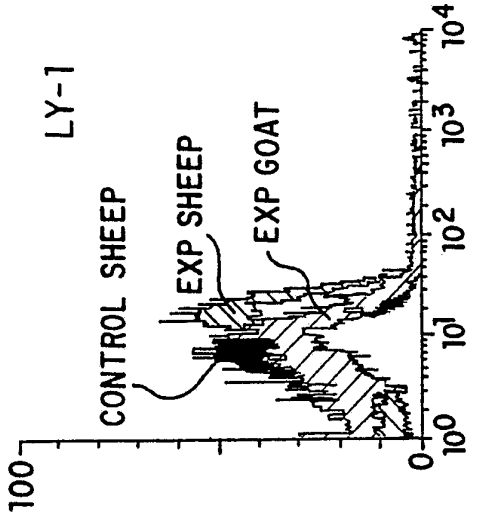

FIG. 9B

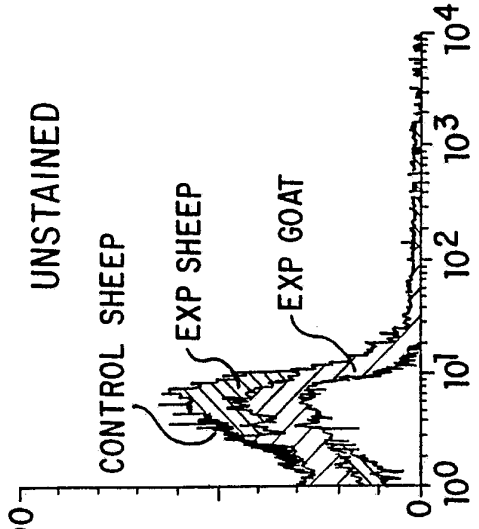

FIG. 9C

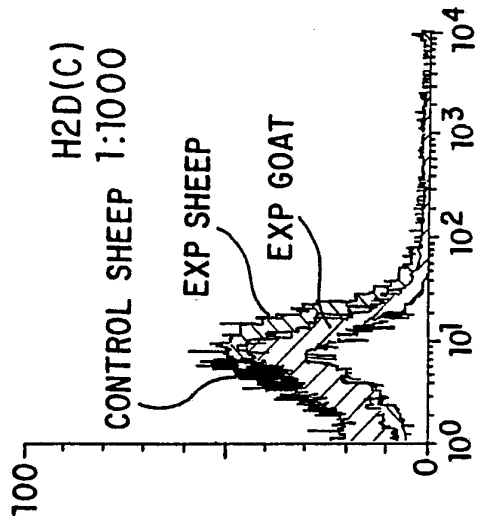

FIG. 9D

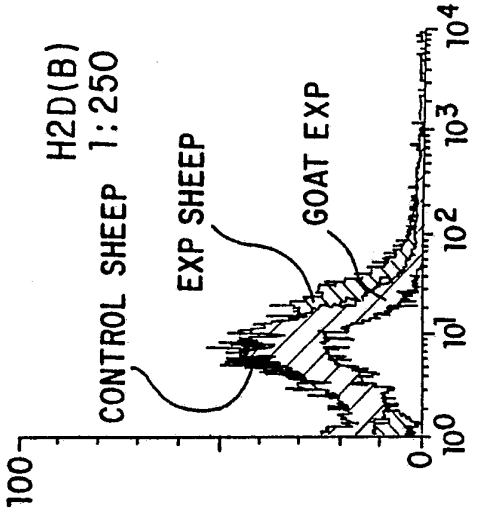

FIG. 9E

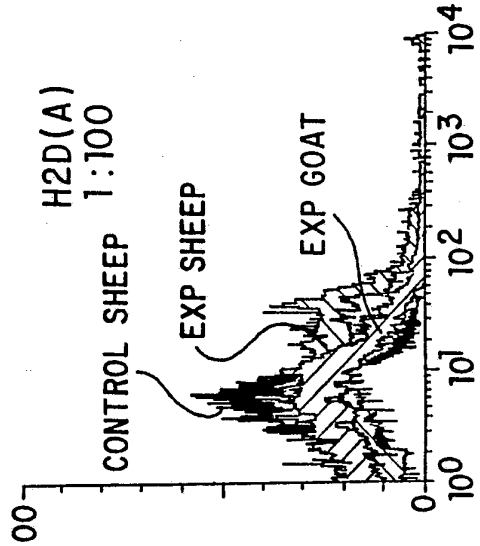

FIG. 9F

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/05918

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 5/00; A61K 35/00
US CL :435/240.2; 424/93A, 582

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.21,240.2; 424/93A, 582, 520; 800/2, DIG. 2

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

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

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US,A, 5,032,407 (Wagner et al.) 16 July 1991, entire document.	1-24
Y	US,A, 4,497,796 (Salser et al.) 05 February 1985, entire document.	9-20, 23, 24
A	Blood Cells, Volume 1, Issued 1991, Tavassoli, "Embryonic and fetal hemopoiesis: an overview," pages 269-281.	1-24
A	International Journal of Cell Clonings, Volume 5, Issued 1987, Kanamara et al., "Characteristics of murine yolk sac erythroid progenitors and their population expansion in liquid culture", pages 134-141.	1-24
Y	Exp. Hemat., Volume 5, Issued 1977, Weinberg et al., "Factors regulating yolk sac hematopoiesis in diffusion chambers: various types of sera, cyclophosphamide, irradiation and long-term culture", pages 374-384, see the entire document.	1-24

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

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

Date of the actual completion of the international search 01 SEPTEMBER 1992	Date of mailing of the international search report 30 SEP 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer JASEMINE C. CHAMBERS Telephone No. (703) 308-0196

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/05918

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Proceedings of the National Academy of Sciences, USA, Volume 86, Issued October 1989, Toles et al., "Hemopoietic stem cells in murine embryonic yolk sac and peripheral blood", pages 7456-7459, see the entire document.	17, 18 1-4, 9-16, 19, 20, 23, 24
Y	Science, Volume 233, Issued 15 August 1986, Flake et al., "Transplantation of fetal hematopoietic stem cells in utero: the creation of hematopoietic chimeras", pages 776-778, see the entire document.	9-20, 23, 24
Y	Cell, Volume 42, Issued August 1985, Dick et al., "Introduction of a selectable gene into primitive stem cells capable of long-term reconstitution of the hemopoietic system of W/W^v mice", pages 71-79, see the entire document.	9-20, 23, 24
Y	Science, Volume 230, Issued 20 December 1985, Eglitis et al., "Gene expression in mice after high efficiency retroviral-mediated gene transfer," pages 1395-1397, see the entire document.	9-20, 23, 24
Y	Differentiation, Volume 36, Issued 1987, Globerson et al., "In vitro differentiation of mouse embryonic yolk sac cells", pages 185-193, see the entire document.	1-24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/05918

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

USPTO Automated Patent System (file USPAT, 1975-1992), DIALOG (files 154, 55, 311, 312).
Search terms: yolk sac, stem cells, mouse, differentiation, injection, transfer, review, embryo, mammal, transplant, inventors' names