Abstract: A composition is described comprising expanded human blood stem cells and human red blood cells containing HbF. The composition is to be administered to a human having sickle cell disease and experiencing symptoms of the disease. A method for preparing the composition is also described.
COMPOSITION FOR REDUCING PROBLEMS ASSOCIATED WITH SICKLE CELL DISEASE USING LOW TEMPERATURE STORAGE

Cross-Reference to Related Applications

This application claims priority from United States Patent Application Number 60/735,737, filed November 12, 2005, and entitled "Composition For Reducing Problems Associated With Sickle Cell Disease Using Low Temperature Storage".

Field of the Invention

This invention relates generally to reducing symptoms associated with sickle cell disease. The invention in part utilizes red blood cells containing fetal hemoglobin (HbF) as well as expanded stem cells after storage at temperatures of for instance from -120°C to -196°C to reduce these symptoms.

Background

Sickle cell disease is a genetic problem in Chromosome 11 where the Beta chain of hemoglobin is coded. There is one amino acid substitution, a valine for glutamic acid in the beta 6th position that forms sickle beta chains. Two sickle beta chains combined with two alpha chains and four iron containing heme groups form sickle hemoglobin.

In its simplest terms, normal red blood cells are shaped somewhat like donuts and flow freely through the veins carrying red blood cells. In a human having sickle cell disease, many of the red blood cells are deformed and assume the shape of sickles or spurs. They tend to attach to the walls of the veins and cannot move freely through the veins. This limits their oxygen carrying ability and creates problems for the individual.
Sickle cell disease predominantly affects African Americans, Arabs, Greeks, Italians, Latin Americans, and people native to India, it can affect anyone. There are three common types of sickle cell disease in the United States: Hemoglobin SS or sickle cell anemia; Hemoglobin SC disease; and Hemoglobin sickle beta-thalassemia. Complications from the sickle cells blocking blood flow and early breaking apart include:

1. pain episodes
2. strokes
3. increased infections
4. leg ulcers
5. bone damage
6. yellow eyes or jaundice
7. early gallstones
8. lung blockage
9. kidney damage and loss of body water in urine
10. painful erections in men (priapism)
11. blood blockage in the spleen or liver (sequestration)
12. eye damage
13. low red blood cell counts (anemia)
14. delayed growth.

In the past, sickle cell disease treatment has been: taking the vitamin folic acid (folate) daily to help make new red cells, daily penicillin until age six to prevent serious infection, drinking plenty of water daily (8-10 glasses for adults), avoiding too hot or too cold temperatures, avoiding over exertion and stress, getting plenty of rest, and getting regular check-ups from knowledgeable health care providers. Recent advances have included
a M > :t r a lMtation, but it has carried a significant mortality rate, is painful, and has complications.

The mutation that makes the sickle hemoglobin (HbS) confers at least two abnormal properties:

1) when de-oxygenated, HbS polymerizes to forms rods and fibers that cause the sickle deformation, and

2) when oxygenated, the HbS molecule is more unstable than normal HbA and may spontaneously decompose (to met-hemoglobin, or to globin without heme).

People with sickle trait have one gene making HbS and one gene making HbA, so you would expect equal amounts of HbS and HbA in the RBC. The unstable property of HbS, however, means that not all of the amount of HbS made in the red blood cell (RBC) stays floating around in the RBC, because some of the HbS decomposes. Therefore, the RBC contents for a human with sickle trait has slightly less than 50% HbS, typically something like 55 to 60 percent HbA and 40 to 45 percent HbS. The predominance of HbA inhibits and dilutes the ability of HbS to show its polymerization property, and so sickle trait is not a form of sickle cell disease. People with sickle trait have no anemia, no painful episodes, no special susceptibility to infection, and no implications for life expectancy. Sickle cell trait is not sickle cell disease.

The most common form of sickle cell disease, HbSS, has no genes for HbA present. A minor hemoglobin (HbA2) may be present in a few percent of the total hemoglobin, and fetal hemoglobin may be present in varying amounts (HbF). However, the vast majority of the hemoglobin in the RBC is HbS, and it will polymerize and cause the sickle cell disease manifestations.
Some people with sickle cell disease have HbSC, HbSD, HbS-O-Arab etc. One gene makes HbS and the other gene makes another variant hemoglobin (HbC, HbD, HbO-Arab, etc.) that usually would not cause a disease by itself. There may be equal parts HbS and the other Hb, or slight variation from equal amounts in the RBC. However, the important difference between HbA and these variant hemoglobins is their ability to participate in polymerization with the HbS. When the inside of RBC contains a combination of hemoglobins that will polymerize, then these are types of sickle cell disease, with anemia, pain, spleen and other problems. Differences in disease pattern between these types of sickle cell disease and the pattern for HbSS can be found statistically, but there is so much variability that the exact disease course is impossible to predict for an human with sickle cell disease based on Hb subtype.

HbS-beta-thalassemia is more complicated. The RBC for people with HbS-beta-zero-thalassemia is the result of one gene making HbS and the other hemoglobin gene is defective and cannot make anything (beta-zero thalassemia). The hemoglobin produced by these two genes is HbS, and then there are minor amounts of HbA2 and HbF. Again, the HbS can polymerize and there are sickle cell disease manifestations statistically similar to HbSS. Another type is HbS-beta-plus-thalassemia, in which one gene makes HbS and the other gene is defective but makes a little bit of HbA. There is less HbA produced than in sickle trait, and so the end result in the RBC is more HbS (70 to 90 percent of the total hemoglobin) than HbA (10 to 30 percent of the total hemoglobin in the RBC). This amount of HbA is not enough to dilute the HbS and cannot inhibit polymerization completely, so the RBC can still sickle and this is a subtype of sickle cell disease.
Sickle cell disease is a genetic problem, there is unlikely to be a "cure" for the disease. One may only confine it to "permanent remission" or eliminate its negative results on the body.

Sickle cell disease manifestations and symptoms are extremely rare in infants. Infants have substantial amounts of fetal hemoglobin (HbF) that suppresses the sickle cell manifestations. The present invention takes advantage of that trait to eliminate or suppress sickle cell symptoms.

WO 2006/081435 and WO 2006/093857 disclose a method of providing readily available material derived from, respectively, cord blood and peripheral blood, and a composition thereof.

Summary of the Invention

The present invention is directed in part to a composition comprising expanded human blood stem cells having a three-dimensional geometry and cell-to-cell support and cell-to-cell geometry that is essentially the same as naturally-occurring human blood stem cells and human red blood cells containing HbF. The composition may further comprise differentiated and other blood cells. One human, having been screened for and identified as having a sickle cell disease (although the disease may not have yet manifested itself), is the source of all blood cells, whether stem cells, red blood cells containing HbF, or other blood cells, of the composition. To prepare the composition, blood must be collected from the human's peripheral blood system prior to passing the age of 5 years, and/or must be collected from the umbilical cord of the human, as discussed below.
Composition contains an amount of expanded human stem cells that is two times, more preferably seven times, the number per volume of the original, naturally-occurring blood collected from the human.

Without being bound by theory, it is believed that by administering a composition the expanded human blood stem cells and red blood cells containing HbF to the human later in life, when sickle cell disease symptoms have begun to manifest themselves, in vivo differentiation of the expanded human blood stem cells in the presence of the red blood cells containing HbF will alleviate the symptoms of the disease. A composition of the present invention may also comprise red blood cells containing HbF, without ex vivo expanded human blood stem cells, to stimulate human blood stem cells already present in the circulation to differentiate into red blood cells containing HbF and alleviate sickle cell disease symptoms thereby.

The present invention relates to a composition for reducing symptoms associated with sickle cell disease comprising human red blood cells containing HbF and expanded adult stem cells, preferably TVEMF-expanded adult stem cells. The composition comprises expanded human blood stem cells from a human blood source, wherein said expanded human blood stem cells are in a number per volume that is at least about 2 times greater than the number per volume in naturally occurring human blood (preferably the source blood), preferably at least about 7 times greater, and wherein the expanded human blood stem cells have a three-dimensional geometry and cell-to-cell support and cell-to-cell geometry that is essentially the same as naturally-occurring human blood stem cells; and human red blood cells containing HbF from the blood source in a number per volume that is at least about 80% of the number per volume of human red blood cells containing HbF in naturally occurring human blood (preferably the source blood, that is, the blood used to prepare the composition), preferably at least two times greater than the number per volume of human red blood cells containing HbF in naturally occurring human blood; wherein the blood source is selected from the group consisting of cord blood of one human, infant peripheral blood of one human, and a mixture of cord blood and infant peripheral blood of one human. Preferably, the expanded blood stem cells are TVEMF-expanded. While red blood cells containing HbF do not replicate and therefore are not expanded in a bioreactor according to the present invention, blood stem cells may expand and/or differentiate in the bioreactor,
wřeresaiet'expihSTCiř replicates the stem cells and said differentiation differentiates stem cells into differentiated red blood cells containing HbF. The invention is also directed to a composition comprising only red blood cells containing PIbF, where the red blood cells containing HbF are in an amount that is at least two times the number per volume of red blood cells containing HbF in the naturally occurring source blood, and preferably in an amount of at least 20,000 cells per dose. (Red blood cells may be separated from other cells after expansion and/or differentiation in a bioreactor, for instance as described below by leukapheresis, and cryopreserved as a composition of this invention).

The invention is also directed to a process for preparing the composition from cord blood and/or infant peripheral blood, alone or in combination, (references to "blood" in this application generally refer to cord and/or infant peripheral source blood from one human) in part by expanding and/or differentiating stem cells of the blood in a rotatable bioreactor, preferably a rotatable TVEMF-bioreactor, and cryopreserving the expanded cells and other components at -120°C to -196°C, or even -130°C to -150°C. The red blood cells containing HbF remain mixed with expanded stem cells (and/or their differentiated progeny) throughout the expansion and/or differentiation process, and typically remain mixed during cryopreservation and are present together in the composition to be administered to a patient. Preferably, all or part of the composition is maintained at said cryopreserving temperature at least until the onset of sickle cell disease symptoms in the human that is the source of the blood and therefore blood stem cells and red blood cells containing HbF of the present composition. Preferably, the expansion process increases the number of adult stem cells per volume at least seven times the number of stem cells per volume in the source blood, while maintaining cells' three-dimensional geometry and cell-to-cell geometry and cell-to-cell support. The invention is also directed to a method of treating sickle cell disease in part by increasing the temperature of the cryopreserved composition to an acceptable temperature for administering the composition to a human suffering from symptoms of sickle cell disease, and administering the composition to the human.

The present invention is directed to a composition for treating sickle cell disease symptoms. Also, the present invention is directed to the use of red blood cells containing
HBF; $Ib; $ in combination with expanded stem cells, in the preparation of a medicament for the treatment of sickle cell disease symptoms.

**BRIEF DESCRIPTION OF THE DRAWINGS**

In the drawings,

Figure 1 is a cross-sectional elevated side view of the assembly of a preferred embodiment of a rotatable bioreactor;

Figure 2 is a cross-sectional elevated side view of a preferred embodiment of a rotatable bioreactor;

Figure 3 is a side perspective of a preferred embodiment of a rotatable bioreactor;

Figure 4 schematically illustrates a preferred embodiment of a culture medium flow loop;

Figure 5 is the orbital path of a typical cell in a non-rotating reference frame;

Figure 6 is a graph of the magnitude of deviation of a cell per revolution; and

Figure 7 is a representative cell path as observed in a rotating reference frame of the culture medium.

**DETAILED DESCRIPTION OF THE DRAWINGS**

In the simplest terms, a rotatable bioreactor according to the present invention comprises a chamber that, in operation, can be controllably rotated about a horizontal (or at least substantially horizontal) axis, and has an interior portion and an exterior portion. The interior portion of the culture chamber defines a space that may removably receive a cell mixture such as a blood cell mixture of the present invention. The culture chamber has at least one aperture so that, when in use, the cell mixture may be placed into the interior portion of the chamber. The aperture (or one or more other apertures) may also be used for
the exposure of culture medium and the removal of cell samples; preferably such aperture is fitted for use with a syringe. In a preferred embodiment of the rotatable bioreactor, an electrically conductive coil is wrapped around the exterior portion of the culture chamber and a TVEMF source is operatively connected to the electrically conductive coil so that, in use, the TVEMF source delivers a TVEMF to the interior portion of the culture chamber to TVEMF-expand and/or —differentiate the cells therein. When the rotatable bioreactor is rotating about its axis, the cell culture inside the bioreactor is three-dimensional, as discussed throughout this application.

In the drawings, referring now to Figure 1, Figure 1 is a cross sectional elevated side view of the assembly of a preferred embodiment of a rotatable bioreactor 10. In this preferred embodiment a motor housing 12 is supported by a base 14. A motor 16 is affixed inside the motor housing 12 and connected by a first wire 18 and a second wire 20 to a control box 22 that houses a control device therein whereby the speed of the motor 16 can be incrementally controlled by turning the control knob 24. Extending from the motor housing 12 is a motor shaft 26. A rotatable mounting 28 removably receives a rotatable bioreactor holder 30 that removably receives a culture chamber 32 preferably disposable and cylindrical (or at least substantially cylindrical), which is affixed, preferably removably, within the rotatable bioreactor holder 30, preferably by a screw 34. The culture chamber 32 is mounted, preferably removably, to the rotatable mounting 28. The rotatable mounting 28 is received by the motor shaft 26. When the control knob 24 is turned on, the culture chamber 32 is rotated about a horizontal (or at least substantially horizontal) axis at a speed that preferably fosters, supports, and maintains the cells’ three-dimensional geometry and cell-to-cell support and cell-to-cell geometry while at the same time minimizing or even preventing cell collision with walls of the interior portion of the rotatable bioreactor and with other cells.

The culture chamber of the rotatable bioreactor of the present invention may preferably be disposable wherein it can be discarded and a new one used in later cell cultures. The culture chamber of the rotatable bioreactor may also preferably be sterilized, for instance in an autoclave, after each use and re-used for later cell cultures. A disposable culture chamber could be manufactured and packaged in a sterile environment thereby enabling it to be used by the medical or research professional much the same as other disposable medical devices are used.
"Figure 2 illustrates another preferred embodiment of the rotatable bioreactor 10. Reference numbers 112 to 134 correspond to Figure 1 references 12 to 34, respectively. Figure 2 is an elevated cross-sectional assembled side view of the rotatable bioreactor 10. Figure 2 further has an electrically conductive coil 135 wrapped around the exterior portion of the culture chamber 132. The electrically conductive coil may preferably be made of any electrically conductive material that conducts electricity including, but not limited to, the following conductive materials: silver, gold, copper, aluminum, iron, lead, titanium, uranium, a ferromagnetic metal, and zinc, or a combination thereof. The electrically conductive coil may also preferably comprise salt water. The electrically conductive coil may also preferably be a solenoid. Furthermore, the electrically conductive coil may preferably be contained in an electric insulator comprising, but not limited to, rubber, plastic, silicones, glass, and ceramic. The electrically conductive coil may be wrapped around the exterior portion of the culture chamber, and therefore, the culture chamber may support a shape of the electrically conductive coil, preferably having an oval (or at least substantially oval) cross-section, more preferably an elliptical (or at least a substantially elliptical) cross-section, and most preferably a circular (or at least a substantially circular) cross-section. By "wrapped around" it is intended that the electrically conductive coil encompasses the culture chamber so that, in use, a uniform (or at least substantially uniform) TVEMF is delivered to the interior portion of the culture chamber, to expose the cells of the three-dimensional culture therein to the TVEMF. The electrically conductive coil may preferably be integral with the culture chamber or adjacent to the culture chamber. By "integral with" it is intended that the electrically conductive coil is affixed to the culture chamber. By "adjacent to" it is meant that the electrically conductive coil is either removably touching or close to the culture chamber.

An electrically conductive coil that is integral with a disposable culture chamber is installed into the rotatable bioreactor along with the disposable culture chamber and operatively connected to the TVEMF source. By "operatively connected" it is meant that when, in use, the TVEMF source is turned on, a TVEMF is delivered to the interior portion of the culture chamber via the electrically conductive coil. When the disposable culture chamber is discarded, the electrically conductive coil is discarded therewith.

In the preferred embodiment of the rotatable bioreactor illustrated in Figure 2, there is a first and a second conductive wire having a first and second end. At the first end, the first conductive wire 140 and the second conductive wire 142, both of which are integral with the
electrically conductive coil 135, are operatively connected to a TVEMF source 144 having a
source knob 145 which, in use, can be turned on to generate a TVEMF. At the second end
the wires 140, 142 are connected to at least one ring to facilitate in part the rotation of the
electrically conductive coil 135, preferably a first ring 146 and a second ring 148
respectively. In this preferred embodiment, when the control knob 124 is turned on, the
culture chamber 132 and the electrically conductive coil 135 are rotated simultaneously.
Furthermore, the electrically conductive coil 135 remains affixed to, preferably removably,
and encompassing, the culture chamber 132, while at the same time supplying a TVEMF to
the cells in the rotatable bioreactor 132.

Figure 3 illustrates a side perspective view of the rotatable bioreactor wound by an
electrically conductive coil 135 also depicted in the preferred embodiment of Figure 2
wherein the electrically conductive coil 135 is wrapped around, and encompassing, a culture
chamber 132.

The culture chamber of a rotatable bioreactor may preferably be fitted with a culture
medium flow loop 400 preferably for the support of respiratory gas exchange in, supply of
nutrients in, and removal of metabolic waste products from a three-dimensional TVEMF
culture. A preferred embodiment of a culture medium flow loop 400 is illustrated in Figure
4, having a culture chamber 401, an oxygenator 402, an apparatus for facilitating the
directional flow of the culture medium, preferably by the use of a main pump 404, and a
supply manifold 406 for the selective input of culture medium requirements such as, but not
limited to, nutrients 408, buffers 410, fresh medium 412, cytokines 414, growth factors 416,
and hormones 418. In this preferred embodiment, the main pump 404 provides fresh culture
medium from the supply manifold 406 to the oxygenator 402 where the culture medium is
oxygenated and passed through the culture chamber 401. The waste in the spent culture
medium from the culture chamber 401 is removed, preferably by the main pump 404, and
delivered to the waste 420 and the remaining volume of culture medium not removed to the
waste 420 is returned to the supply manifold 406 where it may preferably receive a fresh
charge of culture medium requirements before recycling by the pump 404 through the
oxygenator 402 to the culture chamber 401.

In this preferred embodiment of a culture medium flow loop 400, adjustments are
made in response to chemical sensors (not shown) that maintain constant conditions within
... corrects pH. Oxygen, nitrogen, and carbon dioxide are dissolved in a gas exchange system (not shown) in order to support cell respiration. The culture medium flow loop 400 adds oxygen and removes carbon dioxide from a circulating gas capacitance. Although Figure 4 is one preferred embodiment of a culture medium flow loop that may be used in the present invention, the invention is not intended to be so limited. The input of culture medium requirements such as, but not limited to, oxygen, nutrients, buffers, fresh medium, cytokines, growth factors, and hormones into a rotatable bioreactor can also be performed manually, automatically, or by other control means, as can be the control and removal of waste and carbon dioxide.

As various changes could be made in rotatable bioreactors such as are contemplated in the present invention, without departing from the scope of the invention, it is intended that all matter contained herein be interpreted as illustrative and not limiting.

**Detailed Description of the Invention**

The present invention is related to treating symptoms of sickle cell disease by providing a rapidly available composition comprising expanded (adult) stem cells and red blood cells containing HbF. The stem cells and red blood cells containing HbF are cord blood and/or infant peripheral blood collected from a human before the human passed the age of 5, and the cells therein preferably expanded and/or differentiated prior to storing at -120°C to -196°C until the sickle cell disease becomes symptomatic. The composition is then raised to a temperature suitable for administration to the human, and is administered to the human.

The invention may be more fully described by the preferred embodiments as hereinafter described, but is not intended to be limited thereto.

The following definitions are meant to aid in the description and understanding of the defined terms in the context of the present invention. The definitions are not meant to limit these terms to less than is described throughout this application. Furthermore, several definitions are included relating to TVEMF - all of the definitions in this regard should be considered to complement each other, and not construed against each other. Also, while
TVEMP-expansion is preferred, generally reference to expansion or differentiation may be performed with or without TVEMF.

As used throughout this application, the term "sickle cell disease" includes sickle cell anemia, hemoglobin SC disease and Hemoglobin sickle beta-thalassemia, as well as other such symptomatic disorders including but not limited to those described throughout this application. Sickle cell disease is preferably identified in a human whose blood is collected, expanded and/or differentiated (preferably with a TVEMF present), and prepared into a composition of the present invention by genetically identifying the disease (for instance by identifying a mutated gene), or otherwise clinically identifying the disease (for instance by identifying sickle shaped red blood cells or mutant hemoglobin characteristic of a sickle cell disease). The composition may then be administered to the human to treat sickle cell disease symptoms.

As used throughout this application, the term "adult stem cell", or "stem cell", refers to a human pluripotent cell that is undifferentiated and that may give rise to more differentiated cells. With regard to the present invention, an adult stem cell is preferably CD34+/CD38-. Adult stem cells are also known as somatic stem cells, and are not embryonic stem cells directly derived from an embryo. Stem cells are a subpopulation of blood cells. Adult stem cells of the present invention are adult stem cells from human cord blood and/or (infant) peripheral blood.

As used throughout this application, the term "peripheral blood stem cell" refers to an adult stem cell from peripheral blood, and the term "cord blood stem cell" refers to an adult stem cell from cord blood.

As used throughout this application, the term "peripheral blood" according to the present invention is interchangeable with the term "infant peripheral blood", and refers to systemic blood of an infant or young child; that is, blood that circulates, or has circulated, systemically in a human of not more than about 5 years of age. The human is not a fetus. For the purposes of the present invention, there is no reason to distinguish between blood located at different parts of the same circulatory loop. (Infant) peripheral blood according to the present invention preferably refers to peripheral blood collected from a human of not more than 2 years of age, more preferably not more than one year of age, more preferably not
more than 6 months of age, as such blood includes red blood cells relatively rich in HbF. For the purposes of this invention, (infant) peripheral blood may include blood collected from a human of up to 5 years of age, although preferably less than 4 years of age, and more preferably less than 2 years of age, and most preferably less than 1 year of age, as discussed above. Peripheral blood according to this invention does not refer to blood taken from a human individual of more than 5 years of age, as the red blood cells of such blood typically no longer contain HbF. Preferably, the amount of red blood cells containing HbF in a blood source (cord and/or peripheral blood) acceptable for use in the present invention is about 10,000 cells/ml blood. Preferred amounts of blood that may be used to prepare a composition are disclosed throughout the application.

As used throughout this application, the term "peripheral blood cell" refers to a cell from peripheral blood, and the term "cord blood cell" refers to a cell from cord blood. The term "blood cell" according to this invention refers to one or both of peripheral blood cell and cord blood cell. Blood cells capable of replication may undergo TVEMF-expansion and/or — differentiation in a rotatable TVEMF-bioreactor. Red blood cells are also blood cells (peripheral or cord); while most red blood cells containing HbF are anucleate and are not capable of replication, their presence during the expansion of stem cells and other cells in a rotatable TVEMF-bioreactor as a necessary component of the present invention.

As used throughout this application, the term "HbF", or "fetal hemoglobin", refers to a type of hemoglobin found in the fetus during the last 7 months of pregnancy and, after birth, in human children up to 4 or 5 years of age. HbF is preferably contained within red blood cells in the present invention, but may also be present outside of red blood cells, for instance if the integrity of a red blood cell membrane is compromised and HbF is released from the cell.

As used throughout this application, the term "red blood cells containing HbF" refers to human RBCs having HbF inside the confines of their cell membranes. The term containing should be construed broadly, as comprising HbF; for instance, red blood cells including HbF and HbA are red blood cells containing HbF according to the present invention.
Reference to "naturally-occurring blood" or naturally-occurring stem cells, and the like, is to blood or stem cells prior to expansion or differentiation, in the same or similar condition as when or before collected from the human subject. The reference may be used to compare, for instance, expanded or differentiated cells removed from a rotatable bioreactor with cells prior to being expanded or differentiated in the bioreactor. However, if the original blood or other substance is not available, then naturally-occurring blood and so forth may refer to average or typical characteristics of human (cord or infant peripheral) blood, blood stem cells, and so forth.

As used throughout this application, the term "blood cell mixture" refers to a mixture of cord blood cells and/or peripheral blood cells (including at least stem cells and red blood cells containing HbF) with a substance that allows the cells to expand, such as a medium for growth of cells, that may be placed in a rotatable TVEMF-bioreactor (i.e. in the interior portion of a cell culture chamber). The blood cells may be present in the blood cell mixture simply by mixing whole cord or infant peripheral blood with a substance such as a cell culture medium. Also, the blood cell mixture may be made with a cellular preparation from cord and/or infant peripheral blood, such as a "buffy coat", as described throughout this application, containing blood stem cells in combination with red blood cells from the same human, and preferably from the same blood preparation. Preferably, the blood cell mixture comprises CD34+/CD38- blood stem cells and Dulbecco's medium (DMEM). Preferably, at least half of the blood cell mixture is a cell culture medium such as DMEM.

There are several benefits of using cord blood to prepare compositions of the present invention. Where available, it is preferred to use cord blood in the present invention, either without infant peripheral blood or in combination with infant peripheral blood. Cord blood stem cells are young; they are also more likely to be healthier cells because they have had fewer opportunities to be affected by damaging environmental toxins that may change DNA. Also because they are young, cord blood stem cells may have greater plasticity and may more easily differentiate into red blood cells containing HbF. Because of their youth, cord blood stem cells may a little less stable than older stem cells and may be more susceptible to damage, for instance from cryopreservation, than more aged stem cells.
Cord blood is "Mt'bell-rich. Typically, approximately 1-2% of cord blood mononuclear (cells having one nucleus) cells are stem cells. This makes cord blood one of the richest sources of stem cells. Cord blood collected from a Caesarean section is typically even a little richer in stem cells than cord blood collected immediately after vaginal birth. Cord blood and infant peripheral blood are both available sources of stem cells. Stem cells from body tissue such as bone marrow are not readily available. A cord blood collection from a typical human infant immediately after birth will typically yield 50 to 100 ml cord blood.

As used throughout this application, the term "TVEMF" refers to "Time Varying Electromagnetic Force". As discussed throughout the application, the TVEMF of this invention is preferably a square wave (following a Fourier curve). The size of the electrically conductive coil, and number of times it is wound around the culture chamber of the rotatable bioreactor, are such that when a TVEMF is supplied to the electrically conductive coil a TVEMF is generated within the three-dimensional culture in the interior portion of the culture chamber of the rotatable bioreactor. The TVEMF is preferably selected from one of the following: (1) a TVEMF with a force amplitude less than 100 gauss and slew rate greater than 1000 gauss per second, (2) a TVEMF with a low force amplitude bipolar square wave at a frequency less than 100 Hz., (3) a TVEMF with a low force amplitude square wave with less than 100% duty cycle, (4) a TVEMF with slew rates greater than 1000 gauss per second for duration pulses less than 1 ms., (5) a TVEMF with slew rate bipolar delta function-like pulses with a duty cycle less than 1%, (6) a TVEMF with a force amplitude less than 100 gauss peak-to-peak and slew rate bipolar delta function-like pulses and where the duty cycle is less than 1%, (7) a TVEMF applied using a solenoid coil to create uniform force strength throughout the cell mixture, (8) and a TVEMF applied utilizing a flux concentrator to provide spatial gradients of magnetic flux and magnetic flux focusing within the cell mixture. The range of frequency in oscillating electromagnetic force strength is a parameter that may be selected for achieving the desired stimulation of the cells in the three-dimensional culture. However, these parameters are not meant to be limiting to the TVEMF of the present invention, and as such may vary based on other aspects of this invention. TVEMF may be measured for instance by standard equipment such as an EN131 Cell Sensor Gauss Meter.

As used throughout this application, the term "expanded blood cells", or "TVEMF-expanded blood cells" where TVEMF is present, refers to human cord blood cells and/or
cells increased in number per volume after being placed in the interior portion of a culture chamber of a rotatable TVEMF-bioreactor and subjected to a TVEMF. The increase in number of cells per volume is the result of cell replication in the rotatable TVEMF-bioreactor, so that the total number of cells increase. The increase in number of cells per volume is expressly not due to a simple reduction in volume of fluid, for instance, reducing the volume of blood from 70 ml to 10 ml and thereby increasing the number of cells per ml. "Differentiated blood cells" and the like refers to cells that undergo differentiation in the rotating rotatable bioreactor.

As used throughout this application, the term "expanded blood stem cells", or "TVEMF-expanded blood stem cells" where TVEMF is present, refers to human cord blood stem cells and/or peripheral blood stem cells increased in number per volume after being placed in a rotatable TVEMF-bioreactor and subjected to a TVEMF. The increase in number of stem cells per volume is the result of cell replication in the rotatable TVEMF-bioreactor, so that the total number of stem cells in the rotatable bioreactor increase. The increase in number of stem cells per volume is expressly not due to a simple reduction in volume of fluid, for instance, reducing the volume of blood from 70 ml to 10 ml and thereby increasing the number of stem cells per ml.

As used throughout this application, the term "expanding", or where TVEMF is present, "TVEMF-expanding" refers to the step of cells in a rotatable TVEMF-bioreactor replicating (splitting and growing) in the presence of TVEMF in a TVEMF-(rotating) bioreactor. Blood stem cells in the presence of red blood cells containing HbF may replicate without undergoing further differentiation, but preferably differentiate to various hematopoietic stages including to prepare new red blood cells containing HbF while rotating in a rotatable bioreactor. "Differentiating", or where TVEMF is present "TVEMF-differentiating", refers to differentiation typically to a more specialized cell type occurring in a rotatable (preferably TVEMF-) bioreactor. More specifically, "differentiation" means at least the process of a stem cell or other more differentiated hematopoietic cell transforming into a more specialized cell. For the purposes of the present invention, preferably a stem cell will differentiate into a red blood cell containing HbF. Preferably about 70% to about 98% of stem cells replicate (expand) and about 30% to about 50%, preferably 40%, differentiate while rotating in a rotatable bioreactor (preferably while being exposed to a TVEMF in a rotatable TVEMF bioreactor) of the present invention. Even more preferably, expansion of
bëM "SfeWcell? ‟p δ vide1i Fnumber per volume that is about seven times the number per volume of the original source of naturally occurring blood, and differentiation occurs in about 40% of the stem cells.

As used throughout this application, the term "expansion", or where TVEMF is present, "TVEMF-expansion", refers to the process of increasing the number of blood cells in a rotatable TVEMF-bioreactor, preferably blood stem cells. In one preferred embodiment, the increase in number of blood stem cells, is at least 7 times the number per volume of the original peripheral blood source, regardless of the number of cells that differentiate into red blood cells containing HbF. The expansion of blood stem cells in a rotatable TVEMF-bioreactor according to the present invention provides for blood stem cells that maintain, or have the same or essentially the same, three-dimensional geometry and cell-to-cell support and cell-to-cell geometry as blood stem cells prior to TVEMF-expansion. Other aspects of TVEMF-expansion may also provide the exceptional characteristics of the blood stem cells of the present invention. Not to be bound by theory, TVEMF-expansion not only provides for high concentrations of blood stem cells that maintain their three-dimensional geometry and cell-to-cell support. Not to be bound by theory, TVEMF may affect some properties of stem cells during TVEMF-expansion, for instance up-regulation of genes promoting growth, or down regulation of genes preventing growth. Without being bound by theory, TVEMF-expansion in the presence of HbF may cause a deactivation of sickle cell mutations in stem cells or other hematopoietic cells of the present invention.

As used throughout this application, the term "expanded cell", or where TVEMF is present "TVEMF-expanded cell", refers to a cell that has been subjected to the process of TVEMF-expansion and has undergone (or been the result of) replication. Similarly, a "differentiated cell" is a cell that has undergone differentiation in a rotating rotatable bioreactor.

Throughout this application, reference to the treatment of sickle cell disease symptoms may relate to treatment of one or more symptoms of sickle cell disease. The present invention may also include treatment of minor symptoms, and even prevention/prophylaxis of a sickle cell disease symptoms by early introduction of the present
cotopoietin-expanded stem cells and red blood cells containing HbF, before overt symptoms or problems in the human's health are noticed.

As used throughout this application, the term "toxic substance" or related terms may refer to substances that are toxic to a cell, preferably a peripheral blood stem cell; or toxic to a human. In particular, the term toxic substance refers to dead cells, macrophages, as well as substances that may be unique or unusual in blood (for instance, sickle cells or other tissue or waste). Other toxic substances are discussed throughout this application. Removal of toxic substances from blood is well-known in the art, in particular art relating to the introduction of blood products to a human.

As used throughout this application, the term "autologous" refers to a situation in which the source of blood stem cells prior to expansion and/or differentiation and red blood cells containing HbF is the same human as the human to whom a composition of the present invention is to be or is administered.

As indicated throughout this invention, the present invention is directed only to an autologous situation in a human.

As used throughout this application, the term "CD34+" refers to the presence of a surface antigen (CD34) on the surface of a blood cell. CD34 protein is typically present on the surface of hematopoietic stem cells in all states of development.

As used throughout this application, the term "CD38-" refers to the lack of a surface antigen (CD38) on the surface of a blood cell. CD38 is not present on the surface of stem cells of the present invention.

As used throughout this application, the term "cell-to-cell geometry" refers to the geometry of cells including the spacing, distance between, and physical relationship of the cells relative to one another. For instance, TVEMF-expanded stem cells of this invention stay in relation to each other as in the body. The expanded cells are within the bounds of natural spacing between cells, in contrast to for instance two-dimensional expansion containers, where such spacing is not kept. Similarly, differentiated cells
As used throughout this application, the term "cell-to-cell support" refers to the support one cell provides to an adjacent cell. For instance, healthy tissue and cells maintain interactions such as chemical, hormonal, neural (where applicable/appropriate) with other cells in the body. In the present invention, these interactions are maintained within normal functioning parameters, meaning they do not for instance begin to send toxic or damaging signals to other cells (unless such would be done in the natural blood environment).

As used throughout this application, the term "three-dimensional geometry" refers to the geometry of cells in a three-dimensional state (same as or very similar to their natural state), as opposed to two-dimensional geometry for instance as found in cells grown in a Petri dish, where the cells become flattened and/or stretched.

For each of the above three definitions, relating to maintenance of cell-to-cell support and geometry and three dimensional geometry of stem cells of the present invention, the term "essentially the same" means that normal geometry and support are provided in TVEMF-expanded cells of this invention, so that the cells are not changed in such a way as to be for instance dysfunctional, unable to differentiate for instance into red blood cells containing HbF, or toxic or harmful to other cells.

Other statements referring to the above-defined terms or other terms used throughout this application are not meant to be limited by the above definitions, and may contribute to the definitions. Information relating to various aspects of this invention is provided throughout this application, and is not meant to be limited only to the section to which it is contained, but is meant to contribute to an understanding of the invention as a whole.

In operation, blood cells are placed into the interior portion of the culture chamber of a rotatable bioreactor (preferably a TVEMF-bioreactor). In one preferred embodiment, the culture chamber is rotated over a period of time, while at the same time a TVEMF is generated in the culture chamber by the TVEMF source. By "while at the same time," it is
Of the delivery of the TVEMF may be before, concurrent with, or after rotation of the culture chamber is initiated. Upon completion of the period of time, the expanded and/or differentiated cells are removed from the interior portion of the culture chamber. In a more complex rotatable bioreactor, a culture medium enriched with culture medium requirements preferably including, but not limited to, growth medium, buffer, nutrients, hormones, cytokines, and growth factors, which provides sustenance to the cells, can be periodically refreshed and removed.

In use, a rotatable bioreactor of the present invention, preferably a TVEMF-bioreactor, provides a stabilized culture environment into which cells may be introduced, suspended, assembled, grown, and/or maintained with improved retention of delicate three-dimensional structural integrity by simultaneously minimizing the fluid shear stress, providing three-dimensional freedom for cell and substrate spatial orientation, and increasing localization of cells in a particular spatial region for the duration of the expansion. In use, the present invention also provides these three criteria (hereinafter referred to as "the three criteria above"), and at the same time, exposes the cells to a TVEMF. Of particular interest to the present invention is the dimension of the culture chamber, the sedimentation rate of the cells, the rotation rate, the external gravitational field, and the TVEMF.

The stabilized culture environment referred to in the operation of present invention is that condition in the culture medium, particularly the fluid velocity gradients, prior to introduction of cells, which will support a nearly uniform suspension of cells upon their introduction thereby creating a three-dimensional culture upon addition of the cells. In a preferred embodiment, the culture medium is initially stabilized into a near solid body horizontal rotation about an axis within the confines of a similarly rotating chamber wall of a rotatable bioreactor. The chamber walls are set in motion relative to the culture medium so as to initially introduce essentially no fluid stress shear field therein. Cells are introduced to, and move through, the culture medium in the stabilized culture environment thus creating a three-dimensional culture. The cells move under the influence of gravity, centrifugal, and coriolus forces, and the presence of cells within the culture medium of the three-dimensional culture induces secondary effects to the culture medium. The significant motion of the culture medium with respect to the culture chamber, significant fluid shear stress, and other fluid motions, is due to the presence of these cells within the culture medium.
sediment at a slow rate preferably under 0.1 centimeter per second. It is therefore possible, at this early stage of the three-dimensional culture, to select from a broad range of rotational rates (preferably of from about 2 to about 30 RPM) and chamber diameters (preferably of from about 0.5 to about 10 inches). Preferably, the slowest rotational rate is advantageous because it minimizes equipment wear and other logistics associated with handling of the three-dimensional culture.

Not to be bound by theory, rotation about a substantially horizontal axis with respect to the external gravity vector at an angular rate optimizes the orbital path of cells suspended within the three-dimensional culture. In operation, while the culture chamber is rotating, the cells expand and/or differentiate to form a mass of cell aggregates, three-dimensional tissues, and/or tissue-like structures, which increase in size as the three-dimensional culture progresses. The progress of the three-dimensional culture is preferably assessed by a visual, manual, or automatic determination of an increase in the diameter of the three-dimensional cell mass in the three-dimensional culture. An increase in the size of the cell aggregate, tissue, or tissue-like structure in the three-dimensional culture may require appropriate adjustment of the rotation speed in order to optimize the particular paths. The rotation of the culture chamber optimally controls collision frequencies, collision intensities, and localization of the cells in relation to other cells and also the limiting boundaries of the culture chamber of the rotatable bioreactor. In order to control the rotation, if the cells are observed to excessively distort inwards on the downward side and outwards on the upwards side then the revolutions per minute ("RPM") may preferably be increased. If the cells are observed to centrifugate excessively to the outer walls then the RPM may preferably be reduced. Not to be bound by theory, as the operating limits are reached, in terms of high cell sedimentation rates or high gravity strengths, the operator may be unable to satisfy both of these conditions and may be forced to accept degradation in performance as measured against the three criteria above.

The cell sedimentation rate and the external gravitations field place a lower limit on the fluid shear stress obtainable, even within the operating range of the present invention, due to gravitationally induced drift of the cells through the culture medium of the three-dimensional culture. Calculations and measurements place this minimum fluid shear stress very nearly to that resulting from the cells' terminal sedimentation velocity (through the
Centrifugal and coriolis induced motion [classical angular kinematics provide the following equation relating the Coriolis force to an object's mass (m), its velocity in a rotating frame ($v_r$) and the angular velocity of the rotating frame of reference ($\Omega$): $F_{\text{Coriolis}} = -2m(\Omega \times v_r)$] along with secondary effects due to cell and culture medium interactions, act to further degrade the fluid shear stress level as the cells expand and/or differentiate.

Not to be bound by theory, but as the external gravity field is reduced, much denser and larger three-dimensional structures can be obtained. In order to obtain the minimal fluid shear stress level it is preferable that the culture chamber be rotated at substantially the same rate as the culture medium. Not to be bound by theory, but this minimizes the fluid velocity gradient induced upon the three-dimensional culture. It is advantageous to control the rate and size of tissue formation in order to maintain the cell size (and associated sedimentation rate) within a range for which the rate of expansion is able to satisfy the three criteria above. However, preferably, the velocity gradient and resulting fluid shear stress may be intentionally introduced and controlled for specific research purposes such as studying the effects of shear stress on the three-dimensional cell aggregates. In addition, transient disruptions of the expansion process are permitted and tolerated for, among other reasons, logistical purposes during initial system priming, sample acquisition, system maintenance, and culture termination.

Rotating cells about an axis substantially perpendicular to gravity can produce a variety of sedimentation rates, all of which according to the present invention remain spatially localized in distinct regions for extended periods of time ranging from seconds (when sedimentation characteristics are large) to hours (when sedimentation differences are small). Not to be bound by theory, but this allows these cells sufficient time to interact as necessary to form multi-cellular structures and to associate with each other in a three-dimensional culture. Preferably, cells undergo expansion and/or differentiation for at least 4 days, more preferably from about 7 days to about 14 days, most preferably from about 7 days to about 10 days, even more preferably about 7 days, and for other periods of time for instance as discussed throughout this application. Preferably, TVEMF-expansion may continue in a rotatable bioreactor to produce a concentration of cells per volume that is at least 7 times the original concentration of cells per volume that were placed in the rotatable bioreactor.
Culture chamber dimensions also influence the path of cells in the three-dimensional culture of the present invention. A culture chamber diameter is preferably chosen which has the appropriate volume, preferably of from about 15ml to about 2L for the intended three-dimensional culture and which will allow a sufficient seeding density of cells. Not to be bound by theory, but the outward cells drift due to centrifugal force is exaggerated at higher culture chamber radii and for rapidly sedimenting cells. Thus, it is preferable to limit the maximum radius of the culture chamber as a function of the sedimentation properties of the tissues anticipated in the final three-dimensional culture stages (when the largest cell aggregates with high rates of sedimentation have formed).

The path of the cells in the three-dimensional culture has been analytically calculated incorporating the cell motion resulting from gravity, centrifugation, and coriolis effects. A computer simulation of these governing equations allows the operator to model the process and select parameters acceptable (or optimal) for the particular planned three-dimensional culture. Figure 5 shows the typical shape of the cell orbit as observed from the external (non-rotating) reference frame. Figure 6 is a graph of the radial deviation of a cell from the ideal circular streamline plotted as a function of RPM (for a typical cell sedimenting at 0.5 cm per second terminal velocity). This graph (Figure 6) shows the decreasing amplitude of the sinusoidally varying radial cells deviation as induced by gravitational sedimentation. Figure 6 also shows increasing radial cells deviation (per revolution) due to centrifugation as RPM is increased. These opposing constraints influence carefully choosing the optimal RPM to preferably minimize cell impact with, or accumulation at, the chamber walls. A family of curves is generated which is increasingly restrictive, in terms of workable RPM selections, as the external gravity field strength is increased or the cell sedimentation rate is increased. This family of curves, or preferably the computer model which solves these governing orbit equations, is preferably utilized to select the optimal RPM and chamber dimensions for the expansion of cells of a given sedimentation rate in a given external gravity field strength. Not to be bound by theory, but as a typical three-dimensional culture is expanded and/or differentiated, the tissues, cell aggregates, and tissue-like structures increase in size and sedimentation rate, and therefore, the rotation rate may preferably be adjusted to optimize the same.
three-dimensional culture, the cell orbit (Figure 5) from the rotating reference frame of the culture medium is seen to move in a nearly circular path under the influence of the rotating gravity vector (Figure 7). Not to be bound by theory, but the two pseudo forces, coriolis and centrifugal, result from the rotating (accelerated) reference frame and cause distortion of the otherwise nearly circular path. Higher gravity levels and higher cell sedimentation rates produce larger radius circular paths which correspond to larger trajectory deviations from the ideal circular orbit as seen in the non-rotating reference frame. In the rotating reference frame it is thought, not to be bound by theory, that cells of differing sedimentation rates will remain spatially localized near each other for long periods of time with greatly reduced net cumulative separation than if the gravity vector were not rotated; the cells are sedimenting, but in a small circle (as observed in the rotating reference frame). Thus, in operation the present invention provides cells of differing sedimentation properties with sufficient time to interact mechanically and through soluble chemical signals. In operation, the present invention provides for sedimentation rates of preferably from about 0 cm/second up to 10 cm/second.

Furthermore, as discussed throughout the application, in operation the culture chamber of the present invention has at least one aperture preferably for the input of fresh culture medium and a cell mixture and the removal of a volume of spent culture medium containing metabolic waste, but not limited thereto. Preferably, the exchange of culture medium can also be via a culture medium loop wherein fresh or recycled culture medium may be moved within the culture chamber preferably at a rate sufficient to support metabolic gas exchange, nutrient delivery, and metabolic waste product removal. This may slightly degrade the otherwise quiescent three-dimensional culture. It is preferable, therefore, to introduce a mechanism for the support of preferred components including, but not limited to, respiratory gas exchange, nutrient delivery, growth factor delivery to the culture medium of the three-dimensional culture, and also a mechanism for metabolic waste product removal in order to provide a long term three-dimensional culture able to support significant metabolic loads for periods of hours to months.

The present invention exposes the cells to a TVEMF that not only provides for high concentrations of cells that maintain their three-dimensional geometry and cell-to-cell support but in addition, may affect some properties of cells during expansion, for instance up-regulation of genes promoting growth, or down regulation of genes preventing growth. The
electromagnetic field: generated by a TVEMF source. In operation, the electrically conductive coil of a rotatable bioreactor is rotatable with the culture chamber, meaning about the same axis as the culture chamber and in the same direction. Also, the electrically conductive coil is fixed in relation to a culture chamber of a rotatable TVEMF-bioreactor. The electrically conductive coil may be integral with, meaning affixed to and wrapped around the exterior portion of the culture chamber of the electrically conductive coil of the culture chamber of the rotatable bioreactor. The TVEMF source is operatively connected to the rotatable bioreactor.

In addition to the qualitatively unique cells that are produced by the operation of the present invention, not to be bound by theory, an increased efficiency with respect to utilization of the total culture chamber volume for cell and tissue culture may be obtained due to the substantially uniform homogeneous suspension achieved. Advantageously, therefore, the present invention, in operation, provides an increased number of cells in the same rotatable bioreactor with less human resources.

The method of the present invention provides these three criteria above, and at the same time, facilitates and supports expansion such that a sufficient expansion (increase in number per volume, diameter in reference to tissue, or concentration) is detected in a sufficient amount of time. The present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned herein, as well as those inherent therein. Without departing from the scope of the invention, it is intended that all matter contained herein be interpreted as illustrative and not limiting.

The present invention is directed to a composition, process for preparing the composition, and method of treating a human experiencing symptoms of sickle cell disease. This invention may be more fully described by the preferred embodiment(s) as hereinafter described, but is not intended to be limited thereto.

**Operative Method - Preparing a TVEMF-expanded blood stem cell composition, and using the composition**

The following methodology describes preferred embodiments of the present invention. A method is described for preparing TVEMF-expanded blood stem cells in the
presence Wired blBo'd c'ells'' containing HbF (all from the same human) that can be thereafter administered to the human to treat symptoms of sickle cell disease.

Cord blood and/or infant peripheral blood is to be collected from a human, for instance as described throughout this application and as known in the art.

There are two typical ways of collecting cord blood; blood bag collection and syringe collection. Blood bag collection involves a health care provider inserting a needle into the umbilical vein and, with the assistance of gravity, draining the blood into a bag. Once the blood has stopped flowing, the bag will be sealed and labeled by the health care provider. This method is usually done before the placenta is delivered.

Syringe collection is similar to blood bag collection except that the cord blood is drawn into syringes containing anticoagulants (a substance that prevents the blood from clotting). The blood is stored in the syringes instead of in blood bags. This method can be done before or after the placenta is delivered. It is thought to be a more reliable way of collecting blood than blood bag collection. It also allows for more blood to be collected than is possible with blood bag collection. Regardless of which method is utilized, or whether another process for collecting cord blood is utilized, the whole process of collection may take as little as five minutes to perform, or even less. Preferably, the cord blood is collected within 10 to 15 minutes after birth. Waiting longer than this may result in less cord blood being collected, and therefore, fewer cord blood stem cells collected.

In the case of cord blood banking, or storage, once the cord blood arrives at the storage facility the cord blood is tested to make sure it does not carry any infectious or genetic diseases, like hepatitis, HIV/AIDS, leukemia, or an immune disorder. If there are any such problems with the cord blood, it may either be considered unsuitable for storage, or, in some instances, the blood may still be stored with the associated risks noted. If the blood is needed in the future parents can assess whether or not the need for the cord blood stem cells outweighs the associated risks carried with the cord blood.

Infant peripheral blood is preferably collected via a syringe, as well known in the art. Infant peripheral blood may be collected in small quantities over a period of time, for instance over the first year of age, and such small quantities pooled together and cryopreserved for later use, or the quantities may be expanded and/or differentiated immediately upon collection and cryopreserved thereafter, individually or in a collective pool. Peripheral blood would only be removed from a human in an amount that would not be threatening to the subject. Preferably, about 10 to about 500 ml peripheral blood is collected; more preferably, 100-300 ml, even more preferably, 150-200 ml, at one time or
bypoolMg's several collections. The collection of peripheral blood according to this invention is not meant to be limiting, but can also include for instance other means of directly collecting peripheral blood, or pooling peripheral blood from one or more collections. As the peripheral blood according to this invention is from children under 5 years of age, withdrawal of blood may be in periodic and smaller amounts to be pooled at a later point in the process of making, cryopreserving and administering the composition of this invention. Peripheral blood is preferably drawn into one or more syringes containing anticoagulants. The blood may be stored in the syringe or transferred to another vessel.

Cord and/or peripheral blood may be stored by cryopreservation after collection and before expansion and/or differentiation in a rotatable bioreactor, although preferably blood cells are not cryopreserved until after expansion and/or differentiation in a rotatable bioreactor in the present invention. Typically, whether cryopreserved after collection or expanded and/or differentiated certain components of the blood are removed from whole blood to ease further processes. For instance, blood may be separated into three parts: white blood cells ("buffy coat"), red blood cells containing HbF, and plasma. This may be done for instance in a centrifuge (an apparatus that spins the container of blood until the blood is divided) or by sedimentation (the process of injecting sediment into the container of blood causing the blood to separate). Once the blood is divided with the red blood cells (RBC) containing HbF on the bottom, white blood cells (WBC) in the middle, and the plasma on top, the white blood cells and red blood cells containing HbF may be recombined. The buffy coat contains the blood stem cells of interest. The buffy coat may be further processed by removing white blood cells or other cells therein that are not stem cells, preferably while retaining most of the original stem cells, for instance as discussed below.

Another method for separating blood cells is to subject all of the collected blood to one or more (preferably three) rounds of continuous flow leukapheresis in a separator such as a Cobe Spectra cell separator. Such processing will separate peripheral blood cells having one nucleus from other peripheral blood cells. The stem cells are part of the group having one nucleus. The red blood cells have no nucleus and are separated from the stem cells, although the two cell types will be reunited for expansion and/or differentiation. Other methods for the separation of blood cells are known in the art. Such methods should preserve the integrity of blood cells, to minimize stem cell or red blood cell containing HbF losses during this process.
FiřTfiorepfeleiTec embodiment of this invention, only the "buffy coat" (which includes blood stem cells, as discussed throughout this application) (with RBCs containing HbF) described above is the cellular material placed in the rotatable TVEMF-bioreactor. Other embodiments include removing other non-stem cells from the buffy coat, to prepare different blood cell preparation(s). Such a blood cell preparation may even have, as the only remaining blood component, CD34+/CD38- peripheral blood stem cells. For instance, RBCs containing HbF may be isolated by centrifugation or leukapheresis, as described above, and stem cells isolated through negative separation techniques, such as but not limited to sedimentation and centrifugation. Many negative separation methods are well-known in the art. However, positive selection techniques may also be used, and are preferred in this invention. Methods for removing various components of the blood and positively selecting for CD34+/CD38- are known in the art, and may be used so long as they do not lyse or otherwise irreversibly harm the blood stem cells. For instance, an affinity method selective for CD34+/CD38- may be used. Preferably, a "buffy coat" as described above is prepared from collected blood, and the CD34+/CD38- cells therein separated from the buffy coat then reunited with RBCs containing HbF for TVEMF-expansion.

After blood is collected from a human (preferably genetically identified as having sickle cell disease), the blood may be used, whole or in cellular part (comprising at least stem cells and RBCs containing HbF), to prepare a blood cell mixture. Preferably, the remaining cells including peripheral blood stem and red blood cells containing HbF are placed with an appropriate media in a rotatable TVEMF-bioreactor (see "blood cell mixture") such as that described herein. The collected blood, or desired cellular parts including at least stem cells and RBCs containing HbF, as discussed above, must be placed into a rotatable bioreactor for expansion and/or differentiation (preferably TVEMF-) to occur. As discussed above, the term "blood cell mixture" comprises a mixture of blood (or desired cellular parts) with a substance that allows the cells to expand and/or differentiate, such as a medium for growth of cells, that will be placed in a rotatable TVEMF-bioreactor. Cell culture media, media that allow cells to grow and expand, are well-known in the art. Preferably, the substance that allows the cells to expand is cell culture media, more preferably Dulbecco's medium. The components of the cell media must, of course, not kill or damage the stem cells or RBCs containing HbF. Other components may also be added to the blood cell mixture prior to or during TVEMF-
expansion. For instance, the blood or desired blood cells may be placed in the rotatable bioreactor with Dulbecco's medium and further supplemented with 5% (or some other desired amount, for instance in the range of about 1% to about 10%) of human serum albumin. Other additives to the blood cell mixture, including but not limited to growth factor, cytokine, hormone and other substances that may enhance TVEMF-expansion may also be added to the peripheral blood outside or inside the rotatable bioreactor before being placed in the rotatable bioreactor. As indicated throughout this application, cord blood is typically collected in an amount of about 50 ml to about 100 ml. Preferably, the entire volume of a peripheral blood collection from a human is in an amount of about 10 ml to about 500 ml, more preferably about 100 ml to about 300 ml, even more preferably about 150 to about 200 ml peripheral blood is mixed with a cell culture medium such as Dulbecco's medium (DMEM) and supplemented with 5% human serum albumin to prepare a blood cell mixture for TVEMF-expansion. The cord blood and/or peripheral blood may be combined together or treated separately. For smaller children and infants, smaller amounts of blood should be collected, so as not to endanger the child's health. For instance, for a 50 to 100 ml quantity of blood, preferably about 25 to about 100 ml DMEM/5% human serum albumin is used, so that the total volume of the blood cell mixture is about 75 to about 200 ml when placed in the rotatable bioreactor. As a general rule, the more blood that may be collected, the better; for instance, if a collection from one human results in more than 200 ml, the use of all of the stem cells in that peripheral blood is preferred. Where a larger volume is available, for instance by pooling peripheral blood (from the same human), all may be expanded and/or differentiated together, or in parts, as desired.

The term "placed into a rotatable TVEMF-bioreactor" is not meant to be limiting - the blood cell mixture may be made entirely outside of the rotatable bioreactor and then the mixture placed inside the rotatable bioreactor. Also, the blood cell mixture may be entirely mixed inside the rotatable bioreactor. For instance, the blood (or cellular portion thereof) may be placed in the rotatable bioreactor and supplemented with Dulbecco's medium and 5% human serum albumin either already in the rotatable bioreactor, added simultaneously to the rotatable bioreactor, or added after the peripheral blood to the rotatable bioreactor.
A preferred mixture of the present invention to be placed in a rotatable TVEMF-bioreactor comprises the following: CD34+/CD38- stem cells isolated from the buffy coat of a blood sample; RBCs containing HbF (if separated, then recombined with the stem cells), and Dulbecco's medium which, with the CD34+/CD38- cells, is about 150-250 ml, preferably about 200 ml total volume. Even more preferably, at least one of G-CSF (Granulocyte-Colony Stimulating Factor), interleukin-2 (IL-2), erythropoietin (EPO) and a hormone is included in the blood cell mixture, more preferably in an amount sufficient to enhance expansion of blood stem cells and/or differentiation into red blood cells containing HbF. Even more preferably, the amount of G-CSF, IL-2, EPO and/or hormone present in the blood cell mixture prior to expansion and/or differentiation is about 25 to about 200 ng/ml blood cell mixture, more preferably about 50 to about 150 ng/ml, and even more preferably about 100 ng/ml.

The rotatable TVEMF-bioreactor vessel (containing the blood cell mixture including the blood stem cells and RBCs containing HbF) is rotated at a speed that provides for suspension of the cells to maintain their three-dimensional geometry and their cell-to-cell support and cell-to-cell geometry. Preferably, the rotational speed is 5-120 rpm; more preferably, from 10-30 rpm. These rotational speeds are not intended to be limiting; rotational speed will depend at least in part on the type of rotatable bioreactor and size of cell culture chamber and sample placed therein. During the time that the cells are in the rotatable TVEMF-bioreactor, they are preferably fed nutrients and fresh media (for instance, DMEM and 5% human serum albumin; see above discussions of fluid carriers), exposed to hormones, cytokines, and/or growth factors (preferably G-CSF); and toxic materials are removed. The toxic materials removed from blood cells in a rotatable TVEMF-bioreactor include toxic granular material of dying cells and toxic material of granulocytes and macrophages. The TVEMF-expansion of the cells is controlled so that the cells preferably expand (increase in number per volume) at least seven times. Preferably, blood stem cells (with other replicable cells, if present) undergo TVEMF-expansion for at least 4 days, preferably about 7 to about 14 days, more preferably about 7 to about 10 days, even more preferably about 7 days. TVEMF-expansion may continue in a rotatable TVEMF-bioreactor for up to 160 days. While TVEMF-expansion may occur for even longer than 160 days, such a lengthy expansion is not a preferred embodiment of the present invention.
Preferably, WBMF-expansion is carried out in a rotatable TVEMF-bioreactor at a temperature of about 26°C to about 41°C, and more preferably, at a temperature of about 37°C.

One method of monitoring the overall expansion of cells undergoing TVEMF-expansion is by visual inspection. Stem cells are typically dark red in color; red blood cells containing HbF are also red in color. Preferably, the medium used to form the blood cell mixture is light or clear in color. Once the rotatable bioreactor begins to rotate and the TVEMF is applied, the cells preferably cluster in the center of the rotatable bioreactor vessel, with the medium surrounding the colored cluster of cells. Oxygenation and other nutrient additions often do not cloud the ability to visualize the cell cluster through a visualization (typically clear plastic) window built into the rotatable bioreactor. Formation of the cluster is important for helping the stem cells maintain their three-dimensional geometry and cell-to-cell support and cell-to-cell geometry; if the cluster appears to scatter and cells begin to contact the wall of the rotatable bioreactor vessel, the rotational speed is increased (manually or automatically) so that the centralized cluster of cells may form again. A measurement of the visualizable diameter of the cell cluster taken soon after formation may be compared with later cluster diameters, to indicate the approximate number increase in cells in the rotatable TVEMF-bioreactor. Measurement of the increase in the number of cells during TVEMF expansion may also be taken in a number of ways, as known in the art for conventional bioreactors. An automatic sensor could also be included in the rotatable TVEMF-bioreactor to monitor and measure the increase in cluster size.

The TVEMF-expansion process may be carefully monitored, for instance by a laboratory expert, who may check cell cluster formation to ensure the cells remain clustered inside the rotatable bioreactor and will increase the rotation of the rotatable bioreactor when the cell cluster begins to scatter. An automatic system for monitoring the cell cluster and viscosity of the blood cell mixture inside the rotatable bioreactor may also monitor the cell clusters. A change in the viscosity of the cell cluster may become apparent as early as 2 days after beginning the TVEMF-expansion process, and the rotational speed of the rotatable TVEMF-bioreactor may be increased around that time. The rotatable TVEMF-bioreactor speed of rotation may vary throughout TVEMF-expansion. Preferably, the rotational speed is timely adjusted so that the cells undergoing TVEMF-expansion do not contact the sides of the rotatable TVEMF-bioreactor vessel.
'Also; a labtiratiōn expert may, for instance once a day, during TVEMF-expansion, or once every two days, manually (for instance with a syringe) insert fresh media and preferably other desired additives such as nutrients and growth factors, as discussed above, into the rotatable bioreactor, and draw off the old media containing cell wastes and toxins. Also, fresh media and other additives may be automatically pumped into the rotatable TVEMF-bioreactor during TVEMF-expansion, and waste automatically removed.

Stem cells may increase to at least seven times their original number about 7 to about 14 days after being placed in the rotatable TVEMF-bioreactor and TVEMF-expanded. Preferably, the TVEMF-expansion occurs for about 7 to 10 days, and more preferably about 7 days. Measurement of the number of stem cells does not need to be taken during TVEMF-expansion therefore. As indicated above and throughout this application, TVEMF-expanded peripheral blood stem cells of the present invention have the same or essentially the same three-dimensional geometry and cell-to-cell support and cell-to-cell geometry as naturally-occurring, non-expanded peripheral blood stem cells.

Upon completion of TVEMF-expansion, the cellular material in the rotatable TVEMF-bioreactor comprises the stem cells of the present invention, in a composition of the present invention. Various substances may be removed from or added to the composition for further use, however, the composition must include expanded human blood stem cells and human red blood cells containing HbF.

The composition comprises expanded blood stem cells, preferably in an amount of at least seven times the number per volume of blood stem cells per volume as in the peripheral blood from which it originated. For instance, preferably, if a number X of peripheral blood stem cells was placed in a certain volume into a rotatable bioreactor, then after expansion, the number of peripheral blood stem cells in the rotatable bioreactor will be at least 7X (barring removal of cells during the expansion process). Where TVEMF-expanded blood stem cells are present in the composition, such cells should be present at least in an amount of 2 times the number of blood stem cells in the naturally-occurring blood. Preferably, TVEMF-expanded blood stem cells are in a range of about 4 times to about 25 times the number per volume of blood stem cells in naturally-occurring blood.

The present invention is also directed to a composition comprising expanded human blood stem cells, wherein said blood stem cells are present in a number per volume that is at least 7 times greater than the naturally-occurring peripheral blood; and wherein the peripheral blood stem cells have a three-dimensional geometry and cell-to-cell support and cell-to-cell geometry that is the same or similar to or essentially the same as stem cells.
A composition of the present invention may include an acceptable carrier; including but not limited to plasma, blood, albumin, cell culture medium, growth factor, hormone, buffer or cryopreservative. "Pharmaceutically acceptable carrier" means an agent that will allow the introduction of the stem cells into a human. Such carrier may include substances mentioned herein, including in particular any substances that may be used for blood transfusion, for instance blood, plasma, albumin; also, saline or buffer (preferably buffer supplemented with albumin), preferably from the human to which the composition will be introduced. The term "introduction" of a composition to a human is meant to refer to "administration" of a composition to the human. Preferably, administration of a composition of the present invention to a human is performed by intravenous injection. However, other forms of administration may be used, including for instance injection directly into an organ or near a site needing intensive treatment, rectal administration (particularly for a colonic disorder), and other methods for instance such as those well-known in the art, preferably to introduce the composition to an immediate area if needed.

Even more preferably, injection occurs with an acceptable amount G-CSF, for instance in an amount of 0.3ng to 5ug, more preferably 1ng/kg to 100ng/kg, even more preferably 5ng/kg to 20ng/kg, and even more preferably 6ng/kg. Administration of stem cells in a composition of the present invention may occur with pharmaceutically acceptable carriers as described in the general state of the art. The amount of stem cells expanded according to the present invention to be administered in a composition is a therapeutically effective amount (also discussed below) of preferably at least 1000 stem cells, more preferably at least 10⁴ stem cells, even more preferably at least 10⁵ stem cells, and even more preferably in an amount of at least 10⁷ to 10⁹ stem cells, or even more stem cells such as 10¹² stem cells. Administration of such numbers of expanded stem cells may be in one or more doses. As indicated throughout this application, the number of stem cells administered to a human may be limited to the number of stem cells originally available in source blood, as multiplied by expansion according to this invention. Without being bound by theory, it is believed that stem cells not used by the body after administration will simply be removed by natural body systems. "Acceptable carrier" generally refers to any substance the peripheral blood stem cells of the present invention may survive in, i.e. that is not toxic to the cells, whether after TVEMF-expansion, prior to or after cryopreservation, prior to introduction (administration) into a human. Such carriers are well known in the art, and may include a wide variety of substances, including
SubstMice's described for such a purpose throughout this application. For instance, plasma, blood, albumin, cell culture medium, buffer and cryopreservative are all acceptable carriers of this invention. The desired carrier may depend in part on the desired use.

The preferred amount of human red blood cells containing HbF in a composition, and the preferred amount to be administered to a human to treat sickle cell disease symptoms, is about 1 ml of composition comprising from about 20,000 cells/ml composition to about 100,000 cells/ml composition. A normal amount of HbF in naturally-occurring cord blood or infant peripheral blood is about 10,000 cells/ml; the amount of red blood cells containing HbF in a composition according to the present invention is preferably at least twice the normal amount of HbF in normal blood. Also preferably, about 0.5 to about 20 ml of such composition, more preferably about 0.75 ml to about 15 ml, and more preferably about 1 ml to about 10 ml, may be administered in a single dose when the human suffering from sickle cell disease symptoms is in need of treatment.

With further regard to administration of a composition of the present invention to a patient, the usual age of onset of sickle cell disease symptoms is from 4 to 13 years. Preferably, a composition of the present invention will have been prepared and cryopreserved for at least one year, preferably 4 years. Once symptoms of the disease become apparent, treatment with a composition may be administered preferably once a day, once a week, once every two weeks, once a month, once every three months or even once every year, depending on the severity of the disease and symptoms. A prophylactic approach would be to administer a composition of the present invention every three months from age 4 to 13 of a person identified as having sickle cell disease, but where symptoms have not yet occurred, to minimize or delay onset of symptoms. For multiple administrations, cryopreserved compositions may be cryopreserved in small quantities suitable for instance for a one-time administration. Preferably, a composition of the present invention, once thawed and ready to administer to the human subject, is administered within 24 hours of such thawing and readiness, preferably within 12 hours, more preferably within 4 hours and most preferably within 1 hour.

The composition is preferably free of toxic granular material, for example, dying cells and the toxic material or content of granulocytes and macrophages. The composition is cryopreserved by decreasing the temperature of the composition to a temperature of from -130°C to -196°C and maintaining the cryopreserved composition at that temperature.
range until needed for therapeutic or other use. As discussed below, preferably, as much toxic material as is possible is removed from the composition prior to cryopreservation.

Another embodiment of the present invention relates to a method of treating sickle cell disease (as discussed above) with a composition of, preferably, TVEMF-expanded cord blood or infant peripheral blood stem cells and of red blood cells containing HbF. The composition will be prepared and cryopreserved until onset of the disease. The cells may be introduced into a human body, preferably injected intravenously, and for instance replacing sickle cells with healthy cells and/or repressing the formation of other sickle cells with HbF. Preferably, the composition to be introduced into the human body is free of toxic material and other materials that may cause an adverse reaction to the administered TVEMF-expanded blood stem cells. The cells are also readily available for treatment or research where such treatment or research requires the human’s cord blood or infant peripheral blood cells.

A preferred embodiment of the present invention may be performed as follows:

A) Collection and maintenance of cells

Human infant peripheral blood (75 ml; about 0.75 x 10^6 cells/ml) may be collected from a human infant by syringe as described above and suspended in about 75 ml Iscove's modified Dulbecco's medium (IMDM) (GIBCO, Grand Island, NY) supplemented with 20% of 5% human albumin (HA), 100 ng/ml recombinant human G-CSF (Amgen Inc., Thousand Oaks, CA), and 100 ng/ml erythropoietin (Amgen). Because the donors is an infant, the collected blood will have red blood cells containing HbF. The blood cell mixture may be placed in a rotatable TVEMF-bioreactor as shown in Figures 2 and 3 herein. TVEMF-expansion may occur at 37°C, 6% CO₂, with a normal air O₂/N ratio. The rotatable TVEMF-bioreactor may be rotated at a speed of 10 rotations per minute (rpm) initially, then adjusted as needed, as described throughout this application, to keep the peripheral blood cells suspended in the rotatable bioreactor. A time varying current of 6mA may be applied to the rotatable bioreactor. The square
The TVEMF applied to the blood cell mixture may be about 0.5 Gauss. (frequency: about 10 cycles/sec).

Culture media in the blood cell mixture in the rotatable TVEMF-bioreactor may be changed/freshened every one to two days. At day 10, the cells may be removed from the rotatable TVEMF-bioreactor and washed with PBS and analyzed. Control data should also be acquired by comparing the expanded and/or differentiated blood cells to a sample of the original infant human peripheral blood that was not been expanded. It is expected that TVEMF-expansion and/or -differentiation of infant peripheral blood cells resulted in roughly a 10-fold increase in the number of cells over 10 days, as compared to non-expanded control.

B) Analysis of TVEMF-expanded cells

Total cell counts of control and expanded and/or differentiated samples may be obtained with a counting chamber (a device such as a hemocytometer used by placing a volume of either the control cell suspension or expanded sample on a specially-made microscope slide with a microgrid and counting the number of cells in the sample). CD34+ cells may be separated from other cells therein with a Human CD34 Selection Kit (EasySep positive selection, StemCell Technologies), and counted with a counting chamber as indicated above and confirmed with FACSscan flow cytometer (Becton-Dickinson). CFU-GEMM and CFU-GM may be counted by clonogenic assay. Cell viability (where a viable cell is alive and a non-viable cell is dead) may be determined by trypan blue exclusion test. Other cells in the resultant expanded and/or differentiated blood cell mixture may be tested by methods already known in the art.

Operative Method - Cryopreservation
The following methodology is meant to further describe the present invention. As mentioned above, cord and/or infant peripheral blood is to be collected from a human. The blood stem cells and red blood cells containing HbF (with other cells and media as desired) may be placed in a rotatable TVEMF-bioreactor as a blood cell mixture, preferably subjected to a time varying electromagnetic force and, where possible, expanded and/or differentiated. RBCs containing HbF remain with the stem cells preferably throughout the entire process, and at least while the stem cells are expanding and/or differentiating in the bioreactor.

The TVEMF-expanded cells are then removed from the bioreactor and cryopreserved. After removal from the bioreactor, the expanded and/or differentiated blood cell mixture is transferred into at least one cryopreservation container containing at least one cryoprotectant in sufficient quantity to cryopreserve the cells in the mixture. The cells in the mixture are preferably first washed with a solution (for instance, a buffer solution or the desired cryopreservative solution) to remove media and other components present during TVEMF-expansion and/or differentiation, and then preferably mixed in a solution that allows for cryopreservation of the cells. Such solution is commonly referred to as a cryopreservative, cryopreservation solution or cryoprotectant. The cells are transferred to an appropriate cryopreservative container and the container decreased in temperature to generally from -120°C to -196°C, preferably about -130°C to about -150°C, and maintained at that temperature. Preferably, this decrease in temperature is done slowly and carefully, so as to not damage, or at least to minimize damage, to the stem cells during the freezing process. When needed, the temperature of the cells (about the temperature of the cryogenic container) is raised to a temperature compatible with introduction of the cells into the human body (generally from around room temperature to around body temperature), and the TVEMF-expanded cells may be introduced into a human body, for instance as discussed throughout this application.
Freezing cells is ordinarily destructive. Not to be bound by theory, on cooling, water within the cell freezes. Injury then may occur by osmotic effects on the cell membrane, cell dehydration, solute concentration, and ice crystal formation. As ice forms outside the cell, available water is removed from solution and withdrawn from the cell, causing osmotic dehydration and raised solute concentration that may eventually destroy the cell. (For a discussion, see Mazur, P., 1977, Cryobiology 14:251-272.)

Different materials have different freezing points. Preferably, a peripheral blood stem cell composition ready for cryopreservation contains as few contaminating substances as possible, to minimize cell wall damage from the crystallization and freezing process.

These injurious effects can be reduced or even circumvented by (a) use of a cryoprotective agent, (b) control of the freezing rate, and (c) storage at a temperature sufficiently low to minimize degradative reactions.

The inclusion of cryopreservation agents is preferred in the present invention. Cryoprotective agents which can be used include but are not limited to a sufficient amount of dimethyl sulfoxide (DMSO) (Lovelock, J. E. and Bishop, M. W. H., 1959, Nature 183:1394-1395; Ashwood-Smith, M. J., 1961, Nature 190:1204-1205), glycerol, polyvinylpyrrolidone (Rinfret, A. P., 1960, Ann. N.Y. Acad. Sci. 85:576), polyethylene glycol (Sloviter, H. A. and Ravdin, R. G., 1962, Nature 196:548), albumin, dextran, sucrose, ethylene glycol, i-erythritol, D-ribitol, D-mannitol (Rowe, A. W., et al., 1962, Fed. Proc. 21:157), D-sorbitol, i-inositol, D-lactose, choline chloride (Bender, M. A., et al., 1960, J. Appl. Physiol. 15:520), amino acid-glucose solutions or amino acids (Phan The Tran and Bender, M. A., 1960, Exp. Cell Res. 20:651), methanol, acetamide, glycerol monacetate (Lovelock, J. E., 1954, Biochem. J. 56:265), and inorganic salts (Phan The Tran and Bender, M. A., 1960, Proc. Soc. Exp. Biol. Med. 104:388; Phan The Tran and Bender, M. A., 1961, in Radiobiology, Proceedings of the Third Australian Conference on Radiobiology, Ilbery, P. L. T., ed., Butterworth, London, p. 59). In a preferred embodiment, DMSO is used. DMSO, a liquid, is nontoxic to cells in low concentration. Being a small molecule, DMSO freely permeates the cell and protects intracellular organelles by combining with water to modify its freezability and prevent damage from ice formation. Adding plasma (for instance, to a concentration of 20-25%) can augment the protective effect of DMSO. After addition of DMSO, cells should be kept at 0°C or below, since DMSO concentrations of about 1% may be toxic at temperatures above 4°C. My selected preferred cryoprotective agents are, in combination with TVEMF-expanded peripheral blood stem cells for the total composition: 20 to 40% dimethyl sulfoxide solution in 60 to 80% amino acid-glucose solution, or 15 to 25% hydroxyethyl starch solution, or 4 to
of dextran T10, or 15 to 25% polyethylene glycol or 75 to 85% amino acid-glucose solution. The amount of cryopreservative indicated above is preferably the total amount of cryopreservative in the entire cryopreserved composition (not just the amount of substance added to a composition).

While other substances, other than peripheral blood cells and a cryoprotective agent, may be present in a composition of the present invention to be cryopreserved, preferably cryopreservation of a TVEMF-expanded peripheral blood stem cell composition of the present invention occurs with as few other substances as possible, for instance for reasons such as those discussed regarding the mechanism of freezing, above.

Preferably, a TVEMF-expanded peripheral blood stem cell composition of the present invention is cooled to a temperature in the range of about -120°C to about -196°C, preferably about -130°C to about -196°C, and even more preferably about -130°C to about -150°C.

A controlled slow cooling rate is critical. Different cryoprotective agents (Rapatz, G., et al., 1968, Cryobiology 5(1): 18-25) and different cell types have different optimal cooling rates (see e.g. Rowe, A. W. and Rinfret, A. P., 1962, Blood 20:636; Rowe, A. W., 1966, Cryobiology 3(1):12-18; Lewis, J. P., et al., 1967, Transfusion 7(1):17-32; and Mazur, P., 1970, Science 168:939-949 for effects of cooling velocity on survival of peripheral cells (and on their transplantation potential)). The heat of fusion phase where water turns to ice should be minimal. The cooling procedure can be carried out by use of, e.g., a programmable freezing device or a methanol bath procedure.

Programmable freezing apparatuses allow determination of optimal cooling rates and facilitate standard reproducible cooling. Programmable controlled-rate freezers such as Cryomed or Planer permit tuning of the freezing regimen to the desired cooling rate curve. Other acceptable freezers may be, for example, Sanyo Modi MDF-1 155ATN-152C and Model MDF-2136ATN -135C, Princeton CryoTech TEC 2000. For example, for blood cells such as CD34+/CD38- cells in 10% DMSO and 20% plasma, the optimal rate is 1 to 3°C/minute from 0°C to -200°C.

In a preferred embodiment, this cooling rate can be used for the cells of the invention. The cryogenic container holding the cells must be stable at cryogenic temperatures and allow for rapid heat transfer for effective control of both freezing and thawing. Sealed plastic vials (e.g., Nunc, Wheaton cryules) or glass ampules can be used for multiple small amounts (1-2 ml), while larger volumes (100-200 ml) can be frozen in polyolefin bags (e.g., Delmed) held between metal plates for better heat transfer during cooling. (Bags of bone marrow cells have
bderi'successfully'frozen'by placing them in -80°C freezers that, fortuitously, gives a cooling rate of approximately 3°C/minute).

In an alternative embodiment, the methanol bath method of cooling can be used. The methanol bath method is well suited to routine cryopreservation of multiple small items on a large scale. The method does not require manual control of the freezing rate nor a recorder to monitor the rate. In a preferred aspect, DMSO-treated cells are precooled on ice and transferred to a tray containing chilled methanol that is placed, in turn, in a mechanical refrigerator (e.g., Harris or Revco) at -130°C. Thermocouple measurements of the methanol bath and the samples indicate the desired cooling rate of 1 to 3°C/minute. After at least two hours, the specimens will reach a temperature of -80°C and may be placed directly into liquid nitrogen (-196°C) for permanent storage.

After thorough freezing, TVEMF-expanded stem cells can be rapidly transferred to a long-term cryopreservation storage vessel (such as a freezer). In a preferred embodiment, the cells can be cryopreserved stored in liquid nitrogen (-196°C) or its vapor (-165°C). The storage temperature should be below -120°C, preferably below -140°C. Such storage is greatly facilitated by the availability of highly efficient liquid nitrogen refrigerators, which resemble large Thermos containers with an extremely low vacuum and internal super insulation, such that heat leakage and nitrogen losses are kept to an absolute minimum.

The preferred apparatus and procedure for the cryopreservation of the cells is that manufactured by Thermogenesis Corp., Rancho Cordova, CA, utilizing their procedure for lowering the cell temperature to below -130°C. The cells are held in a Thermogenesis plasma bag during freezing and storage.

Other freezers are commercially available. For instance, the "BioArchive" freezer not only freezes but also inventories a cryogenic sample such as blood or cells of the present invention, for instance managing up to 3,626 bags of frozen blood at a time. This freezer has a robotic arm that will retrieve a specific sample when instructed, ensuring that no other examples are disturbed or exposed to warmer temperatures. Other freezers commercially available include, but are not limited to, Sanyo Model MDF-1 155 ATN-152C and Model MDF-2136 ATN-135C, and Princeton CryoTech TEC 2000.

After the temperature of the TVEMF-expanded peripheral blood stem cell composition is reduced to below -120°C, preferably below -130°C, they may be held in an apparatus such as a Thermogenesis freezer. Their temperature is maintained at a temperature of about -120°C to -196°C, preferably -130°C to -150°C. The temperature
of a &eacute;bsef"M'-TVBMF-expanded peripheral blood stem cell composition of the present invention should not be above -120°C for a prolonged period of time.

Cryopreserved TVEMF-expanded human blood stem cells, or a composition thereof, according to the present invention may be frozen for an indefinite period of time, to be thawed when needed. Preferably, a composition according to the present invention will be cryopreserved from about 4 to about 20 years. Even longer time periods may work, perhaps even as long as the lifetime of the blood donor.

When needed, bags with the cells therein may be placed in a thawing system such as a Thermogenesis Plasma Thawer or other thawing apparatus such as in the Thermoline Thawer series. The temperature of the cryopreserved composition is raised to room temperature. In another preferred method of thawing cells mixed with a cryoprotective agent, bags having a cryopreserved TVEMF-expanded peripheral blood stem cell composition of the present invention, stored in liquid nitrogen, may be placed in the gas phase of liquid nitrogen for 15 minutes, exposed to ambient air room temperature for 5 minutes, and finally thawed in a 37°C water bath as rapidly as possible. The contents of the thawed bags may be immediately diluted with an equal volume of a solution containing 2.5% (weight/volume) human serum albumin and 5% (weight/volume) Dextran 40 (Solplex 40; Sifra, Verona, Italy) in isotonic salt solution and subsequently centrifuged at 400 g for ten minutes. The supernatant would be removed and the sedimented cells resuspended in fresh albumin/Dextran solution. See Rubinstein, P. et al., Processing and cryopreservation of placental/umbilical cord blood for unrelated bone marrow reconstitution. Proc. Natl. Acad. Sci. 92:10119-1012 (1995) for Removal of Hypertonic Cryoprotectant; a variation on this preferred method of thawing cells can be found in Lazzari, L. et al., Evaluation of the effect of cryopreservation on ex vivo expansion of hematopoietic progenitors from cord blood. Bone Marrow Trans. 28:693-698 (2001).

After the cells are raised in temperature to room temperature, they are available for administration to treat sickle cell disease symptoms in the human whose blood was used to prepare the composition. The thawed composition may be introduced directly into the human, if the cells are in a pharmaceutically acceptable carrier. If the cryopreservative is not a pharmaceutically acceptable carrier, cryoprotectant should be removed from the cells and replaced with a pharmaceutically acceptable carrier. The solution in which the thawed cells are present may be completely washed away, and exchanged with another, or added to or otherwise manipulated as desired. Various additives may be added to
the thawed compositions prior to introduction into a human body, preferably soon to immediately prior to such introduction. Such additives include but are not limited to a erythropoietin, growth factor, a cytokine, a hormone, a suitable buffer or diluent. Preferably, erythropoietin is added. Even more preferably, G-CSF is added in an amount of about 20 to about 40 micrograms/kg body weight, and even more preferably in an amount of about 30 micrograms/kg body weight. Similarly, other growth factors or additives may be added in these amounts. Also, prior to introduction, the now-thawed cryopreserved cells may be mixed with the human's own plasma, blood or albumin, or other materials that for instance may accompany blood transfusions.

Intravenous injection of a sufficient amount of a composition of the present invention comprising expanded blood stem cells and red blood cells containing HbF should be able to be used to treat symptoms of sickle cell disease, as discussed throughout this application.

A TVEMF-expanded blood stem cell composition of the present invention should be introduced into a human, in an amount sufficient to treat sickle cell disease symptoms. Preferably, at least 20 ml of a composition according to the present invention, having $10^7$ to $10^9$ stem cells per ml and about 20,000 red blood cells containing HbF /ml composition to about 100,000 cells/ml composition is used for any treatment, preferably all at once. These amounts are particularly preferred in a 75-80 kg human. A dose may also be reduced for instance to 10,000 red blood cells containing HbF /ml at a rate of 40 ml/ min for a desired amount of minutes or volume, for instance for about 1 to about 5 minutes. The amount of TVEMF-expanded peripheral blood stem cells in a composition being introduced into a human depends in part on the number of cells present in the source peripheral blood material (in particular if only a fairly limited amount is available). A preferred range of TVEMF-expanded peripheral blood stem cells introduced into a human may be, for instance, about 10 ml to about 50 ml of a TVEMF-expanded peripheral blood stem cell composition having $10^7$ to $10^9$ stem cells per ml, or potentially even more. While it is understood that a high concentration of any substance, administered to a human, may be toxic or even lethal, it is unlikely that introducing all of the TVEMF-expanded peripheral blood stem cells, for instance after TVEMF-expansion at least 7 times, will cause an overdose in TVEMF-expanded peripheral blood stem cells. Where peripheral blood from several donors or multiple collections from the same donor is used, the number of peripheral blood stem cells introduced into a human may be higher. Also, the dosage of TVEMF-cells that may be introduced to the human is not limited by the amount of peripheral blood provided from collection from one human; multiple administrations, for instance once a day or twice a day,
or once a week, or once a month, for a time certain or indefinitely, or other administration time frames, may more easily be used. Preferably, a dose of a composition of this invention is administered to the human whose blood cells are the source of the composition when suffering from a symptom of sickle cell disease, once a week for at least 3 weeks, preferably for at least 6 weeks. Also, where a human having sickle cell disease symptoms is to be treated, the severity of the symptom(s) may warrant the use of more composition than for milder symptoms, or the use of a smaller dose.

It is to be understood that, while the embodiment described above generally relates to cryopreserving TVEMF-expanded peripheral blood stem cells, TVEMF-expansion may occur after thawing of already cryopreserved, non-expanded, or non-TVEMF-expanded, peripheral blood stem cells. Also, if cryopreservation is desired, TVEMF-expansion may occur both before and after freezing the cells. Blood banks, for instance, have cryopreserved compositions comprising peripheral blood stem cells in frozen storage, in case such is needed at some point in time. Such compositions may be thawed according to conventional methods and then TVEMF-expanded as described herein, including variations in the TVEMF-process as described herein. Thereafter, such TVEMF-expanded peripheral blood stem cells are considered to be compositions of the present invention, as described above. TVEMF-expansion prior to cryopreserving is preferred, for instance as if a traumatic injury occurs, a human's peripheral blood stem cells have already been expanded and do not require precious extra days to prepare.

Also, while not preferred, it should be noted that TVEMF-expanded peripheral blood stem cells of the present invention may be cryopreserved, and then thawed, and then if not used, cryopreserved again. Prior to the cells being frozen, are preferably TVEMF-expanded (that is, increased in number, not size). The cells may also be expanded after being frozen and then thawed, even if already expanded before freezing.

Expansion of peripheral blood stem cells may take several days. In a situation where it is important to have an immediate supply of peripheral blood stem cells, such as a life-or-death situation or in the case of a traumatic injury, especially if research needs to be accomplished prior to reintroduction of the cells, several days may not be available to await the expansion of the peripheral blood stem cells. It is particularly desirable, therefore, to have such expanded peripheral blood stem cells available from birth forward in anticipation of an emergency where every minute in delaying treatment can mean the difference in life or death.
Also, it is to be understood that the TVEMF-expanded peripheral blood stem cells of the present application may be introduced into the human that is the source of the peripheral blood, after TVEMF-expansion, with or without cryopreservation.

During the entire process of expansion, preservation, and thawing, blood stem cells of the present invention maintain their three-dimensional geometry and their cell-to-cell support and cell-to-cell geometry.

It is expected that the composition of the present invention, comprising a highly concentrated mixture of red blood cells containing HbF and expanded adult stem cells (or red blood cells containing HbF alone) will essentially eliminate the symptoms of sickle cell disease.

While preferred embodiments have been herein described, those skilled in the art will understand the present invention to include various changes and modifications. The scope of the invention is not intended to be limited to the above-described embodiments.
I claim:

1. A composition comprising expanded human blood stem cells from a blood source, wherein said expanded human blood stem cells are in a number per volume that is at least 2 times greater than the number per volume in naturally occurring human blood, preferably at least 7 times greater, and wherein the expanded human blood stem cells have a three-dimensional geometry and cell-to-cell support and cell-to-cell geometry that is essentially the same as naturally-occurring human blood stem cells; and human red blood cells containing HbF from the blood source in a number per volume that is at least 80% of the number per volume of human red blood cells containing HbF in naturally occurring human blood, preferably at least two times greater than the number per volume of human red blood cells containing HbF in naturally occurring human blood; wherein the blood source is selected from the group consisting of cord blood of one human, infant peripheral blood of one human, and a mixture of cord blood and infant peripheral blood of one human; and wherein the composition preferably further comprises at least one of a growth factor, erythropoietin, GCSF and IL-2.

2. The composition of claim 1, further comprising an acceptable carrier.

3. The composition of claim 2, wherein said acceptable carrier is at least one of the group consisting of plasma, blood, albumin, cell culture medium, buffer and cryopreservative.

4. The composition of claim 3, wherein said composition further comprises at least one substance of the group consisting of a growth factor and a hormone.

5. The composition of claim 3, wherein said acceptable carrier is at least a cryopreservative in an amount sufficient for cryopreservation of the expanded human blood stem cells and the human red blood cells containing HbF, and wherein said composition is at a temperature of from about -120°C to about -196°C.

6. The composition of claim 5, wherein the composition is at a temperature of from about -130°C to about -150°C.

7. The composition of claim 5, wherein the cryopreservative is selected from the group consisting of about 20% to about 40% dimethyl sulfoxide solution in about 60% to about 80% amino acid-glucose solution; about 15% to about 25% hydroxyethyl starch solution; about 4% to about 6% glycerol, about 3% to about 5% glucose and about 6% to about 10% dextran TIO; about 15% to about 25% polyethylene glycol; and about 75% to about 85% amino acid-glucose solution.
8. The composition of claim 3, wherein said acceptable carrier is at least a cryopreservative in an amount sufficient for cryopreservation of the expanded human blood stem cells and the human red blood cells containing HbF, and the composition is at a temperature sufficient to cryopreserve the expanded human blood stem cells and the human red blood cells containing HbF.

9. The composition of claim 8, wherein the cryopreservative is selected from the group consisting of about 20% to about 40% dimethyl sulfoxide solution in about 60% to about 80% amino acid-glucose solution; about 15% to about 25% hydroxyethyl starch solution; about 4% to about 6% glycerol, about 3% to about 5% glucose and about 6% to about 10% dextran T10; about 15% to about 25% polyethylene glycol; and about 75% to about 85% amino acid-glucose solution.

10. The composition of claim 1, further comprising a pharmaceutically acceptable carrier.

11. The composition of claim 10, wherein said composition is free of toxic material.

12. The composition of claim 1, wherein said composition is free of toxic material.

13. The composition of claim 1, wherein the human red blood cells containing HbF are in a number of human red blood cells containing HbF per volume that is at least 2 times greater than the number of human red blood cells containing HbF per volume in naturally-occurring human blood.

14. The composition of claim 1, wherein the composition further comprises at least one substance of the group consisting of granulocyte-colony stimulating factor, interleukin-2 and erythropoietin.

15. The composition of claim 1, wherein the composition is to be administered to a human suffering from a symptom of sickle cell disease.

16. The composition of claim 11, wherein the composition is to be administered to a human suffering from a symptom of sickle cell disease.

17. A composition comprising TVEMF-expanded human blood stem cells, and human red blood cells containing HbF, wherein the TVEMF-expanded human blood stem cells and the human red blood cells containing HbF are from a same human.

18. The composition of claim 17, further comprising an acceptable carrier.

19. The composition of claim 18, wherein said acceptable carrier is at least one of the group consisting of plasma, blood, albumin, cell culture medium, buffer and cryopreservative.

20. The composition of claim 19, wherein said composition further comprises at least one substance of the group consisting of a growth factor, and a hormone.
21. The compositions of claim 19, wherein said acceptable carrier is at least a cryopreservative in an amount sufficient for cryopreservation of the expanded human blood stem cells and the human red blood cells containing HbF, and wherein said composition is at a temperature of from about -120C to about -196C.

22. The composition of claim 21, wherein the composition is at a temperature of from about -130C to about -150C.

23. The composition of claim 21, wherein the cryopreservative is selected from the group consisting of about 20% to about 40% dimethyl sulfoxide solution in about 60% to about 80% amino acid-glucose solution; about 15% to about 25% hydroxyethyl starch solution; about 4% to about 6% glycerol, about 3% to about 5% glucose and about 6% to about 10% dextran TIO; about 15% to about 25% polyethylene glycol; and about 75% to about 85% amino acid-glucose solution.

24. The composition of claim 19, wherein said acceptable carrier is at least a cryopreservative in an amount sufficient for cryopreservation of the expanded stem cells and the red blood cells containing HbF, and the composition is at a temperature sufficient to cryopreserve the expanded stem cells and the red blood cells containing HbF.

25. The composition of claim 24, wherein the cryopreservative is selected from the group consisting of about 20% to about 40% dimethyl sulfoxide solution in about 60% to about 80% amino acid-glucose solution; about 15% to about 25% hydroxyethyl starch solution; about 4% to about 6% glycerol, about 3% to about 5% glucose and about 6% to about 10% dextran TIO; about 15% to about 25% polyethylene glycol; and about 75% to about 85% amino acid-glucose solution.

26. The composition of claim 17, further comprising a pharmaceutically acceptable carrier.

27. The composition of claim 26, wherein said composition is free of toxic material.

28. The composition of claim 17, wherein said composition is free of toxic material.

29. The composition of claim 17, wherein the human red blood cells containing HbF are in a number of human red blood cells containing HbF per volume that is at least 2 times greater than the number of human red blood cells containing HbF per volume in naturally-occurring human blood.

30. The composition of claim 17, wherein the composition further comprises at least one substance of the group consisting of granulocyte-colony stimulating factor, interleukin-2 and erythropoietin.

31. The composition of claim 17, wherein the composition is to be administered to a human suffering from a symptom of sickle cell disease.
32. The composition of claim 27, wherein the composition is to be administered to a human suffering from a symptom of sickle cell disease.

33. A process for preparing the composition of claim 1, comprising the steps of
   a. collecting blood from a human, wherein the blood is selected from at least one of the group consisting of cord blood of the human and infant peripheral blood of the human, and wherein the blood comprises stem cells and red blood cells containing HbF;
   b. preparing and placing a blood cell mixture comprising at least stem cells and red blood cells containing HbF in a culture chamber of a rotatable bioreactor;
   c. rotating the culture chamber to prepare expanded stem cells and preferably new red blood cells containing HbF; and
   d. stopping rotation of the rotatable bioreactor to prepare an expanded and preferably differentiated, rotated blood cell mixture.

34. The process of claim 33, wherein the human is identified as having a genetic sickle cell disease.

35. The process of claim 33, further comprising the step of
   e. washing cells in the expanded and preferably differentiated, rotated blood cell mixture, adding a cryopreservative to the rotated blood cell mixture and transferring the washed cells to a cryopreservative container.

36. The process of claim 35, further comprising the step of
   f. lowering the temperature of the cryopreservative container to a temperature of from about -120°C to about -196°C at a controlled rate to cryopreserve the expanded blood cell mixture.

37. The process of claim 36, wherein the lowering of the temperature is to about -130°C to about -150°C.

38. The process of claim 36, further comprising the step of
   g. maintaining the temperature of the cryopreservative container at about -120°C to about -196°C for a period of time.

39. The process of claim 38, wherein the period of time is at least 1 year.

40. The process of claim 38, wherein the period of time is at least 7 years.

41. The process of claim 38, wherein the period of time is at least 14 years.
A process for preparing the composition of claim 17, comprising the steps of:

a. collecting blood from a human, wherein the blood is selected from at least one of the group consisting of cord blood of the human and infant peripheral blood of the human, and wherein the blood comprises stem cells and red blood cells containing HbF;

b. placing a blood cell mixture comprising at least stem cells and red blood cells containing HbF in a culture chamber of a rotatable TVEMF-bioreactor;

c. rotating the culture chamber to prepare expanded stem cells and preferably new red blood cells containing HbF; and

d. stopping rotation of the rotatable bioreactor to form a rotated blood cell mixture.

43. The process of claim 42, wherein the human is identified as having a genetic sickle cell disease.

44. The process of claim 42, further comprising the step of

h. washing cells of the rotated mixture, adding a cryopreservative to the mixture and transferring the cells to a cryopreservative container.

45. The process of claim 44, further comprising the step of

i. lowering the temperature of the cryopreservative container to a temperature of from about -120°C to about -196°C at a controlled rate to cryopreserve the expanded blood cell mixture.

46. The process of claim 45, wherein the lowering of the temperature is to about -130°C to about -150°C.

47. The process of claim 45, further comprising the step of

j. maintaining the temperature of the cryopreservative container at about -120°C to about -196°C for a period of time.

48. The process of claim 47, wherein the period of time is at least 1 year.

49. The process of claim 47, wherein the period of time is at least 7 years.

50. The process of claim 38, wherein the period of time is at least 14 years.

51. The process of claim 42, wherein the TVEMF is selected from one of the following:

1. a TVEMF with a force amplitude less than 100 gauss and slew rate greater than 1000 gauss per second, (2) a TVEMF with a low force amplitude bipolar square wave at a frequency less than 100 Hz., (3) a TVEMF with a low force amplitude square wave with less than 100% duty cycle, (4) a TVEMF with slew rates greater than 1000 gauss per second for duration pulses less than 1 ms., (5) a TVEMF with slew rate bipolar delta function-like pulses with a duty cycle less than 1%, (6) a TVEMF with a force amplitude less than 100 gauss peak-to-peak and slew rate bipolar delta function-like pulses and where the duty cycle is less than 1%, (7) a TVEMF applied using a solenoid coil to create uniform force strength throughout the cell mixture, and (8) a TVEMF applied utilizing a flux concentrator to provide spatial gradients of magnetic flux and magnetic flux focusing within the cell mixture.
52. The process of claim 51, wherein the TVEMF-expanding continues until the number per volume of TVEMF-expanded blood stem cells is about 2 times to about 10 times the number per volume of blood stem cells in the collected blood.

53. The process of claim 42, wherein the rotatable TVEMF-bioreactor has an integral TVEMF source.

54. The process of claim 42, wherein the rotatable TVEMF-bioreactor has an adjacent TVEMF source.

55. The process of claim 42, further comprising the step of removing toxic material from at least one of the group consisting of the blood cell mixture and the expanded blood cells.

56. A method of treating a human suffering from a symptom of sickle cell disease comprising the steps of
   a. increasing the temperature of a cryopreserved composition of claim 5 to a temperature suitable for administration of the composition to a human;
   b. preparing the composition for administration to a human; and
   c. administering a therapeutically effective amount of the composition to a human.

57. The method of claim 56, wherein the temperature suitable for administration is about 25°C to about 38°C.

58. The method of claim 56, wherein the administering step is intravenous administration.

59. A composition comprising red blood cells containing HbF from the blood of one human, wherein said blood is cord blood, infant peripheral blood or a mixture thereof, wherein the red blood cells containing HbF are present in an amount of at least about 2 times the number per volume than the number per volume occurring in the naturally occurring source blood.

60. A composition according to claim 59, prepared by the process of claim 42, further comprising the step of separating red blood cells from other cells in the expanded and/or differentiated blood cell mixture.

61. A composition for the treatment of sickle cell disease symptoms, comprising expanded human blood stem cells from a blood source, wherein said expanded human blood stem cells are in a number per volume that is at least 2 times greater than the number per volume in naturally occurring human blood, preferably at least 7 times greater, and wherein the expanded human blood stem cells have a three-dimensional geometry and cell-to-cell support and cell-to-cell geometry that is essentially the same as naturally-occurring human blood stem cells; and
   human red blood cells containing HbF from the blood source in a number per volume that is at least 80% of the number per volume of human red blood cells containing HbF in naturally occurring human blood, preferably at least two times greater than the number per volume of human red blood cells containing HbF in naturally occurring human blood;
wherein the blood source is selected from the group consisting of cord blood of one human, infant peripheral blood of one human, and a mixture of cord blood and infant peripheral blood of one human; and wherein the composition preferably further comprises at least one of a growth factor, erythropoietin, GCSF and IL-2.

62. The use of expanded and/or differentiated human blood stem cells and red blood cells containing HbF in the preparation of a medicament for the treatment of sickle cell disease symptoms.

63. The composition of claim 1, wherein the source of blood is infant peripheral blood.
Magnitude of Deviation of Cells Across Streamlines

Note: Centrifugal deviation accumulates with time, gravity induced deviation varies sinusoidally

FIG. 6