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(54) Title: ISOLATION OF FETAL ERYTHROCYTES

(57) Abstract

A method for isolating nucleated fetal erythrocytes (NFEs) from a maternal blood sample by separating erythrocytes in the maternal blood sample from all other nucleated cells therein; and isolating NFEs from nonnucleated maternal erythrocytes. Preferably the maternal blood is peripheral maternal blood. The isolated NFEs can then be analyzed for genetic disorders and the like, such as by *in situ* hybridization.

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ISOLATION OF FETAL ERYTHROCYTES

Background of the Invention

The invention relates to a procedure for the isolation and enrichment of nucleated fetal erythrocytes (NFE) from maternal blood during the early stages of pregnancy, particularly for subsequent prenatal diagnosis such as by in situ hybridization.

Several devices and methods have been developed for separation of blood components, particularly for separation of red blood cells, platelets, leukocytes and plasma from one another. See, for example, U.S. Patents 4,880,548, 4,923,620 and 4,925,572 and the discussion therein. These methods and devices have been directed at separation of whole blood components for blood banking of packed erythrocytes or packed platelets from which fibrinogen and/or fibrin gels, microaggregates, and leukocytes have been removed, transfusion or therapeutic, particularly emergency, administration of individual concentrated components usually in relatively large volumes, i.e., one or more units (450 ml. each).

The number of NFE present in maternal blood is very low (estimates are from 1 in 10,000 to 1 in 1,000,000). The

current state of the art does not permit the isolation of these cells mainly because of the presence of large numbers of maternal cells.

SUMMARY OF THE INVENTION

The invention provides a method used to separate nucleated fetal erythrocytes (NFE) from the peripheral blood of women in their first or second trimester of pregnancy. Since this constitutes a non-invasive procedure with no danger to either mother or product, the test can be used for all pregnant women regardless of age and risk factors. Blood collection is done at the doctor's office, clinic or elsewhere and cell isolation and analysis can be performed in similar facilities and in clinical genetics laboratories. The NFE isolation method of the invention is used to provide samples of isolated fetal erythrocytes fro analysis by technologies like in situ hybridization for the prenatal diagnosis of genetic disorders.

Accordingly, the method provided by the invention is one for isolating nucleated fetal erythrocytes (NFEs) from maternal blood by separating NFEs in a sample of maternal blood from all other nucleated cells therein; and isolating NFEs from nonnucleated maternal erythrocytes. Preferably the maternal blood sample is from peripheral maternal blood. The isolated NFEs are analyzed for genetic disorders and the like such as by in situ hybridization.

PREFERRED EMBODIMENTS

Thus, in a principal aspect, the invention relates to a method for isolating nucleated fetal erythrocytes (NFEs) from a sample of maternal blood. This method comprises separating NEFs in a sample maternal blood from all other nucleated cells therein; and isolating the NFEs from nonnucleated maternal erythrocytes. Preferably, the sample is of peripheral maternal blood and is collected in amounts of about 5 to 50 ml of maternal blood.

In a first aspect, separated the erythrocytes in the maternal blood sample are from other nucleated cells by passing the whole blood sample through a leukocyte depletion device and collecting the erythrocytes that have passed through the filter. Suprisingly, the nucleated fetal erythrocytes pass through with the nonnucleated maternal erythrocytes rather than being retained with the nucleated leukocytes. This leukocyte depletion device is preferably a small version of the type made and sold by Pall Corporation, Glen Cove, NY and described in detail in U.S. Patent No. 4,925,572.

When a liquid is brought into contact with the upstream surface of a porous medium and a small pressure differential is applied, flow into and through the porous medium may or may not occur. A condition in which no flow occurs is that in which the liquid does not wet the material of which the porous structure is made. A series of liquids can be prepared, each with a surface tension of about 3 dynes/cm higher compared with the one preceding. A drop of each may then be placed on a porous surface and observed to determine whether it is absorbed quickly, or remains on the surface.

Similar behavior is observed for porous media made using other synthetic resins, with the wet-unwet values dependent principally on the surface characteristics of the material from which the porous medium is made, and secondarily, on the pore size characteristics of the porous medium. For example, fibrous polyesters (specifically polybutylene terephthalate (hereinafter "PBT") sheets) which have pore diameters less than about twenty micrometers were wetted by a liquid with a surface tension of 50 dynes/cm, but were not wetted by a liquid with a surface tension of 54 dynes/cm.

In order to characterize this behavior of a porous medium, the term "critical wetting surface tension" (CWST has been defined as described below. The CWST of a porous medium may be determined by individually applying to its surface, preferably dropwise, a wries of liquids with surface tensions varying by 2 to 4 dynes/cm, and observing the absorption or nonabsorption of each liquid. The CWST of a porous medium, in units of dynes/cm, is deaned as the mean value of the surface tension of the liquid which is absorbed and that of a liquid of neighboring surface tension which is not absorbed.

In measuring CWST, a series of standard liquids for testing are prepared with surface tensions varying in a sequential manner by 2 to 4 dynes/cm. Wetting is defined as absorption into or obvious wetting of the porous medium by at least 90% of the drops of a given standard within 10 minutes. Non-wetting is, defined by non-absorption or non-wetting of at least 90% of the drops of a given standard in 10 minutes. Testing is continued using liquids of successively higher or lower surface tension, until a pair has been identified, one wetting and one non-wetting, which

are the most closely spaced in surface tension. The CWST is then within that range and, for convenience, the average of the two surface tensions is used as a single number to specify the CWST. Appropriate solutions with varying surface tensions are reported in U.S. Patent No. 4,925,572.

In whole blood, the red cells are suspended in blood plasma, which has a surface tension of 73 dynes/cm. Hence, if whole blood is placed in contact with a porous medium, spontaneous wetting will occur if the porous medium has a CWST of 73 dynes/cm or higher.

One embodiment of the invention used a device for the depletion of the leukocyte content of a blood sample comprising at least first, second, and third preformed porous elements with the second element interposed between the first and third elements. Each successive element has a smaller pore diameter than that preceding it. The first element is for removing gels, the second element is for removing microaggregates, and the third element is for removing leukocytes. This embodiment of the leukocyte depletion device has a third element which has a pore diameter in the range from about 4 to about 8 micrometers, preferably in the range of from about 4 to about 5.5 micrometers.

In another embodiment, the device has a first element which comprises a needled fibrous structure, and can be hot compressed to a controlled thickness. The average pore diameter of this first element can be such that, when prewetted by isopropyl alcohol, a differential pressure of 4 to 7 cm of water column induces air flow through it at the rate of 0.5 cm/second.

Also, the device can include at least two interposed or laminated elements of porous media which stepwise span in approximate geometric progression the pore diameter range of from about 25 to about 10 micrometers.

For example, the device can include at least two interposed elements of porous media which have progressively stepwise decreasing pore diameters spanning the range from about 25 to about 10 micrometers, or can include a single element in which the pore diameter varies stepwise from about 25 micrometers to the range of from about 10 to about 15 micrometers.

One or more elements of the device is preferably treated with a surfactant. The surfactant induces a surface tension of about 75 to 45 dynes/cm in a blood sample. As a further alternative, at least one element can be surface modified by an energy source while in contact with a monomer containing at least one hydroxyl moiety and one moiety capable of activation by an energy source, together with a monomer containing at least one hydrophobic moiety and one moiety capable of activation by an energy source.

Most particularly, the device has an element in which the means for removing leukocytes includes a filtration means. Preferably, this element is preformed of synthetic fibers whose surface has a modified CWST in excess of 53-63 dynes/cm. These fibers can be surface modified by exposure to an energy source while in contact with monomers as described above.

The invention can additionally use a device for depletion of leukocytes from a blood product comprising at least one element in which a fibrous medium has been

radiation grafted to obtain a critical wetting surface tension in excess of 53 dynes/cm and thereafter hot compressed to form a non-friable coherent body. This device can have a CWST in the range of about 55 to 75 dynes/cm, and can also have the fibrous surface modified by exposure to an energy source while in contact with monomers as described above.

The invention can also use a device for leukocyte depletion comprising a preformed element of synthetic fibers modified to a CWST in the range of about 55 to 75 dynes/cm, and can also have the fibrous surface modified by exposure to an energy source while in contact with monomers as described above.

The invention particularly uses a device for the leukocyte depletion of a blood sample comprising a housing including an inlet and an outlet and defining a fluid flow path between the inlet and the outlet, an upstream porous element for removing gels, at least one intermediate porous element for removing microaggregates, and a downstream porous element for removing leukocytes, the elements being secured within the housing by an interference fit.

In another aspect, the nucleated fetal erythrocytes are isolated from nonnucleated maternal erythrocytes by centrifugal separation through a pore gradient material. Usually about 90%, i.e., 4.5 to 45 ml of the fluid volume of the sample, now freed of leukocytes, passes through the leukocyte depletion device, will the small remaining amount of fluid volume is retained in the device. The material to be centrifugally separated is preferably diluted 3-7 fold in a physiological fluid, such as phosphate buffered saline. Preferably the gradient is of a polysaccharide, for example,

a 4-20% sucrose gradient. Examples of such pore gradient materials include Ficoll and Percoll gradient materials available from SIGMA Chemical Co., St. Louis, MO.

Another useful method for isolating the nucleated from nonnucleated erythrocytes comprises lysing the nonnucleated maternal erythrocytes, such as with ammonium chloride. The erythrocyte-containing fluid volume is mixed with ammonium chloride, usually about 0.1-0.2M aqueous concentration in about a 1:4 (v/v) ratio.

The method of the invention for isolating the nucleated erythrocytes from nonnucleated maternal erythrocytes can be supplemented by the known method of reacting erythrocytes with solid phase bound antibody specifically bindable with the nucleated erythrocytes and separating the unbound nonnucleated erythrocytes therefrom. This would be performed subsequent to the fetal erythrocyte isolation method of the invention. A suitable antibody can be of any of the types of polyclonal, monoclonal, single chain, Fab_2 region fragments or other variants that retain the desired specificity. The antibody is bound directly or through any typical linkage group to a solid phase of known type including slides, beads, microparticles or microcapsules, magnetic beads or the like. One preferred specificity or ligand for such an antibody is the transferrin receptor. Antibodies to the transferrin receptor can be obtained from Becton-Dickinson, Piscataway, NJ.

Another aspect of the invention provides a method of assaying nucleic acid in nucleated fetal erythrocytes. This method includes separating NFEs in a sample of maternal blood from all other nucleated cells therein; isolating the

NFEs from nonnucleated maternal erythrocytes; and assaying nucleic acid associated with the NFEs so isolated.

In a preferred embodiment of this aspect the nucleic acid are assayed in nucleated fetal erythrocytes having substantially intact membranes and the nucleic acids are intact fetal chromosomes or fragments thereof.

EXAMPLE 1
Nucleated Fetal Erythrocyte Isolation

Fifty (50) ml of pooled umbilical cord blood were filtered using a leukocyte removal filter (RC 50 from Pall Biomedical Products Corporation, Glen Cove, NY). An empty 60 ml syringe barrel was connected to the filter by a short length of tubing and the blood subsequently passed through the RC 50 filter. The collected fraction containing the erythrocytes (26 ml) was diluted in five times its volume of PBS and overlaid on a histopaque 1.077 gradient (SIGMA, St. Louis, MO). The gradient was centrifuged for 40 minutes at 300 g and the cells at the interface were removed, washed once in PBS, and centrifuged to form pellets. The pellets were combined, resuspended in PBS, and the cells were placed into slides by centrifugation using a cytocentrifuge.

The slides were fixed in ethanol/methanol (3:1) for two minutes, stained with Gimsa stain, and observed under the microscope. Nucleated erythrocytes were visually scored and counted. The only nucleated cells seen were nucleated erythrocytes. From the counts it was calculated that approximately 5000 nucleated erythrocytes were recovered.

WHAT IS CLAIMED IS:

1. A method for isolating nucleated fetal erythrocytes from maternal blood which comprises:

separating NFEs in a sample of maternal blood from all other nucleated cells therein; and

isolating the NFEs from nonnucleated maternal erythrocytes.

2. The method of claim 1 wherein the blood sample is of peripheral maternal blood.

3. The method of claim 1 wherein the sample comprises about 5 to 50 ml of maternal blood.

4. The method of claim 1 wherein separating the erythrocytes in the maternal blood sample from other nucleated cells comprises passing the blood through a device which retains all nucleated cells except the NFEs and collecting the erythrocytes that have passed through the device.

5. The method of claim 4 wherein the device is a leukocyte depletion device.

6. The method of claim 1 wherein isolating the NFEs from nonnucleated maternal erythrocytes comprises centrifugal separation through a pore gradient material.

7. The method of claim 1 wherein isolating the NFEs from nonnucleated maternal erythrocytes comprises lysing the nonnucleated erythrocytes.

8. The method of claim 7 wherein the nonnucleated maternal erythrocytes are lysed with ammonium chloride.

9. The method of claim 7 wherein isolating the NFEs from nonnucleated maternal erythrocytes comprises reacting the erythrocytes with solid phase-bound antibody specifically bindable with the nucleated erythrocytes and separating the unbound nonnucleated erythrocytes therefrom.

10. A method of assaying biopolymers in nucleated fetal erythrocytes comprising:

separating erythrocytes in a sample of maternal blood from all other nucleated cells therein;

isolating NFEs from nonnucleated maternal erythrocytes; and

assaying nucleic acids associated with the NFEs so isolated.

11. The method of claim 10 wherein the nucleic acids are assayed in NFEs having intact membranes.

12. The method of claim 10 wherein the sample is a sample of peripheral maternal blood.

13. The method of claim 10 wherein separating the erythrocytes in the maternal blood sample from other nucleated cells comprises passing the whole blood through a device which retains all cells except the NFEs and collecting the erythrocytes that have passed through the device.

14. The method of claim 12 wherein the device is a leukocyte depletion device.

15. The method of claim 10 wherein isolating the NFEs from nonnucleated maternal erythrocytes comprises centrifugal separation through a pore gradient material.

16. The method of claim 10 which further comprises enriching the concentration of the NFEs so isolated.

INTERNATIONAL SEARCH REPORT

Ir ational application No.
PCT/US94/00644

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68; G01N 33/543, 33/49
US CL :435/2, 6, 7.25; 436/17, 177, 178, 518; 530/388.7, 389.6, 413, 427
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/2, 6, 7.25; 436/17, 177, 178, 518; 530/388.7, 389.6, 413, 427

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

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

APS, MEDLINE, BIOSIS
search terms: nucleated fetal erythrocytes, leukocyte depletion

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	American Journal of Obstetrics and Gynecology, Price, et al., Volume 165, Number 6, Part 1, issued December 1991, "Prenatal diagnosis with fetal cells isolated from maternal blood by multiparameter flow cytometry", pages 1731-1737, see pages 1732-1733.	1-3,6,10-12 15,16 ---------- 4,5,7-9,13,14
Y	US, A, 4,925,572 (PALL) 15 May 1990, see entire document.	4,5,13,14
Y	US, A, 4,185,964 (LANCASTER) 29 January 1980, see entire document.	7,8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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