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[54] **HEMATOLOGIC REFERENCE CONTROL**

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[57] **ABSTRACT**

A hematologic reference control provided for laboratory use in the calibration and control of automated and manual hematologic analyses. The reference control comprises a suspension of washed, stabilized human red blood cells in a nonproteinaceous aqueous suspension fluid that replaces the plasma in human blood. Stability in the reference control is attained by conditioning the cells by the inclusion in the aqueous suspension fluid of materials tending to make the cells assume a spherical shape without substantial change in the mean cell volume of the cells as well as imparting to the cells a resistance to the normal tendency of degrading with time. The aqueous suspension fluid furthermore produces an environment for the cells inhibiting biological activity. In a preferred embodiment there is further included in the reference control a minor amount of fixed human red blood cells, processed to have a substantially increased mean cell volume. The fixed cells are resistant to a change in cell volume, and to dissolution under the action of lysing agents producing lysing of the stabilized cells. The fixed red blood cells in the reference control substitute for the white cell population in human blood.

8 Claims, No Drawings

HEMATOLOGIC REFERENCE CONTROL

This invention concerns generally what is referred to herein as a hematologic reference control, such comprising a suspension of human red blood cells in an aqueous suspension fluid usable in a hematology laboratory in the obtaining of reference values in connection with assays performed on human blood using manual and automated procedures.

A need exists for such a reference control which is stable, in that the same can be used over a long period of time without substantial change occurring in the reference values obtained therefrom. Preferably the reference control also should resemble human blood, with respect to pertinent characteristics, such as coloring, specific gravity, potassium and sodium ion concentrations, pH, and the manner in which the constituents thereof remain in suspension.

A general object, therefore, is to provide a reference control exhibiting a stability that substantially exceeds that of controls presently known.

Another object of the invention is to provide a reference control which, with respect to pertinent physical characteristics, has a close resemblance to human blood.

More specifically an object is to provide a reference control including a population of human red blood cells suspended in a suspension fluid, with the cells conditioned in their suspended state so as to be resistant to degradation. The cells nevertheless do respond to traditional lysing agents, whereby the reference control may be used in a procedure which requires lysing of red blood cells.

Another object is to provide a reference control which includes a group of specially processed human red blood cells, referred to as fixed cells, incorporated into the reference control to substitute for the white blood cell population, unstable with time, normally found in human blood. These specially processed cells, by reason of a swollen condition produced therein, and by reason of a fixing of the cells in their swollen condition, possess a specific gravity significantly less than that of normal red blood cells and similar to that of white blood cells, and will remain in this condition with substantially no change during the life of the reference control. The fixing of the cells furthermore makes them resistant to dissolution or degradation under the influence of the usual lysing agents used in hematologic test procedures. The fixed cells in the reference control tend to remain in suspension in a manner similar to white cells of human blood. Since the fixed cells are of human origin, certain advantages result such as ease of clean up, not attained with the use of other materials such as synthetic particles for the replacement of the white cells in human blood.

The reference control of the instant invention may be readily formulated to provide reference values usable in making assays of normal human blood. Additionally, if desired, the formulation of the reference control may be such as to provide reference values for abnormal ranges of blood.

The foregoing and other objects and advantages of the instant invention will become more fully apparent from a reading of the following description, to be taken in conjunction with the specific examples set forth included to illustrate the invention specifically.

Speaking in general terms, in a reference control according to a preferred embodiment of the instant invention, human red blood cells are initially washed with an aqueous washing fluid, for the purpose of washing the cells free of plasma, anticoagulant, white blood cells and other debris, and to initiate stabilization of the cells, in a manner more fully to be described. The washed red blood cells are suspended in an aqueous suspension fluid, formulated to have many of the physical characteristics of human blood plasma. The suspension fluid is free of protein, to inhibit such things as bacterial growth in the fluid and surface accumulation of protein, and free of materials which normally favor biological activity in the cells. The suspension fluid further includes certain salts dissolved therein, serving to condition the cells, whereby the cells transform into substantially spherical bodies with somewhat toughened cellular membranes. The cells, however, maintain substantially their original mean cell volume. The cells in this condition exhibit maximum stability over the life of the reference control. Finally, there is included in the suspension fluid a minor amount of swollen, fixed red blood cells, these specially processed swollen red cells substituting for the white cell population found in the usual human blood.

WASHING FLUID

The washing fluid which is used in the initial processing of the so-called stabilized cells of the reference control, in addition to cleansing the cells to rid them of traces of plasma, platelets and other debris, is effective to initiate stabilization of the cells by performing a number of different functions. Thus, the washing fluid may include a sulfhydryl group reactant exemplified by iodoacetamide, in sufficient quantity to react with and thus deactivate the free sulfhydryl groups found on normal red blood cells, to inhibit glycolysis and to prevent such sulfhydryl groups from antagonizing merthiolate or other similar antiseptic included in the suspension fluid for the purpose of inhibiting bacterial or fungal growth. Stabilization of the cell membranes, and protection against hemolysis, is gained by the inclusion of an ethylene dinitrilo tetraacetic acid salt or salts, these functioning as a chelating material effective to remove multivalent positive ions from the cells, as exemplified by the calcium ion. The washing liquid may further include a small amount of aldehyde, for the purpose of toughening the cell membranes, and material or materials promoting clumping or aggregating of the cells whereby after washing their separation from the washing fluid is promoted. Materials of this latter category comprise Dextran, and sodium bicarbonate. An alkali tartrate may be included as a membrane strengthener. Thus, a typical washing fluid may comprise the following materials, preferably in the ranges indicated, dissolved in deionized or distilled water:

Material	Molarity
Dextran (molecular weight 150,000 to 350,000)	0.00009–0.00015
2-Iodoacetamide	0.00040–0.00070
Sodium bicarbonate	0.02000–0.04000
Sodium tartrate	0.05000–0.15000
Ethylene dinitrilo tetra acetic acid, dipotassium salt (EDTA, potassium salt)	0.00180–0.00270
Ethylene dinitrilo tetra acetic acid, tetra sodium	

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Material	Molarity
salt (EDTA, sodium salt)	0.00040–0.00080
Glutaraldehyde	0–0.00008
Deionized or distilled water to desired volume	

Commercially available packed human red blood cells may be washed with the washing fluid indicated above, as by introducing the blood cells at room temperature into a separatory vessel containing the washing fluid, and then gently mixing to prepare a suspension with the cells dispersed throughout the fluid. About 1 liter of washing fluid may be used in the washing of a unit of packed cells containing about 330 ml of material. After the mixing, the mixture is allowed to rest, which causes the red cells to aggregate and to precipitate, with such promoted by the Dextran and sodium bicarbonate in the washing fluid. The supernatant, containing white blood cells, platelets, plasma, unprecipitated red blood cells and other debris is syphoned off. The washed cells are then collected in a receptacle by draining them from the vessel, preferably with discarding of the first cells drained and discarding of the cells forming the top surface region of the precipitated cells.

SUSPENSION FLUID

The suspension fluid which substitutes for plasma in natural blood is an artificial nonplasma-type fluid substantially free of chlorine ions. The fluid is buffered, to maintain a pH relatively unaffected by the red cells introduced into the fluid, and is formulated to have bactericidal and fungicidal qualities, so that prolonged storage will not result in growth of contaminating organisms. The suspension fluid also imparts a resistance to degradation to washed red blood cells introduced into the fluid, and is formulated to have certain characteristics resembling that of human plasma, such as specific gravity, pH, and potassium and sodium ion concentrations.

The suspension fluid further includes a material tending to make the washed red blood cells substantially spherical in shape, without substantially changing the mean cell volume of the cells. As so conditioned, the cells have a stabilized shape while possessing substantially the specific gravity of normal red blood cells.

A typical formulation for a suspension fluid as contemplated herein comprises the following dissolved in deionized or distilled water, preferably in the ranges indicated:

Material	Molarity	
Dextran (molecular weight 10,000 to 50,000)	0.00075	–0.00225
Ethanol	1.60	–1.95
Merthiolate	0.00020	–0.00030
Tetracycline hydrochloride	0	–0.00030
Tris Hydroxymethyl-2 Aminoethane sulfonic acid (TES)	0	–0.0175
N,N-Bis (2-Hydroxyethyl)-2-aminoethane-sulfonic acid (BES)	0	–0.0188
N-2-Hydroxyethyl-piperazine-N-2-ethane sulfonic acid (HEPES)	0	–0.0168
2-Naphthol-3,6-disulfonic acid, disodium salt	0.0125	–0.0250

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Material	Molarity	
Sodium tartrate	0.0370	–0.0500
Ethylene dinitrilo tetra acetic acid, dipotassium salt (EDTA, potassium salt)	0.00180	–0.00270
Ethylene dinitrilo tetra acetic acid, tetrasodium salt (EDTA, sodium salt)	0.00040	–0.00080
Sodium hydroxide to pH of 7.0–7.5	0.004	–0.013
Deionized or distilled water to desired volume		

The potassium ion concentration is preferably selected to be within the range of 3 to 6 milliequivalents per liter, and the sodium ion concentration between 120 and 160 milliequivalents per liter. The Dextran in the above formulation imparts to the suspension fluid a specific gravity (1.01–1.03) approximating that of normal plasma. By the inclusion of the ethanol, an antiseptic is introduced and also a membrane-active compound serving to strengthen the membranes of washed cells introduced into the fluid which stabilizes the cell. The merthiolate and tetracycline hydrochloride are antibacterial and antifungal in activity. The sodium tartrate imparts resistance to cell degradation. The sodium and potassium salts of EDTA are chelating agents, and introduce strength to cell membranes. The BES, TES and HEPES are introduced for buffering purposes. Various ratios of the three compounds are used according to the pH desired, their pK_a points at 20°C. being 7.15, 7.50 and 7.55, respectively.

By the inclusion of the disulfonic acid salt of naphthol, it is observed that washed red blood cells suspended in the fluid tend to round out and assume spherical shapes, without a substantial change in mean cell volume. This spherical shape is stable and maintained over the life of the reference control.

Washed red blood cells, when suspended in a suspension fluid of the type indicated above, while being stable and resistant to degradation of the type that would adversely affect the stability of the reference control for hematologic measurements, nevertheless are susceptible to lysing when subjected to the usual lysing agents employed in hematologic procedures, such agents usually containing saponin or membrane-active detergent compounds.

As previously indicated, this invention contemplates a small fraction of so-called fixed red blood cells in the reference control which substitute for white cell population in the usual blood. These are processed by first treating them with a swelling fluid, to induce swelling of the cells to a significantly greater mean cell volume. The swelling, however, is not so great as to produce hemolyzing and bursting of the cells. The swollen cells are then treated with a fixing fluid, which in effect subjects the cells to a tanning operation. The cells when so fixed maintain their swollen condition, and are resistant to the usual lysing agents used in hematologic procedures.

SWELLING FLUID

A swelling fluid as contemplated herein may comprise the following, preferably in the ranges indicated, dissolved in deionized or distilled water.

Material	Molarity
Sodium bicarbonate	0.0200 -0.0400
2-iodoacetamide	0-0.00070
Potassium sodium tartrate	0.0200 -0.0400
Dextran (clinical grade, 150,000 to 350,000 M.W.)	0.00009-0.00015
Deionized or distilled water to desired volume	

Swelling of the red blood cells when such are immersed in the swelling fluid is the result of osmosis, the swelling fluid having a solute concentration which is reduced from the solute concentration within the cells themselves. Thus, the sodium and potassium concentration in the swelling fluid may typically be in the range of 50 to 60 milliequivalents, with the concentration within a typical cell being in the neighborhood of 140 milliequivalents. With the concentration of water outside the cells being greater than the concentration inside the cells, there is a tendency for the water concentration to equilibrate, with water moving inside the cells to swell them. The normal specific gravity of a cell may be within the range of 1.10 to 1.12. With swelling to approximately 50 percent greater volume, the specific gravity is reduced, to be within the range of 1.06 to 1.08.

Referring to the formulation for the swelling fluid set forth above, the pH of the fluid normally would be within the range of 7.0 to 9.0. The presence of the sodium bicarbonate and Dextran is to promote aggregation of the swollen red cells, to promote their separation from the swelling fluid. The iodoacetamide, while not strictly necessary, is included to inhibit glycolysis and to inactivate sulfhydryl groups, and performs a function similar to the functioning discussed in connection with the washing fluid. The potassium sodium tartrate is a membrane strengthening agent, and functions to inhibit bursting of the cells under swelling conditions.

FIXING FLUID

Human red cells swollen to approximately 150 percent their normal mean cell volume by the swelling fluid set forth above are fixed permanently in this swollen condition with a fixing fluid. The fixing fluid is essentially a tanning medium, rendering the cells stable and impervious to the usual lysing agents. A typical fluid may have the following composition:

Material	Molarity
Potassium sodium tartrate	0.0075 - 0.0095
Glutaraldehyde	0.0015 - 0.0025
Deionized or distilled water to desired volume	

It should be noted that there is a relatively high concentration of the aldehyde in the above formulation, such being the tanning agent. The potassium sodium tartrate is preferably included, to maintain the swollen cells in their swollen condition during the tanning of the cells with the aldehyde.

PRODUCTION OF ALDEHYDE FIXED RED BLOOD CELLS

In preparing the fixed red blood cells, a unit of packed human blood cells (about 330 ml) may be deposited in a separatory funnel and a liter of swelling fluid added thereto. With gentle swirling, a suspension is produced. During swirling, the cells swell to about 150 percent their original mean cell volume, the unit of blood thereby increasing to about 500 ml.

The swollen cells then may be permitted to settle or precipitate for 1 hour. The swollen cells may be drained from the base of the funnel. Preferably, the first few milliliters of the packed cells drained are discarded as well as the cells making up the top layer in the separatory funnel in order to obtain a pure sample.

The swollen cells so produced may be introduced into 2 liters of fixing fluid which is being rapidly stirred. The rapid stirring is continued for a period of about 24 hours. The molarity of the glutaraldehyde is then approximately doubled from the molarity of the original fixing fluid, and stirring continued for another 24 hours. While this rapid stirring continues, some energy may be introduced at about 100 watts/cm^2 to physically clean the cells and destroy those of marginal stability. The fixed cells produced are then gently centrifuged, and the supernatant discarded. The swollen fixed cells may then be washed with deionized or distilled water, or a physiological salt solution if desired. A suspension may then be prepared from the remaining clean, fixed cells through addition of some suspension fluid, which conveniently may be about twice the volume of the fixed cells. A cell count of the fixed cells in the fixed cell suspension so produced may be performed by a counting device such as a Coulter Counter, a conventional commercially available automatic blood counter which counts cells electronically as such are caused to pass through an orifice in the machine. For a discussion of a Coulter Counter Model S, reference is made to the article of Pinkerton, et al., entitled "An Assessment of the Coulter Counter Model S" appearing in the Journal of Clinical Pathology 1970, 23, 68–76.

PREPARATION OF REFERENCE CONTROL

Describing the preparing of a typical reference control, and one usable to provide reference values generally paralleling those found in normal blood specimens, a measured volume of washed, unfixed, red blood cells prepared as described above was gently mixed with suspension fluid also prepared as described above, to produce a suspension of the washed, unfixed, red blood cells. The volume of suspension fluid added was 0.7 times the volume of the washed red blood cells, since this results in a red blood cell concentration in the final control approximating that of normal blood.

A typical fixed cell suspension prepared as described above had a cell count performed thereon using a Coulter Counter Model F, indicating a cell count of fixed red cells of 1.5 million per mm^3. In making the cell count, a voltage threshhold was used in the counter similar to the threshhold used when making white cell counts of normal blood.

Employing the formula $V=(WS/F-W)$, wherein V is the volume in mls of fixed cell suspension to be added, W is the desired white cell count in cells per mm^3, S is the volume in mls of suspension of washed unfixed red blood cells to receive V, and F is the fixed cell suspen-

sion count in cells per mm^3, it was determined that 4.2 mls of the fixed cell suspension should be introduced to one liter of washed unfixed red cell suspension to produce the reference control desired. The resulting reference control was allowed to stand for a period of 14 days, to permit the washed unfixed cells to become fully stabilized in the suspension.

A reference control so produced was used in obtaining reference values with a Coulter Counter Model S. Thus, and following typical procedures with such a counter, a red cell count was performed on a fraction of the reference control, yielding a count of 4.82 ± 0.10 million per mm^3. A white blood cell count was performed on another fraction (with lysing of the red cells using cyanmethemoglobin solution) yielding a count of 5.9 times $10^3 \pm 0.4$ thousand cells per mm^3. The hemoglobin concentration as measured by the machine yielded a value of 15.4 ± 0.2 gms per 100 ml sample. The mean cell volume as measured by the counter was 92.2 ± 2.2 cubic microns. The values indicated are the mean plus or minus twice the standard deviation from the mean. The values indicated, which are typical of normal blood, held constant for more than 60 days after first assay. The values obtained were confirmed using known manual techniques.

Typically, the reference control as contemplated herein may be assigned a refrigerated lifetime of at least 60 days from date of assay, during which time the hematologic values obtainable therefrom are expected to remain stable. Usually, longer lifetimes are expected. At room termperatures, stability is good, with the shelf life of the control usually exceeding 1 week. The color of the reference control remains red until well after the expected life of the control, permitting its use as a quality control item. The mean cell volume of the stabilized red cells of a typical reference control as measured directly or by calibrated automated devices stabilizes at about 90 cubic microns. The specific gravity of the usual reference control for standard use is within the range of 1.045 and 1.070, and the pH between 7.0 and 7.5. The potassium ion concentration of the usual control ranges from 3 to 6 milliequivalents per liter, and the sodium ion concentration between 120 and 160 milliequivalents per liter.

It is claimed and desired to secure by Letters Patent:

1. A hematologic reference control comprising a suspension of stabilized human red blood cells in a non-proteinaceous aqueous suspension fluid, said blood cells retaining ability to respond to a lysing agent by dissolution, and being conditioned in said suspension by the inclusion in said suspension fluid of a disulfonic acid salt of naphthol, and an alkaline tartrate.

2. The reference control of claim **1**, which further includes Dextran having a molecular weight within a range of 10,000 to 50,000 dissolved in said suspension fluid, and imparting to the suspension fluid a specific gravity within the range of 1.01 to 1.03.

3. The reference control of claim **1**, which further includes a minor amount of fixed human red blood cells, swollen to significantly greater mean cell volume than the mean cell volume of the stabilized cells and functioning as a substitute for the white cell population in human blood, the fixed cells having been fixed through an aldehyde treatment so as to be resistant to a change in mean cell volume, and resistant to dissolution by a lysing agent producing dissolution of the stabilized cells.

4. The reference control of claim **1**, which further includes Dextran having a molecular weight within a range of 10,000 to 50,000, which is dissolved in the suspension liquid and imparts to the suspension fluid a specific gravity within the range of 1.01 to 1.03, and a minor amount of fixed human red blood cells swollen to significantly greater mean cell volume than the mean cell volume of the stabilized red blood cells and functioning as a substitute for the white cell population in human blood, said fixed cells being fixed through an aldehyde treatment to be resistant to a change in mean cell volume and resistant to dissolution by a lysing agent producing dissolution of the stabilized cells.

5. In a hematologic reference control including a first group of human red blood cells suspended in an aqueous suspension fluid, a second group of human red blood cells suspended in said suspension fluid functioning to substitute for the white cell population of human blood, said second group of red blood cells consisting of cells in a swollen state whereby the cells of the second group have a significantly greater mean cell volume than the mean cell volume of the cells in the first group, the cells of the second group having been fixed in their swollen state whereby said cells are more resistant to change in cell volume and to dissolution than the remainder of the cells.

6. The reference control of claim **5**, wherein fixing of the cells of said second group is by submersing the cells in an aldehyde tanning solution.

7. A swelling fluid for the swelling of human red blood cells to increase their mean cell volume and to promote aggregation of the cells in their swollen state comprising an aqueous solution of sodium bicarbonate, iodoacetamide, an alkali tartrate, and Dextran having a molecular weight within a range of about 150,000 to 350,000.

8. A fixing fluid, for fixing swollen human red blood cells in their swollen state and imparting resistance to dissolution by lysing agents, comprising an aqueous solution of an alkali tartrate and glutaraldehyde.

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