

Evaluation of methods for preparing pure lymphocyte suspensions from peripheral blood

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Methods

Lymphocytes are required in our laboratory for the tissue typing of donors and recipients for renal transplantation. A suspension of lymphocytes is often needed rapidly, and consequently in all methods to be described the emphasis is on achieving a pure suspension in the shortest possible time.

Blood for each procedure is defibrinated by gentle swirling in a conical flask containing one glass bead of 3 mm diameter per 5 ml of blood. If this is not practicable blood may be collected into heparin and then defibrinated in the same manner after the addition of excess thrombin to neutralize the heparin. Blood from patients on chronic dialysis often requires the addition of thrombin to facilitate defibrination because of the residual heparin and other circulating anticoagulants present after dialysis.

Where the suspension is ultimately to be used for tissue typing sterility in these and the following procedures need not be strictly observed, but should the cells be for subsequent culture sterility must be maintained throughout.

A SEPARATION OF LYMPHOCYTES USING A COLUMN OF MICROSPHERULES

1 Defibrinated blood is sedimented in a long tube or cylinder (160 × 12 mm) containing 1/5 volume of a 3% solution of gelatin in saline at 37°C for 30 minutes (Coulson and Chalmers, 1964).

2 The leucocyte-rich supernatant is pipetted into a centrifuge tube and a few drops of chicken antihuman red cell serum are added (Sanderson, 1967) and centrifuged at 1,000 rpm for 10 minutes.

3 Discard all but the last 2-3 ml of the supernatant and resuspend the button of white cells and red cell agglutinates by gentle tapping.

4 The resuspended cells are added to the top of a column of plastic microspherules (Pontyclun Chemical Co. Ltd, London, W4) in a 10 ml syringe. The column is prepared by making a slurry of microspherules in complement-fixation test buffer (CFT) warmed to 37°C. This is then poured into the syringe, the bottom of which is plugged with a square of gauze.

Barbitone	0.575
Sodium chloride	8.5 g
Magnesium chloride	0.168
Calcium chloride	0.028
Barbitone soluble	0.185
Distilled water	1 litre

Constituents of complement fixation test buffer pH 7.2

5 The cells are washed into the column with CFT buffer and incubated at 37°C for 30 minutes. During this time the neutrophils and monocytes adhere to the microspherules and the red cell agglutinates remain on the top of the column.

6 Lymphocytes are then obtained by adding CFT buffer drop by drop to the top of the column and collecting the cell-rich fluid in a centrifuge tube at the base. The fluid must not be forced through the column as this will lead to neutrophil contamination.

7 The eluate is centrifuged at 1,000 rpm for 15 minutes.

8 The supernatant is discarded and a small volume of CFT buffer is then added without disturbing the cell button. The lymphocytes are gently resuspended to a concentration of 1-2 10^6 per ml.

The purity of the lymphocyte suspension thus obtained is 90-98% and the total time taken is 90 minutes.

Comments

The 3% gelatin solution in saline was later discarded in favour of Plasmagel (Laboratoire Roger Bellon, Neuilly, France) because, although gelatin is an excellent sedimenting agent, the solution is difficult to sterilize.

Chick antihuman erythrocyte serum was used in preference to anti-A, B, and H, because these antisera were not found to be sufficiently avid. The amount of agglutinating serum added depends on the titre of the particular serum used.

Hypotonic shock as a method of removing red cells was tried and found to be very efficient in normal healthy individuals. Unfortunately, in some patients it was found that the brief exposure to distilled water not only lysed red cells but also appeared to damage the lymphocytes. In these cases a high background count of dead cells was experienced when the resulting suspension was used in tissue typing.

The use of plastic microspherules was later discarded in favour of a nylon fibre. This was found to be both cheap and easily sterilized by autoclaving, and was an excellent agent for the removal of neutrophils from leucocyte suspensions. In uraemic patients, however, and in patients with a high proportion of neutrophils in the peripheral blood high contamination with neutrophils was experienced.

The main disadvantage of this method is the fact that it takes 90 minutes to obtain a lymphocyte suspension and that cells are lost in the column as well as in the red cell agglutinates. Thus the

total yield of lymphocytes is often lower than could be expected from the total white cell count.

B RAPID TECHNIQUE DEVELOPED FROM METHOD A

1 Defibrinated blood is added to 1/5 volume of Plasmagel in a centrifuge tube and spun at 700 rpm for five minutes.

2 The leucocyte-rich supernatant is added to a column of nylon fibre in a 10 ml syringe which has been previously moistened with CFT buffer at 37°C.

3 The column is incubated at 37°C for 15 minutes.

4 The cells are washed slowly from the column with CFT buffer and a few drops of chick anti-human red cell serum are added.

5 The mixture is spun at 1,000 rpm for 10 minutes.

6 The supernatant is discarded and the lymphocytes are resuspended from the cell button in CFT buffer to a count of $1-2 \times 10^6$ ml.

The purity of the lymphocyte suspension obtained is 90-98%, and total time is 30 minutes.

Comments

This method, though rapid, was found to be satisfactory only in healthy persons. In a diseased state difficulty was experienced in removing the neutrophils because of the shortened incubation time of the cells in contact with the nylon column.

C SEPARATION USING A DENSITY GRADIENT REAGENT (BÖYUM, 1968; HARRIS AND UKAEJIOFO, 1969)

Reagent

Sixty parts of 9% Ficoll in distilled water and 25 parts of 34% Triosil in distilled water. Mix and store at 4°C avoiding exposure to the light.

Procedure

1 Defibrinated blood is diluted 1:4 in CFT buffer.

2 One ml of diluted blood is carefully layered onto 2 ml of density gradient reagent (as above) in a centrifuge tube of $\frac{1}{2}$ in. diameter and centrifuged at 3,000 rpm for 20 minutes.

3 At the end of the centrifugation, the cells at the interface are carefully removed with a Pasteur pipette and washed in a large volume of CFT buffer at 1,000 rpm for 10 minutes.

4 The supernatant is discarded and the cell button resuspended in CFT buffer adjusting the number of lymphocytes to $1-2 \times 10^6$ ml.

The purity of the lymphocyte suspension obtained is 98-100% and the total time 30 minutes.

Comments

In our hands it was found that there was usually a small degree of contamination by red cells.

This is not sufficient to cause difficulty in subsequent tissue typing but it is not ideal. The resulting suspension is a great improvement on those obtained in the two methods using a column. The only disadvantage of the gradient was the fact that in patients with a reduced white cell count difficulty was often experienced in obtaining sufficient numbers of cells. Consequently the following method was developed.

D SEPARATION USING SEDIMENTATION AND A DENSITY GRADIENT REAGENT

1 Defibrinated blood is added to 1/5 volume of Plasmagel and allowed to sediment at an angle of 45° at 37°C for five minutes.

2 Two ml or more of the leucocyte-rich supernate is carefully layered onto approximately 2 ml of density gradient reagent (as for method C) and centrifuged at 3,000 rpm for 20 minutes.

3 The cells at the interface are removed and washed in a large volume of CFT buffer as before by centrifuging at 1,000 rpm for 10 minutes.

4 The cell button is resuspended in CFT buffer and the lymphocytes are adjusted to $1-2 \times 10^6$ ml.

The purity of the lymphocyte suspension obtained is 98-100%, and the total time 35 minutes.

Comments

This is the best method for obtaining a high and pure yield of lymphocytes, even in those patients whose clinical state makes it difficult to obtain a neutrophil-free suspension by other methods. This technique also eliminates red cell contamination and, as yet, we have found no disadvantages whilst its future applications seem numerous.

Preparations of lymphocytes from dog blood can be obtained using this method, and it may also provide an effective means of obtaining lymphocytes from other animals whose lymphocytes are approximately the same size as those in man.

It has been observed that the proportion of cell-rich supernatant to the density gradient reagent is relatively unimportant and thus it is possible to expand this method even further to obtain the larger volumes of cells required for other procedures, such as lymphocyte culture. Where lymphocytes are required for culture work, tissue culture medium 199 should be substituted for CFT buffer.

References

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