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Separation of Lymphocytes, Lymphocyte Subgroups and Monocytes: A Review

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Summary

Several techniques are now available for isolating lymphocytes from blood and other sources, but no single technique can be considered best for all purposes. In selecting a separation procedure, it is recommended that the procedure be as simple as possible, if otherwise satisfactory. The present paper reviews the most widely used techniques, including the separation of lymphocyte subpopulations and monocytes.

Separation of Leucocytes and Lymphocytes

Most techniques are based on the following principles:

1. Differences in size (sedimentation rate) and density,
2. Differences in biological properties (phagocytosis, adherence);
3. Combination of 1 and 2.

Separation by Sedimentation at 1 G

Unfractionated leucocytes may be obtained simply by leaving anticoagulated blood for semimentation in a test tube or beaker at room temperature. The plasma layer containing the leucocytes can be pipetted off after 1 to 2 hours when the majority of red cells have settled.

The sedimentation is more rapid if an erythrocyte-aggregating agent is added (1). Dextran (Dextran 500, Pharmacia, Uppsala/Sweden) or methylcellulose (25 centipoise) dissolved in 0.9% NaCl are usually used in the following proportions:

1. 10 parts blood + 1 (or 2) parts dextran 6%;
2. 10 parts blood + 0.5 (or 1) part methylcellulose 2%.

The sedimentation of erythrocytes then takes 15 to 40 minutes, depending on the height of the blood cell column. A similar cell suspension may be obtained by centrifugation of the blood (6 to 7 minutes at 1700 rpm ~ 500 g). After centrifugation, the leucocytes are found as a white layer (buffy coat layer) on top of the erythrocyte pellet and can be removed with a pipette. In both techniques 30% to 40% of the leucocytes are lost in the erythrocyte pellet. The loss is not selective and can be minimized by resuspending the erythrocytes and repeating the procedure.

Cell suspensions obtained by these procedures may be used for lymphocyte cultures, but it is then apparently neccessary to show separately that contaminating granulocytes (~ 2 per lymphocyte), platelets, and red cells (1 to 10 per leucocyte) do not interfer with functional tests. Platelet contamination can be avoided by using defibrinated blood.

Otherwise, sedimentation (or centrifugation) of blood is commonly used as the first step in techniques designed to obtain pure lymphocyte suspensions.

Isolation of Lymphocytes and Monocytes based on Density Differences

The Isopaque-Ficoll (IF) technique (2, 3, 4) is widely used (5). This method can be used in two different ways; one procedure is illustrated in Figure 1. One part of human blood is mixed with one part of 0.9% NaCl and layered over IF (density = 1.077 g/ml) in a centrifuge tube. After centrifugation at 2000 rpm (~ 700 g) for 15 to 20 minutes at room temperature, the mononuclear cells are found as a thin layer at the interface while granulocytes and erythrocytes have settled to the bottom of the tube. The mononuclear cells are composed of approximately 85% lymphocytes and 15% monocytes;

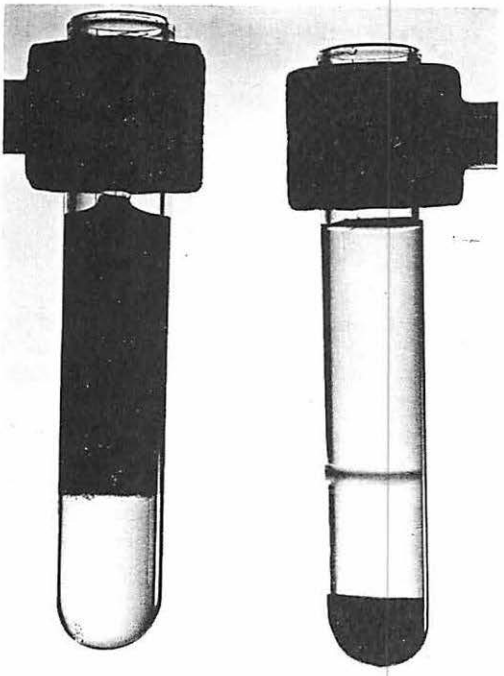

Fig. 1 Isolation of monoculear cells by centrifugation at 20C for 20 minutes at 700 g. A mixture (8 ml) of 1 part blood and 1 (or 2 to 3) part 0.9% NaCl is layered on top of 3 ml Isopaque-Ficoll separation fluid. After centrifugation mononuclear cells and platelets are found on top of the separation fluid; erythrocytes and granulocytes have formed a sediment at the bottom of the tube.

contamination with granulocytes and erythrocytes is negligible. The platelets come together with mononuclear cells, but this contamination can be avoided by using defibrinated blood (6). The yield of mononuclear cells from anticoagulated blood is close to 100%, when the separation is carried out in small tubes (13 to 14 mm diameter); some loss may take place, however, during additional washings (4). For larger blood volumes (> 30 ml), it is more convenient to use dextran sedimentation (or centrifugation) as an initial step. The leucocyte layer is then similarly placed on top of the IF solution and centrifuged as described. With this procedure, a pure suspension of granulocytes (with some erythrocyte contamination) can be harvested from the bottom of the tube. Using two IF solutions with different densities, lymphocytes and granulocytes may be isolated by a one-step procedure (7).

The IF technique is rapid and applicable for blood lymphocyte isolation in most species. In order to get rid of monocytes, the procedure must be modified (see below). In general the technique can be used to remove granulocytes, red cells, and dead cells from any suspension of lymphoid cells. In some species it may, however be necessary to increase the density of the separation fluid (8).

Lymphocytes can also be isolated by flotation on a Ficoll solution (without Isopaque); the yield, however, is lower and the erythrocyte contamination increases (9).

Relatively pure suspensions of blood monocytes have been obtained by using albumin as separation fluid. Suspended leucocytes are mixed with 35% albumin to give a final concentration of 27% (10, 11, 12, 13). Following centrifugation, the monocytes can be collected from the top of the albumin solution. The purity varies from 40% (11) to 70% (10); the contaminating cells are predominantly lymphocytes. The yield is approximately 50%. An even higher purity (80%) with a 30% yield has been reported by using a discontinuous gradient of IF (14).

Lymphocyte Isolation by Combining Phagocytosis (Iron) and Sedimentation or Centrifugation

IF separated cells contain a significant fraction of monocytes. A purer lymphocyte suspension can be obtained by utilizing the ability of granulocytes and monocytes to enguif small iron particles (15, 16). Following incubation of whole blood, or a leucocyte suspension with iron particles, the iron-loaded cells can be removed with a magnet (15, 16, 17); the erythrocytes, by sedimentation (18). Based on these principles, a semi-automated technique has been developed (19). The iron-loaded cells may be removed by sedimentation alone without a magnet since they settle more rapidly due to their increased density. Unphagocytized small iron particles can be removed after this point via a magnetic stirrer (18). These procedures have provided human lymphocyte suspensions with 95% to 97% purity (18, 19); the yield varies from 40% (17) to 90% (19). The erytrhocyte contamination varies

from 2 (17) to 40 (19) per lymphocyte. The erythrocytes can be removed by hyptonic lysis or by adding a hemolytic agent. It should be noted, however, that NH_4Cl lysis reversibly may impair functional properties of lymphocytes (20).

A lower erythrocyte contamination is obtained by a modified technique (4, 21). Buffy coat leucocytes are incubated with iron particles. After 30 minutes the cells are separated with IF; the iron-loaded monocytes settle to the bottom, and a pure lymphocyte suspension (1% monocytes, < 10% erythrocytes) is found at the interface.

Removal of Unwanted Cells by Adherence

Monocytes and granulocytes (and platelets) suspended in serum or heparin-plasma adhere readily to surfaces and may, therefore, be removed. Several procedures have been reported. The leucocytes are applied to a column of glass wool (22, 23), cotton batting (24), nylon fibers (25), or glass beads (26). Usually the cells are incubated for 30 minutes at 37C (26), or they pass to column at a slow rate (23, 25). The effluent contains lymphocytes of 95% to 96% purity. The recovery of lymphocytes amounts to 60% to 70% of the number applied on the column. The cell suspension is almost devoid of platelets, but erythrocytes contaminate the lymphocyte collection in proportion to their number among unseparated cells. The procedure is easy; it has also been reported that, with IF-separated cells as starting material, the monocytes (90% purity) can be detached by washing the column with an EDTA solution (27). One problem, however, is that there may be a selective depletion of B lymphocytes, which are retained on the column in various amounts (see below).

A simple way of using the principle of adherence is to layer a thin film of unseparated leucocytes in a glass bottle (or Petri dish). The nonadherent cell population which is carefully removed after 90 minutes of incubation, comprises 90% to 98% lymphocytes (28). It is imperative that a well-standardized procedure be used in regard to cleaning glassware, to cell concentration, to suspending medium, etc. Under optimal conditions 81% to 100% lymphocyte recovery is obtained. The same procedure may be used for improving monocyte isolation when granulocytes have been initially removed by the albumin (12) or IF centrifugation technique. After incubation, when nonadherent cell have been collected, the remaining adherent monocytes can be detached by gentle scraping with a rubber policeman (12).

Lymphocyte Isolation from Sources other than Blood

Lymphocytes are easily available from different lymphoid organs. In animals an even suspension of spleen cells is obtained simply by cutting the spleen in small pieces and then using a tissue homogenizer. If necessary red cells, granulocytes, and dead cells can be removed by the IF procedure (4, 8). Lymph nodes can be dissected out and minced with a pair of scissors and a Pasteur pipette in order to produce an even cell suspension.

Due to their small size, lymphocytes settle slowly. This principle has been used for isolation of bone marrow lymphocytes. A simple procedure was reported by *Yamana* and *Nairn* (29). Rat bone marrow cells were applied on top of a moistened glass wool column. After 30 minutes sedimentation at 37C, the cells were eluted. The top fraction consisted of 60% lymphocytes and 30% to 35% erythroblasts. A higher degree of purity (80% to 90%) was obtained by sedimentation in a gradient (30, 31). This was accomplished by combining glass wool filtration and dextran gradient centrifugation (32) or by using a serum-sucrose (33) gradient (yield 40% to 50%).

Separation of Lymphocytes Subpopulations

To a large extent lymphocytes can now be characterized by the relationship between surface markers and functional properties. Parallel with the progress in this field, many separation techniques have been developed. The major subpopulations of, B and T lymphocytes, as well as subsets within these gropus (survey 34, 35) can be isolated rather efficiently

These techniques are largely based on differences in specific properties (surface structures) or general properties (electrophoretic mobility, size, density, adherence). The purity of the isolated cells can finally be tested by identifying the surface markers.

Column Fractionation (Adherence)

The adherence is either unspecific or based on on specific receptors. The procedure is generally used to remove unwanted cells, but the adhering cells may also subsequently be eluted.

B lymphocytes adhere nonspecifically by incubation on nylon fiber columns (36, 37, 38), and the cells passing the column comprise more than 90% T lymphocytes (37, 39, 40). The B cells retained on the column contain a significant proportion of T cells. Working with human tonsil lymphocytes (*Greaves* and *Brown* 40), reported a 95% to 96% purity of B lymphocytes with this procedure. In general nylon fiber incubation is a convenient first step for the further characterization of subsets of T lymphocytes.

A more specific adsorption of subpopulations occurs on glass or plastic beads coated with antigens (41), haptens (42), or antibodies specific to membrane determinants on lymphocytes (43). B cells have a high concentration of Ig on their surface, and passage of lymphoid cells through an anti-Ig column may yield a very pure T cell population (44). In addition to B cells, such a column may also adsorb K (killer) cells and subpopulations of T cells (45).

Ig-bearing B cells can also be removed by filtration through Sephadex anti-Fab columns (46) and then recovered by enzymatic digestion of the immunoabsorbent. In principle such affinity columns may possess the potential for depleting as well as selecting cells with specific surface markers or with specificity for a particular antigen.

Rosette Formation

The first use of this procedure was based on the observation that human lymphocytes have receptors (47) for sheep red blood cells (SRBC). During incubation the SRBC adhere to the surface of single T lymphocytes (48) and form a rosette. When centrifuged then with IF, the rosettes settle to the bottom of the tube while nonrosetted cells remain at the interface (48). Since the SRBC in the pellet may be removed by lysis, an almost pure suspension of T cells can be acquired. One advantage of the technique is that it leaves non-SRBC-binding cells (B) intact for further analysis. To achieve a satisfactory B-cell separation, however, it may be necessary to repeat the procedure (49). The stability of the rosettes may be improved by chemical pretreatment of the SRBC (50, 51). In a similar procedure, a human lymphocyte suspension can be depleted of B cells which form rosettes with monkey red blood cells (50). Another potential application comes from the report that mouse red blood cells may be a marker for B cells in man (52). The rosetting technique may be used for many species by coating the red cells in different ways. In this way *Parish* and *Hayward* (53) were able to separate rat lymphocytes with surface Ig as well as with receptors for complement and Fc fragments.

Cytotoxic Antisera

In the mouse the unwanted cell population (B or T) can be rather efficiently removed by means of specific antisera (54). In general, however, such antisera are difficult to prepare.

Separation Based on Physical Properties

It is possible to achieve a satisfactory separation of mouse B and T cells by electrophoresis since T cells have a higher electrophoretic mobility than B cells (55, 56).

An effective separation based on difference in densities or sedimentation properties alone is possible only to a certain extent (57). A significant enrichment of antibody-producing cells (58, 59) or activated (mitogens) cells (55), however, can be obtained by sedimentation at unit gravity or by dextran gradient centrifugation (60). By combining centrifugation on bovine serum albumin with the rosetting procedure, it has been possible to isolate human lymphocytes with precursor (of B and T) characteristics (61, 62).

As a whole, isolation of lymphocyte subpopulations is a rapidly expanding field. We can expect advanced procedures for selecting subgroups to be developed in the future.

References

1 *Skoog, W.A., W.S. Beck:* Studies on the fibrinogen, dextran and phytohemagglutinin methods of isolating leukocytes. Blood 11 (1956) 436-454

2 *Bøyum, A.:* Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand. J. clin. Lab. Invest. 21, Suppl. 97 (1968) 77-89

3 *Bøyum, A.:* Separation of blood leucocytes, granulocytes and lymphocytes. Tissue Antigens 4 (1974) 269-274

4 *Bøyum, A.:* Isolation of lymphocytes, granulocytes and macrophages. Scand. J. Immunol. 5. Suppl. 5 (1976) 9-15

5 Special technical report. Identification, enumeration, and isolation of B and T lymphocytes from human peripheral blood. Scand. J. Immunol. 3 (1974) 521-532

6 *Thorsby, E., A. Bratlie:* A rapid method for preparation of pure lymphocyte suspensions. Histocompatibility testing (1970) 655-666, Munksgaard, Copenhagen.

7 *English, D., B.R. Andersen:* Single-step separation of red blood cells, granulocytes and mononuclear leucocytes on discontinuous gradients of Ficoll-Hypaque. J. Immunol. Meth. 5 (1974) 249-252

8 *Davidson, W.F., C.R. Parish:* A procedure for removing red cells and dead cells from lymphoid cell suspensions. J. Immunol. Meth. 7 (1975) 291-300

9 *Boyle, W., A. Chow:* Isolation of human lymphocytes by a Ficoll barrier method. Transfusion 9 (1969) 151-155

10 *Bennet, W.E., Z.A. Cohn:* The isolation and selected properties blood monocytes. J. Exp. Med. 123 (1966) 145-159

11 *Hay, F.C., G. Torrigani, I.M. Roitt:* The binding of human IgG subclasses to human monocytes. Eur. J. Immunol. 2 (1972) 257-261

12 *Brodersen, M.P., C.P. Burns:* The separation of human monocytes from blood including biochemical observations Proc. Soc. Exp Biol. Med. 144 (1973) 941-944

13 *Holm, G., S. Hammarstrøm:* Heamolytic activity of human blood monocytes. Lysis of human erythrocytes treated with anti-A-serum. Clin. exp. Immunol. 13 (1973) 29-43

14 *Loos, H., B. Blok-Schut, R. van Dorn, R. Hoksbergen, A.B. de la Riviere, L. Meerhof:* A method for the recognition and separation of human blood monocytes on density gradients. Blood 48 (1976) 731-742

15 *Levine, S.:* Magnetic techniques for in vitro isolation of leucocytes. Science 123 (1956) 185-186

16 *Carstairs, K.:* The human small lymphocyte: Its possible pluripotential quality, Lancet 1 (1962) 829-832

17 *Shah, S.A., J.A. Dickson:* Lymphocyte separation from blood. Nature 249 (1974) 168-169

18 *Thierfelder, S.:* A method for the isolation of human lymphocytes. Vox Sang. 9 (1964) 447-454

19 *Lichtenstein, B., L. Paseltiner, R. Weingard, R. Widmark:* A new semi-automated method for the high yield separation of lymphocytes from blood. Technicon Instruments. Corporation, Tarrytown, New York (1971) 1-5

20 *Yust, I., R.W. Smith, J.R. Wunderlich, D.L Mann:* Temporary inhibition of antibody-dependent, cell-mediated cytoxicity by pretreatment of human attacking cells with ammonium chloride. J. Immunol. 116 (1976) 1170-1172

21 *Tebbi, K.:* Purification of lymphocytes. Lancet 1 (1973) 1392

22 *Johnsen, T.M., J.E. Garvin:* Separation of lymphocytes in human blood by means of glass wool column. Proc. Soc. Exp. Biol. Med. 102 (1959) 333-335

23 *Brandt, L., J. Børjeson, Å.Norden, I. Olsson:* Separation of lymphocytes from peripheral blood by means of a glass wool column. A method for in vitro culture of lymphocytes. Acta. Med. Scand. 172 (1962) 459-462

24 *Lamvik, J.O.:* Separation of lymphocytes from human blood. Acta haemat. 35 (1966) 294-303

25 *Greenwalt, T.J., M. Gajewski, J.L. McKenna:* A new method for preparing buffy coat-poor blood. Transfusion 2 (1962) 221-229

26 *Rabinowitz, Y.:* Separation of lymphocytes, polymorphonuclear leukocytes and monocytes on glass columns, including tissue culture observations. Blood 23 (1964) 811-828

27 *Summers, M., M. Worwood, A. Jacobs:* Ferritin in normal erythrocytes, lymphocytes, polymorphs, and monocytes. Brit. J. Heamat. 28 (1974) 19-26

28 *Alderson, E., J.P. Birchall, J.J.T. Owen:* A simple method of lymphocyte surification from human peripheral blood. J. Immunol. Meth. 11 (1976) 297-301

29 *Yamana, S., R.C. Nairn:* Simple separation of lymphoid cells from granulocytic series in rat bone marrow. Immunology. 21 (1971) 1031-1035

30 *Peterson, E.A., W.H. Evans:* Separation of bone marrow cells by sedimentation at unit gravity. Nature 214 (1967) 824-825

31 *Bøyum, A.:* Isolation and removal of lymphocytes from bone marrow of rats and guinea-pigs. Scand. J. Clin. Lab. Invest. 21, Suppl. 97 (1968) 91-106

32 *Morrison, J.H.:* Separation of lymphocytes of marrow by combined glass-wool filtration and dextran-gradient centrifugation. Brit. J. Haemat. 13 (1967) 229-235

33 *Johnson, L.I.:* Bone marrow lymphocytes. Their isolation, colony-forming capacity and graft-versus-host potential. I. The isolation and characterization of subpopulations of bone marrow lymphocytes. Proc. Soc. Ecp. Med. 136 (1971) 1277-1283

34 Separation of T and B lymphocytes subpopulations. Transpl. Rev. 25 (1975)
35 Lymphocytes, isolation, fractionation and characterization. Scand. J. Immunol. 5 (1976) Suppl. 5
36 *Eisen, S.A., H.J. Wedner, W. Parker:* Isolation of pure human peripheral blood T-lymphocytes using nylon wool columns. Immunol. Communications 1 (1972) 571-577
37 *Wisløff, F., S. Frøland:* Antibody-dependent lymphocyte-mediated cytotoxicity in man: No requirement for lymphocytes with membrane-bound immunoglobulin. Scand. J. Immunol. 2 (1973) 151-157
38 *Trizio, D., G. Cudkowicz:* Separation of T and B lymphocytes by nylon wool columns: Evaluation of efficacy by functional assays in vivo. J. Immunol. 113 (1974) 1093-1097
39 *Julius, M.H., E. Simpson, L.A. Herzenberg:* A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol. 3 (1973) 645-649
40 *Greaves, M.F., G. Brown:* Purification of human T and B lymphocytes. J. Immunol. 122 (1974) 420-423
41 *Wigzell, H., B. Anderson:* Cell separation on antigen-coated columns. Elimination of high-rate antibodyforming cells and immunological memory. J. Exp. Med. 129 (1969) 23-36
42 *Truffa-Bachi, P., L. Wofsy:* Specific separation on affinity columns. Proc. Natl. Acad. Sci. 66 (1970) 685-692
43 *Wigzell, H.:* Specific fractionation of lymphocytes using glass or plastic bead columns. Scand. J. Immunol. 5, Suppl. 5 (1976) 23-30
44 *Wigzell, H., K.G. Sundqvist, T.O. Yoshida:* Separation of cells according to surface antigens by the use of antibody coated columns. Fractionation of cells carrying immunoglobulens and blood group antigens. Scand. J. Immunol. 1 (1972) 75-87
45 *Rubin, B., P.N. Jørgensen, F. Güttler, M. Høier-Madsen:* Impact of Ig-anti-Ig column fractionation on T-lymphocyte functions. Scand. J. Immunol. 5 (1976) 345-359
46 *Schlossman, S.F., L. Hudson:* Specific purification of lymphocyte populations on a digestible immunabsorbent. J. Immunol. 110 (1973) 313-315
47 *Brain, P., J. Gordon, W.A. Willetts:* Rosette formation by peripheral blood lymphocytes. Clin. Exp. Immunol. 6 (1970) 681-688
48 *Frøland, S.S.:* Binding of sheep erythrocytes to human lymphocytes. A probable marker of T lymphocytes. Scand. J. Immunol. 1 (1972) 269-280
49 *Jondal, M.:* SRBC rosette formation as human T lymphocyte marker. Scand. J. Immunol. 5, Suppl. 5 (1976) 69-76
50 *Pellegrino, M.A., S. Ferrone, A.N. Theofilopoulos:* Isolation of human T and B lymphocytes by rosette formation with 2-aminoethylisothiouronium bromide (AET)-treated sheep red blood cells and with monkey red blood cells. J. Immunol. Meth. 11 (1976) 273-279
51 *Hokland, P., M. Hokland, I. Heron:* An improved technique for obtaining E rosettes with human lymphocytes and its use for C cell purification. J. Immunol. Meth. 13 (1976) 175-182
52 *Stathopoulos, G., E.V. Elliot:* Formation of mouse or sheep red-blod-cell rosettes by lymphocytes from normal and leukaemic individuals. Lancet 1 (1974) 600-601
53 *Parish, C.R., J.A. Hayward:* The lymphocyte surface. II. Separation of Fc receptor, C'3 receptor and surface immunoglobulin-bearing lymphocytes. Proc. R. Soc. Lond. B. 187 (1974) 65-81
54 *Raff, M.C.:* Surface antigenic markers for distinguishing T and B lymphocytes in mice. Transpl. Rev. 6 (1971) 52-80
55 *Häyry, H., L.C. Andersson:* Fractionation of immunocompetent cells with different physical properties. Scand. J. Immunol. 5, Suppl. 5 (1976) 31-44
56 *Platsoucas, C.D., A.L. Griffith, N. Catsimpoolas:* Density gradient electrophoresis of mouse spleen lymphocytes: Separation of T and B cell fractions. J. Immunol. Meth. 13 (1976) 145-152
57 *Shortman, K., H. von Boehmer, J. Lipp, K. Hopper:* Subpopulations of T-lymphocytes. Transpl. Rev. 25 (1975) 163-210
58 *Mage, M.G., W.H. Evans, E.A. Peterson:* Enrichment of antibody plaque-forming cells of spleen by sedimentation at unit gravity. Proc. Soc. Exp. Biol. Med. 127 (1968) 478-481
59 *Phillips, R.A., R.G. Miller:* Antibody-producing cells: Analysis and purification by velocity sedimentation Cell Tissue Kinet. 3 (1970) 263-274
60 *Shenker, B., I. Gray:* Enrichment of PHA transformed lymphocytes in samples containing mixed populations. J. Immunol. Meth. 13 (1976) 161-166
61 *Gatien, J.G., E.E. Schneeberger, R. Parkman, E. Merler:* Isolation on discontinuous gradients of bovine albumin of a subpopulation of human lymphocytes exhibiting precursor characteristics. Eur. J. Immunol. 5 (1975) 306-312
62 *Gatien, J.G., E.E. Schneeberger, E. Merler:* Analysis of human thymocyte subpopulations using discontinuous gradients of albumin: precursor lymphocytes in human thymus. Eur. J. Immunol. 5 (1975) 312-317

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