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Enrichment of Antibody Plaque-Forming Cells of Spleen by Sedimentation at Unit Gravity (32719)

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Many biological problems, particularly in the area of cell differentiation and maturation, involve the study of complex mixtures of cells, such as spleen or bone marrow. In immunology, such problems as the identification of the cell types involved in various phases of the induction of immune reactions and studies at the molecular level of antibody biosynthesis would be aided by the development of methods for the fractionation of heterogeneous cell populations containing immune cells. One approach to this problem is to exploit physical differences between cells, such as differences in size and density. Szenberg and Shortman (1) have recently studied the distribution of cells active in graft versus host reactions after equilibrium density centrifugation of fowl blood lymphocytes. The present study reports the distribution of hemolytic antibody plaque-forming cells (PFC) and nonplaque formers after sedimentation of spleen cell suspensions from mice immunized with sheep erythrocytes, at unit gravity in a sucrose gradient.

Material and Methods. A method previously developed for bone marrow by Peterson and Evans (2) was employed. It utilizes a cylindrical sedimentation chamber with a conical top and bottom. A chamber of 16-inch diameter (Fig. 1) was used in the present work.

Cell counts were carried out on a Sanborn Electronic Cell Counter, using 4% acetic acid as a diluent for counting nucleated cells. Smears for differential counts were stained with Giemsa, and the criteria of Dunn and of Wintrobe (3) were used to identify the various cell types.

Cells producing high hemolytic efficiency antibodies [H-PFC, considered to be of the

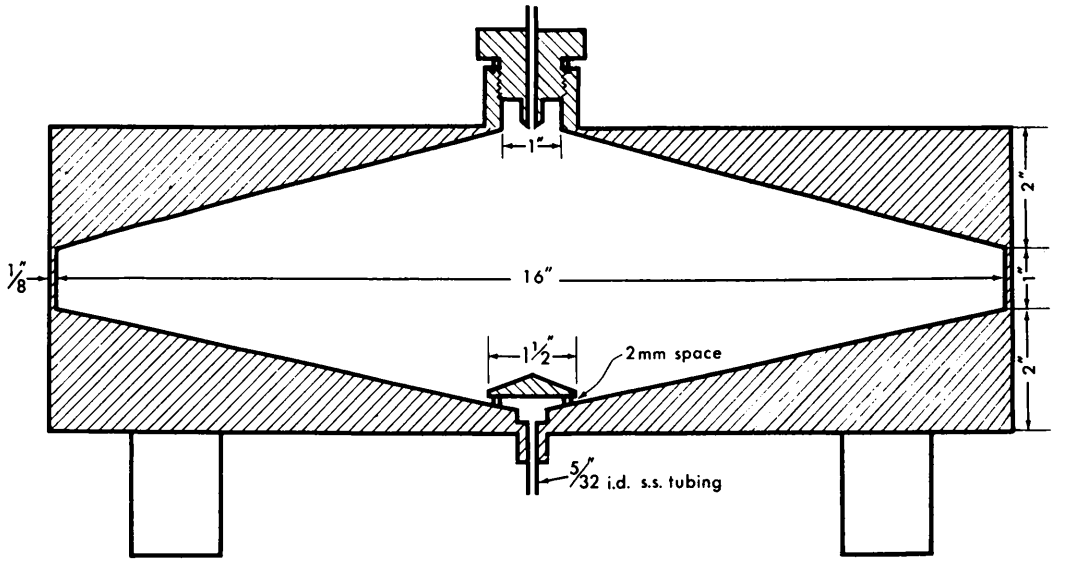

FIG. 1. Sedimentation chamber (constructed of lucite) shown in vertical cross section. Fluid enters and leaves the chamber through 5/32 inch i. d. stainless steel tubing. The threaded plug containing the outlet is sealed with an O-ring. The baffle above the inlet is mounted on three small pegs, only two of which appear in the figure.

γM-immunoglobulin class (4)] to sheep erythrocytes were identified by the method of localized hemolysis in gel of Jerne and Nordin (5), except that DEAE-dextran was omitted (6) and agarose (*Seakem* brand, from Bausch & Lomb, Inc., Rochester N. Y.) was used in place of agar. Cells producing low hemolytic efficiency antibodies (L-PFC, considered to be primarily γG-immunoglobulins) were assayed with a rabbit antiserum to mouse γ-globulin (7).

Balb/C female mice, 7 weeks old, were given a primary immunization of 5×10^8 sheep erythrocytes, ip. A second ip injection of 1.4×10^8 sheep erythrocytes was given to 2 such mice 10–29 days later. Three days after the second injection, the 2 mice were killed; their spleens were removed aseptically, cut into four or five pieces with a scissors, suspended in a test tube in 10 ml of Hanks' balanced salt solution with 0.04% bovine serum albumin, and gently disrupted with a loose-fitting Teflon plunger. The cell suspension was then filtered through 2 layers of silk cloth, and through a porous polyethylene disk having a nominal pore size of 70 μ.

The prepared spleen cell suspension (1.4×10^8 nucleated cells in 50 ml of Hanks' solution containing 0.025% sucrose) was rapidly introduced into the conical bottom of a 16-inch diameter cylindrical sedimentation chamber. This was followed immediately by a gradient of sucrose which was introduced into the sedimentation chamber until the sample layer and the underlying sucrose gradient were both within the cylindrical portion of the sedimentation chamber. The gradient was formed by connecting three vessels (connected to the inlet of the sedimentation chamber) containing 800 ml of 0.30% sucrose in Hanks' solution. The second and third vessels each had 2 liters of Hanks' solution containing 1.00% and 1.38% sucrose respectively. The cells were allowed to sediment for 100 min at 4°. The contents of the chamber were then pumped out into 50-ml conical polypropylene centrifuge tubes, BSA was added to each tube to a final concentration of 0.04%, and the tubes were centrifuged at 1500 rpm for 10 min at 4°. The pellets were gently resuspended in small aliquots of their own supernatants, pooled into fractions as shown in Fig. 2, made up to a convenient volume (usually 3–4 ml), and aliquots were assayed for their content of high and low hemolytic efficiency plaque-forming cells.

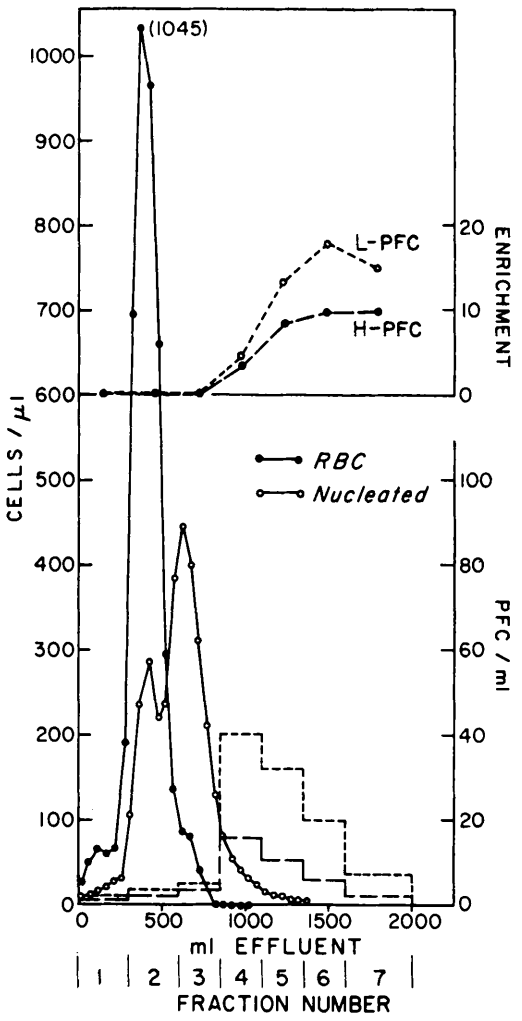

FIG. 2. Sedimentation at unit gravity of spleen cells from a mouse immunized with sheep erythrocytes. Enrichments shown are the values for each fraction. H-PFC: — — — —; L-PFC: - - - -.

Results and Discussion. The sedimentation pattern of the mouse spleen suspension at 1*g* in a sucrose gradient is shown in Fig. 2. The bulk of the plaque-forming cells can be seen to sediment faster than the bulk of the non-plaque forming cells. Recoveries of total nucleated cells and plaque-forming cells are given for each fraction in Table I. Of the cells recovered, over 90% of all nucleated cells were found in fractions 1–3, whereas over 90% of the PFC's were found in fractions 4–7. In the fractions collected, recoveries were 79% of the original nucleated cells, 53% of high efficiency plaque-forming cells, and 74% of low efficiency plaque-forming cells. Enrichments of plaque-forming cells, ranging from 0.08 in fraction 3 to 18 in fraction 6, are shown in the upper portion of Fig. 2. Enrichments are calculated as the ratio of plaque-forming cells to total nucleated cells in a given fraction, divided by their ratio in the initial unfractionated suspension. There was no significant difference in the sedimentation rate of the high vs low efficiency plaque-forming cells.

The differential count for the nucleated cells in each fraction of the gradient is shown in Table I. Fraction 1 had no nucleated cells but contained platelet-like particles. Subsequent fractions were devoid of such particles. Fractions 2 and 3 (near the top of the gradient) contained mostly small lymphocytes. Fraction 4 contained most of the mature granulocytes. Because Fractions 4–7, which contained most of the PFC's, were morphologically heterogeneous, and because PFC's were at most 0.5% of the total nucleated cells, no correlation could be made between morphology and plaque-forming activity.

The plaques found in Fraction 1 (lightest fraction) were not associated with identifiable nucleated cells. They are complement dependent, do not appear on plates with human erythrocytes, are not abolished by penicillin, and are not coincident with bacterial colonies found after 24- and 48-hours incubation at 37°. Some of these plaques (in Fraction 1) had at their centers particles of about 3 μ diameter which, by phase-contrast microscopy appeared to be membrane-enclosed and similar to platelets. They thus appear to be due to antibody released from cell fragments similar to platelets. Hemolytic plaque formation by cell fragments has been reported by Friedman (8).

In preliminary experiments, when very low numbers of total nucleated cells were plated (5×10^4 to 5×10^5/plate), the number of PFC/10^6 nucleated cells was only 10–50% of that found when 5×10^6 or more nucleated cells were plated. This low recovery could be overcome by overlaying the immune cell layer during the initial incubation at 37° with a suspension of 3×10^7 normal spleen cells in

TABLE I. Total Nucleated Cells, Plaque-Forming Cells, and Differential Counts on Fractions from Sedimentation at 1*g* of Spleen Cells from a Mouse Immunized with Sheep Erythrocytes.

Cell type	Initial	Fraction 1	2	3	4	5	6	7
Total nucleated ($\times 10^{-6}$)	1370	17	388	487	73	20	9	6
H-PFC (cells/fraction)	21,400	347	600	854	4040	2590	1365	923
L-PFC (cells/fraction)	41,200	680	1173	1205	9900	7960	4851	2860
Granulocytes[a] (%)	2	0	0	1	23	8	3	2
Erythrocyte precursors (%)	9	0	8	2	11	7	9	15
Small lymphocytes (%)	83	0	91	92	46	51	49	54
Large lymphocytes (%)	5	0	1	5	19	33	37	29
Macrophages (%)	1	0	0	0	1	1	2	0

[a] Granulocytes: include all mature and immature myeloid cells; erythrocyte precursors: include all nucleated cells except pronormoplasts; large lymphocytes: include blasts and plasma cells.

Eagle's medium, or supernatant from such a suspension. Ingraham and Bussard (9) found no such losses over a 30-fold dilution range. However, their most dilute sample had 3×10^6 nucleated cells/ml, whereas our most concentrated samples on plating after sedimentation had 2×10^6 nucleated cells/ml.

Although the sedimentation technique employed in this study yielded enrichments of up to 18-fold, the maximal concentration of antisheep erythrocyte PFC's achieved was still less than 1%. However, many of the other cells in these enriched fractions may be producers of antibodies to other antigens and may differ only in the immunological specificity of their protein product. For this reason, the complete separation of cells producing antibodies to a given antigen can be expected to require the development of immunologic methods.

Summary. Spleen cells from mice immunized with sheep erythrocytes were sedimented at unit gravity in a sucrose gradient. Cells forming hemolytic antibody plaques sedimented more rapidly than the bulk of the nonplaque-forming cells, resulting in a partial separation with enrichments of up to 18-fold over their starting concentration.

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