decreasing its biosynthesis via a pathway that may not directly involve glucose as the precursor.

We are thankful to Dr. H. A. B. Parpia, Director of the Institute, for his interest in the work and to Mrs. Indira A. S. Murti for statistical examination of the data.

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Received Jan. 30, 1968. P.S.E.B.M., 1968, Vol. 128.

Collection of Rosette-Forming Spleen Cells by BPA Density Gradient Centrifugation* (33024)

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Studies on cellular or biochemical aspects of antibody formation are often difficult to interpret because of the low percentage of antibody producing cells among the cells of active lymph nodes or spleens. An attempt to enrich the population of antibody producing cells based on the physical properties of these cells has met with some success (1,2).

It has recently been shown that when spleen cells or lymph node cells (LNC) obtained from animals immunized with sheep red blood cells (SRBC) are mixed with SRBC they form rosettes (3,4) consisting of antibody producing cells and SRBC attached to them. These rosettes could be assumed to have a density intermediate between that of antibody producing cells and that of SRBC. Since bovine plasma albumin (BPA) density gradient centrifugation has been used successfully for separation of cells where differences in density might be involved (2,5,6) this approach was applied to the separation of antibody producing cells from suspensions of cells of active lymph nodes or spleens.

* This study was supported by Research Grant HE 04598 (AI) of the United States Public Health Service.

Materials and Methods. $C_{57}Bl/6$ male mice and Balb female mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. The mice were immunized intraperitoneally with 0.2 ml of 50% SRBC washed in PBS. Rabbits were immunized with 0.5 ml of 50% SRBC injected into each hind footpad, with an interval of 5–6 weeks between first and second injections.

Rosette test. Cells from spleens or popliteal lymph nodes of immunized mice or rabbits were washed once with Eagle's medium supplemented with 10% fetal calf serum (Microbiological Associates). The rosette test was performed in a final volume of 1.0 ml of Eagle's medium containing 20–30 million mouse spleen cells and 2% SRBC, or rabbit LNC with 0.5% SRBC. The cell mixture was incubated for 2 hours at 37°C without shaking

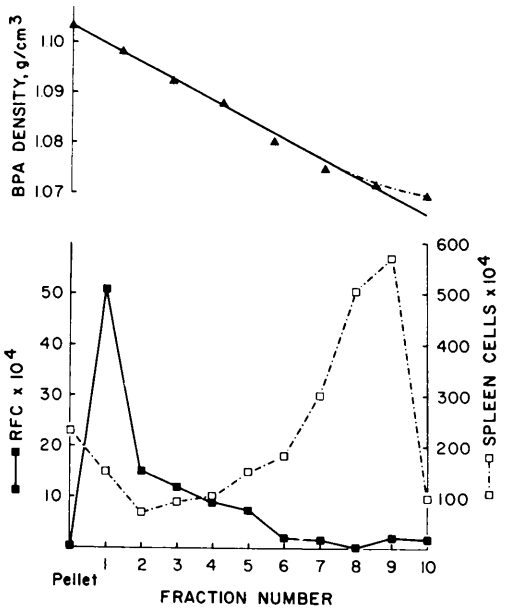

FIG. 1. Incubation mixture of SRBC and anti-SRBC spleen cells: Number of rosettes and of free spleen cells in fractions obtained by density gradient centrifugation in bovine plasma albumin.

and kept overnight at 4°C. The rosette count was done in a hemacytometer and expressed as the number of rosettes per 10^6 nucleated cells. The background level of rosettes was determined by using normal spleen cells or normal LNC.

Preparation of BPA. Bovine plasma albumin (fraction V) was obtained from the Armour Co. and the solution was deionized by the procedure described by Leif and Vinograd (7). The density of BPA obtained by this procedure is approximately 1.109 gm/cm^3 and the pH is 7.2. Solutions of lower density were prepared by diluting the original solution with balanced salt solution NKM (7) 1:1 (v/v). The density of this solution is approximately 1.06 gm/cm^3. Densities of BPA solutions were determined from their refractive indices, measured with a Bausch and Lomb refractometer at room temperature. The density of the BPA solution was calculated from the refractive index according to Leif and Vinograd (7).

Density gradient centrifugation. Linear BPA gradients were prepared in cellulose nitrate tubes (Beckman no. 302232) by mixing 2.5 ml of BPA of density 1.109 gm/cm^3 with 2.5 ml of BPA of density 1.06 gm/cm^3. The concentration of BPA increased linearly down the gradient, with some deviation from linearity at the top of the gradient (Fig. 1). One ml of a rosette preparation was layered on top of the BPA gradient and centrifuged at 5000 rpm for 90 min at 4°C in the SW25 rotor, using 6.5-ml adaptors in a spinco model L ultracentrifuge.

Collection of the fractions. Fractions were collected by piercing the base of the tube using an 18-gauge needle. The number of rosettes in each fraction was determined from rosette counts in a hemacytometer, and the total number of nucleated cells was determined after lysis of the erythrocytes present, by diluting the samples with 1.5% acetic acid.

Results. Production of rosettes. Rosette tests were conducted on cell suspensions obtained from C^{57}Bl/6 mouse spleens and rabbit lymph nodes. The number of rosettes formed was a function of the ratio between the number of LNC or spleen cells and the sheep red blood cells used in the test. In the mouse, the optimal conditions for rosette formation were 20–30 million spleen cells and 1–2% SRBC, in a final volume of 1.0 ml. When rabbit LNC were used the optimal conditions were 20–30 million LNC and 0.5% SRBC in a final volume of 1.0 ml. The cell suspensions were incubated at 37°C without shaking. The number of rosettes increased from 1700/10^6 LNC after 1 hour to 3000/10^6 LNC after 2 hours. When the cell suspension was then kept overnight at 4°C there was a further 2–3-fold increase in the number of rosettes. The number of rosettes varied in different preparations (Table I). Normal mouse spleens cells or normal rabbit LNC mixed with the same concentration of SRBC showed background counts of 0–320 and 0–128 rosettes/10^6 cells, respectively.

Control observations. The possibility that some of the rosettes might have been formed by antibody produced elsewhere and bound to the surface of the spleen cells or LNC was examined in four ways:

One source of antibody to which the cells could have been exposed was that in tissue

TABLE I. Range of Rosette-Forming Cells (RFC) in Mouse Spleen Cells and Rabbit LNC 4 Days after Injection of SRBC.

Species	Type of immunization	No. of expts.	No. of rosettes per 10^6 cells
Mouse	Primary	4	1000– 6000
	Secondary	13	5000–35,000[a]
Rabbit	Primary	15	2000–10,000
	Secondary	10	3000–25,000

[a] In 3 experiments not included in Table I, the number of RFC/10^6 spleen cells was 200,000; 130,000; and 60,000.

fluids in the spleen or lymph node. Normal $C_{57}Bl/6$ spleen cells were incubated in twofold dilutions of pooled $C_{57}Bl/6$ anti-SRBC serum of hemagglutination titer 1280. After 1 hour at 37°C the cells were washed 3 times with PBS and a rosette test was performed. As shown in Table II the number of rosettes did not increase above background level.

Another possible source of antibody was that produced *in vitro* by the entire cell suspension, in the course of incubation for rosette production. To test for adsorption of such antibody to inactive cells as a mechanism of rosette formation, spleen cells from mice obtained 5 days after a primary or secondary injection of SRBC were incubated at 20–30 million/ml at 37°C for 2 hours, and the cells were then removed by centrifugation. Normal $C_{57}Bl/6$ spleen cells were incubated with SRBC in supernatant fluid, as in the standard rosette test. After 2-hours incubation at 37°C and overnight at 4°C, no rosettes could be demonstrated above background level (Table II).

TABLE II. Effect of $C_{57}Bl/_6$ Anti-SRBC Serum on Rosette Formation by Normal $C_{57}Bl/_6$ Spleen Cells.

Dilution of serum[a]	Rosettes per 10^6 spleen cells
1:5	108
1:10	150
1:20	432
1:40	<50
1:80	108
1:160	<50
1:320	<50
Supernatant fluid[b]	108
Normal $C_{57}Bl/_6$ serum	216
Eagle's medium	160

[a] HA titer 1280.

[b] See text.

To test that the rosettes formed under these conditions reflected an active process by the spleen cells, these were incubated for 1 hour at 37° with sodium iodoacetate to inactivate enzymes involved in the glycolytic cycle. The cells were then washed twice with PBS and the rosette test was done. Table III shows a marked decrease in the number of rosettes in relation to the concentration of iodoacetate. (By interpolation a 50% reduction corresponded to $10^{-4.6}$ *M* iodoacetate).

TABLE III. Inhibition of Rosette Formation by Treatment of the Spleen Cells with Iodoacetate.

Sodium iodoacetate (*M*)	RFC per 10^6 cells[a]	% of control
—	5140	100
5×10^{-6}	4560	89
10^{-5}	3440	68
5×10^{-5}	3220	63
10^{-4}	2220	44
5×10^{-4}	1666	33
10^{-3}	666	13
5×10^{-3}	440	9

[a] Spleen cells from $C_{57}Bl/_6$ mice 4 days after secondary injection of SRBC.

In a final test of whether an active process by the cells was the basis of the rosettes observed, use was made of the observation that antibody formation can be specifically suppressed by exposure of antibody producing cells *in vitro* to appropriate antileukocyte sera produced in allogeneic animals (8). For rabbit LNC, 0.2 ml containing 8×10^6 popliteal LNC from SRBC-injected rabbits was incubated with 0.4 ml of rabbit antirabbit leucocyte serum at successive twofold dilutions. After 30 min at 37°C in the presence of complement, these cell suspensions were washed 3 times with PBS and a rosette test was performed. A markedly reduced number of rosettes could be demonstrated. The results are given as rosettes per 10^6 cells and in

TABLE IV. Suppression of Rosette Formation in Rabbit and Mouse Cells by Allogeneic Antileukocyte Sera.

Dilution of antiserum[a]	Rabbit lymph node cells[b]		Mouse spleen cells[c]	
	Rosette count per 10^6 LNC	% of control	Rosette count per 10^6 spleen cells	% of control
—	25,000	100	8750	100
1024	—	—	7812	89
512	15,000	61	6250	72
256	8125	33	5940	69
128	2500	10	625	7
64	2500	10	<100	<1
32	625	3	—	—

[a] Rabbit antileukocyte sera and mouse antispleen sera were used, respectively. The sera were prepared as described elsewhere (8,9).

[b] LNC were obtained from rabbits 4 days after secondary immunization with SRBC.

[c] Spleen cells were obtained from Balb mice 4 days after primary immunization with SRBC.

percentage reduction from the control level in Table IV. Similar results are shown in the Table for mouse spleen cells and mouse allogeneic antiserum.

Fractionation of incubation mixtures on BPA density gradient. A rosette preparation of spleen cells from immunized mice was layered on a BPA density gradient. After centrifugation for 90 min at 5000 rpm, the fractions obtained were examined for the number of rosettes and of nucleated cells. The percentage of rosette-forming cells (RFC) in each fraction was determined (Fig. 1). Some of the fractions contained up to 40% RFC (Table V). In a series of such experiments, the recovery of LNC from the BPA density gradient was 50–100% and the recovery of rosettes was 60–100%. About 40–50% of the rosettes were recovered in the first 3 fractions. The BPA had no apparent effect on the size or on the number of the rosettes being formed. Use of Ficoll or dextran of various molecular weights was not suitable for fractionation of RFC because it showed a tendency to clump RBC and free LNC or spleen cells.

Discussion. This study presents a method for isolation of rosette-forming cells with high yields. Using the fact that because of their size and density the rosettes appear, on centrifugation in BPA density gradient, in a zone between the free spleen cells and the free SRBC, fractions have been obtained with RFC up to 40% of spleen cells, from starting material of 0.5–3% (Table I).

An important question was whether the rosettes observed were produced by an active process of the cells or by antibody adsorbed to the surface of LNC or spleen cells. When normal mouse spleen cells were incubated with mouse anti-SRBC serum, or with medium in which antibody producing cells had been incubated, no increase in rosettes above background level was observed. Storb and Weiser (4), using guinea pig spleen cells and LNC, found a slight increase in rosette numbers after incubation of normal cells with anti-SRBC guinea pig serum. This difference might be due to the difference in species,

TABLE V. Enrichment of Rosette-Forming Cells (RFC) by Fractionation of Rosette Preparations on BPA Density Gradient.

Expt. no.	RFC/10^3 spleen cells in original preparation[a]	RFC/10^3 spleen cells in the most enriched fraction
1	23	250
2	23	210
3	23	230
4	23	340
5	36	370
6	30	378
7	7	500

[a] The spleen cells were obtained from $C_{57}Bl/_6$ mice 4 and 5 days after secondary immunization with SRBC.

since the background level of rosette formation by normal guinea pig cells observed by Storb and Weiser was higher than the background levels found with mouse or rabbit cells in this study.

Strong evidence that the rosettes are actively produced is provided by the "suppression" test. Harris and Harris (8) showed in previous studies that antileukocyte serum, on *in vitro* incubation with stimulated cells, can suppress antibody production. The suppression was expressed as reduction in titers of circulating antibody in recipients of transferred cells, or as reduction in number of hemolytic plaques. Using similar antileukocyte sera a reduction in the number of the rosettes to the background level was observed. It is interesting to note that when rabbit LNC were used in the rosette suppression test the decline in RFC with increasing concentration of the suppressive sera was more gradual than in the Balb spleen cells. The reason probably lies in the fact that the rabbit population is genetically heterogeneous, whereas in the mouse system used there is a single major antigenic difference between the strain of the antibody producing cells and the strain of mice in which the suppressive antiserum was produced. Additional evidence that the rosette formation requires active synthesis of antibody by the rosette-forming cells was given by the effect of iodoacetate in preventing rosette formation by such cells.

Summary. A method for fractionation of rosette-forming cells has been described. Incubation mixtures of SRBC with antibody producing mouse spleen cells in which rosettes had been formed were layered on BPA density gradients for centrifugation, with resulting enrichment of rosettes relative to the original cell suspension. Certain fractions consisted of up to 40% rosette-forming cells. The possibility that rosettes were formed passively under the conditions of this study was ruled out by a series of experiments. Production of rosettes did not result from incubation of normal spleen cells with mouse anti-SRBC serum or with medium in which antibody producing suspensions of cells had been incubated. The production of rosettes could be suppressed by prior incubation of the cells with iodoacetate or with antileukocyte or antispleen cell serum produced in allogeneic animals.

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Received Jan. 31, 1968. P.S.E.B.M., 1968, Vol. 128.