

A POPULATION OF LYMPHOCYTES BEARING A MEMBRANE
RECEPTOR FOR ANTIGEN-ANTIBODY-COMPLEMENT
COMPLEXES

I. SEPARATION AND CHARACTERIZATION*

BY CELSO BIANCO,‡ M.D., RICHARD PATRICK,§ PH.D.,
AND VICTOR NUSSENZWEIG,|| M.D.

*(From the Department of Pathology, New York University School of Medicine, New York
10016 and the Department of Pathology, Health Center, University of Connecticut,
Farmington, Connecticut 06032)*

(Received for publication 15 May 1970)

A few years ago, Uhr and Phillips made the interesting observation that antigen-antibody-complement (Ag-Ab-C) complexes bind to the surface of some, but not all, lymphocytes (1, 2). They reported that flagella-anti-flagella complexes adhere in vitro to the membranes of lymphocytes, and that measures which excluded participation of complement markedly diminished this interaction. The same phenomenon was observed in our laboratory in experiments which had been designed to study the nature and specificity of the binding of erythrocytes sensitized with antibody and complement (EAC)¹ to various types of leukocytes (3-5). This interaction leads to the formation of conspicuous clusters of red cells surrounding the leukocytes ("rosettes"), which can be counted under the microscope.

The observation that lymphocytes obtained from mouse thymus, as opposed to those obtained from other lymphoid organs, did not contain any cells capable of binding EAC (3) suggested that this membrane property could be used as a marker for the characterization of distinct subpopulations of lymphocytes. The results to be reported support this working hypothesis: lymphocytes which bind

* This investigation was supported by National Institutes of Health Grants AI08499 and AI08251

‡ Dr. Celso Bianco's work is supported by a training fellowship from the World Health Organization and from the Fundação de Amparo a Pesquisa de São Paulo, Brazil.

§ Dr. Richard Patrick was a research fellow of the Connecticut Heart Association. Present address: Department of Microbiology, University of South Dakota, Vermillion, S. Dak.

|| Career Investigator of the Health Research Council of the City of New York, Contract I558.

¹ *Abbreviations used in this paper:* A, Antibody; BSA, Bovine serum albumin; C, Complement; CRL, Complement receptor lymphocytes; E, Sheep red blood cells; EA, Erythrocytes sensitized with antibody; EAC, Erythrocytes sensitized with antibody and complement; PMN, polymorphonuclear leukocytes; VBS, Veronal-buffered saline.

EAC (which we provisionally call CRL [complement receptor lymphocytes]) have distinctive properties and a characteristic distribution in lymphoid organs.

Materials and Methods

Bovine serum albumin (BSA) (35% solution) was obtained from Pentex, Inc., Kankakee, Ill. Lots 45 and 46 were used. Sheep red blood cells (E) were obtained weekly from Behring Diagnostics, Woodbury, N.Y. and washed three times in 0.15 M NaCl solution (saline) before use. Amboceptor (Rabbit anti-sheep red blood cells antisera prepared against boiled stroma) was obtained from Certified Blood Donor Service, Inc., Jamaica, N.Y. Mouse complement was prepared as follows: CF1 mice (Carworth Laboratory Animals, New City, N.Y.) were anesthetized with ether; blood was collected by cutting the axillary vessels, allowed to clot at room temperature for 5 min, and centrifuged at 4°C. The serum was separated and frozen at -20°C. Complement was frequently used on the same day that it had been obtained. Guinea pig complement, lyophilized, and fetal calf serum, frozen, were obtained from the Grand Island Biological Co., Grand Island, N.Y. Burro red blood cells were bought from Animal Blood Center, Syracuse, N.Y. They were washed three times in saline before use. Sodium salt of heparin without preservative (Panheparin) was obtained from Abbott Laboratories, North Chicago, Ill. ADP, adenosine-5-diphosphate grade I, sodium salt, was purchased from Sigma Chemical Co., St. Louis, Mo. EDTA, ethylenediaminetetraacetic acid, disodium salt, was obtained from Matheson, Coleman, and Bell, Cincinnati, Ohio. It was dissolved in distilled water and the pH brought to 7.6 with concentrated NaOH. The concentration of the stock solution of Na₃HEDTA was 0.1 M. The osmolarity of a solution containing 0.01 M Na₃HEDTA in culture medium 199 was 285 milliosmol/kg as measured in a Fiske model H osmometer (Fiske Associates, Inc., Uxbridge, Mass.). Agarose, special grade, was purchased from Mann Research Labs, Inc., New York. Nylon wool was purchased as scrubbed nylon fiber (FT-242) from Fenwall Laboratories, Morton Grove, Ill. It was kept in distilled water, which was changed twice a day during 1 wk, and then dried in an incubator. Diluents and culture media used: veronal-buffered saline (VBS), at pH 7.4, was used for complement fixation; it contained 1.5×10^{-4} M Ca⁺⁺ and 5×10^{-4} M Mg⁺⁺. Gey's and Dulbecco's PBS were prepared as indicated in reference 6. Medium 199 was purchased from Microbiological Associates, Bethesda, Md., and 35 mg of sodium bicarbonate added per 100 ml of medium. RPMI 1640 medium was purchased from Grand Island Biological Company, Grand Island, N.Y.

Sensitization of Sheep Red Blood Cells (E) with Antibody (A) and Mouse Complement (C).—Equal volumes of a 5% suspension of washed E and a 1:500 dilution of amboceptor in saline were incubated at 37°C for 30 min. The sensitized cells (EA) were washed and a 5% suspension prepared in VBS. Equal volumes of 5% EA and 1/10 or 1/20 dilutions of mouse complement were then incubated at 37°C for 30 min. Under these conditions only a few of the red cells were lysed and after three washings in VBS a final suspension of EAC was made in medium 199. Suspensions of E and EA were prepared in a similar way.

Preparation of EAC4 and EAC43.—These intermediates were prepared by using purified human complement components, as follows: (a) C1q, C1r, C1s, C2 and C3. These components were prepared from normal human serum as previously described (7). (b) EAC4. This intermediate was prepared by modification of the method of Borsos and Rapp (8, 9). Packed cells were obtained from 10 ml of 5×10^8 cells/ml of optimally sensitized sheep erythrocytes. 0.5 ml each of C1q, C1r, and C1s diluted 1:10 in VBS Ca⁺⁺ (VBS containing 10^{-3} M Ca⁺⁺) was added to the cell button and incubated at 37°C for 15 min. The resulting EAC1 were washed once with warm (37°C) VBS Ca⁺⁺ and resuspended to 10 ml in the same buffer. 0.5 ml of EDTA serum (normal human serum diluted 1:20 in VBS without cations, containing 0.02 M Na₃HEDTA, incubated for 15 min at 37°C and made fresh for each EAC4 preparation) was mixed with the 10 ml of EAC1 and incubated at 32°C for 10 min. The cells were then washed

twice with cold EDTA buffer and twice with cold VBS. The resulting EAC4 cells were strongly positive when tested with an R4 and negative when assayed by immune adherence. (c) EAC43. This intermediate was obtained by washing EAC1423 with EDTA buffer and allowing functional C2 to decay. EAC14 was prepared from EAC4 and C1q, C1r, and C1s exactly as described above for EAC1. EAC1423 was prepared by reacting equal volumes of EAC14 (5×10^3 cells/ml) and mixtures of purified C2 and C3 for 5 min at 37°C. Approximate 20 and 250 μg of C2 and C3 respectively were used per 5×10^8 EAC14. The EAC1423 intermediate was strongly lytic with chelated complement and the subsequent EAC43 was strongly positive when tested in immune adherence.

Human Peripheral Blood Lymphocytes.—Prepared as in reference 10. Venous blood was collected in heparin, allowed to sediment for 2 hr and the plasma separated. ADP was added and the resulting platelet clumps removed by quick filtration through a small amount of nylon wool. The polymorphonuclear leukocytes (PMN) and monocytes were removed by passing the plasma through a column packed with 1.3 g of nylon wool/10 ml of plasma, maintained at 37°C. The cells were then washed twice with medium 199 in a cold centrifuge (200 g) and kept in an ice bath until used. The resulting suspensions contained more than 90% of mononuclear cells.

Cell Suspensions from Lymphoid Tissues.—All operations were performed with cells suspended in culture medium 199 or RPMI. The organs were teased with a pair of forceps in culture medium. The suspension was allowed to sediment for 5 min, and the supernatant collected with a Pasteur pipette. The cells were washed twice by centrifugation at 200 g in a cold centrifuge, resuspended in culture medium 199 or RPMI, and kept in an ice bath until used. The following organs were used as a source of lymphocytes: mouse (CF1 strain)—cervical, axillary, inguinal, and mesenteric lymph nodes, spleen and thymus; rabbit—popliteal lymph nodes; guinea pig (Hartley strain)—axillary and inguinal lymph nodes; rat (Sprague-Dawley)—axillary and inguinal lymph nodes; chicken ($H_1 \times H_{36}$ white Leghorn)—spleen. Bone marrow cells were obtained as follows: femurs of a mouse were freed from the surrounding tissue and the epiphysis cut off with a scalpel. The marrow cells were removed by injecting culture medium through a 26 gauge needle introduced into one of the extremities of the bone. The cells were washed twice and kept in an ice bath.

Standard Assay for Detecting Lymphocytes (CRL) which Bind EAC.—Performed as described by Lay and Nussenzweig (3). Disposable plastic tubes with caps, originally 12 \times 75 mm were cut 12 mm above the bottom in order to hold 1 ml of liquid and allow for only a small air bubble when capped. Such tubes were filled with the mixture of lymphocytes and appropriate red cell suspension and sealed with paraffin. The final concentrations used were 10^6 lymphocytes/ml and 5×10^7 red cells/ml. The tubes were placed in a submersion rotator with horizontal axis and rotated at 20 rpm in a 37°C water bath for 30 min. The tubes were then taken out and placed in an ice bath. Before counting, one tube at a time was placed in a rotator for another 5 min at 20 rpm, at room temperature. Total leukocytes and rosettes were counted separately. A sample of the suspension was distributed in four hemocytometer chambers for the rosette counting. For the leukocyte counting 0.1 ml of a solution of 10% acetic acid and 1% gentian violet in distilled water was added to 0.5 ml of the sample to lyse the red cells and hemocytometer chambers were loaded with the final suspension. Four entire chambers (36 squares — 3.6 mm^3) were scored for rosettes and two chambers (8 squares — 0.8 mm^3) for the white cells. In this way, approximately 600 white cells and several hundred rosettes were counted per tube. The results were expressed as percentage of rosettes in relation to the total number of nucleated cells. The evaluation of the number of rosettes in a given sample is quite accurate. For example, when nine different tubes of the same preparation of mouse lymphocytes and EAC were assayed, the mean was 22.8 and the standard error of the mean was 0.70. During the counting procedure the tubes were left in an ice bath. Up to 4 hr, there was no significant change in the number of rosettes observed. Normal mouse serum or fetal calf serum up to a concentration of

20% could be added to the incubation medium without influencing the results of the assay. In some experiments the standard assay was performed in presence of Na_3HEDTA 0.01 M, in order to inhibit the binding of EAC to PMN cells, monocytes, or macrophages (3, 11).

Adherence of Lymphoid Cells to Nylon Wool.—Lymphocytes were collected from the lymph nodes of three CF1 mice, pooled, washed, and resuspended in medium 199 containing 20% normal CF1 mouse serum. The cell concentration was $2 \times 10^7/\text{ml}$ and 2.5 ml of cell suspension was added to syringes containing prewarmed nylon wool. The amount of nylon wool contained in each syringe (400 mg) was packed in such a way as to absorb all the medium which was added to it. After incubation at 37°C for 30 min, 2.5 ml preheated medium was added to the column, the eluate collected, and cooled in ice. Then the nylon wool was slowly compressed with the piston and a few more cells were collected. The mixture of cells was counted, and after the necessary adjustment in their concentration, the standard assay was performed. Lymphocytes from the thymus, obtained from the same mice, and treated similarly, were filtered simultaneously through other nylon wool columns.

The Labeling of Short- and Long-Lived Lymphocytes.—Performed according to reference 12. CF1 mice were injected intraperitoneally with 0.75 μCi ^3H -thymidine (New England Nuclear Corp., Boston, Mass., 2 Ci/mole) per gram of body weight every 6 hr for 5 days. Using this schedule, short-lived lymphocytes were labeled as well as the long-lived cells which divided during the period of injection. The animals were killed 6 hr after the last injection. For labeling of long-lived lymphocytes, mice were injected with 0.75 $\mu\text{Ci/g}$ body weight of ^3H -thymidine daily for 17 days. The mice were killed 17 days after the last injection. The standard assay for CRL was performed with the lymphoid cells of their peripheral lymph nodes. The tubes were then centrifuged for 5 min at 100 g, and the supernates discarded. The cells were resuspended in two or three drops of fetal calf serum by gentle agitation, smears prepared and fixed with methanol for 10 min. For radioautographs the slides were dipped in NTB3 Kodak Nuclear Track Emulsion (Eastman Kodak Co., Rochester, N.Y.) diluted 1:3 in water and exposed for 2–3 wk. They were developed with Kodak D-19 developer, fixed with Kodak fixer, and stained with Giemsa (2 ml) diluted in acetone (3 ml), phosphate buffer 0.07 M, pH 6.5 (2 ml) and distilled water (31 ml). Each slide was examined twice under the microscope ($\times 1250$). The first time every lymphocyte was counted and scored for the number of grains. Then the same slide was again examined and only CRL (rosettes) were scored for the number of grains. Both experiments (labeling long-lived and short-lived lymphocytes) were performed twice.

Cell Separation by Differential Flotation in Albumin Gradients.—Separation of spleen cells and of lymph node cells were performed as proposed by Raidt et al. (13). The cells were washed once in medium 199. After centrifugation they were resuspended in 33% BSA, placed in the bottom of a 5 ml cellulose nitrate tube, and overlaid with 1 ml quantities of 29, 26, 23, and 10% BSA. Centrifugation was carried out at 4°C for 30 min at 15,000 g in a SW39 head of a Spinco ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.).

Depletion of CRL by Differential Centrifugation of Rosettes.—The separation of lymphocytes with receptor for EAC from the whole cell population was achieved by combining rosette formation with a differential centrifugation method, in view of the fact that rosettes have a higher density than free lymphocytes.

The procedure used for this separation is summarized in Fig. 1. 5×10^7 lymphocytes were mixed with 2×10^9 EAC (experimental tube) or E (control tube) to a final volume of 1 ml in medium 199. The suspension was incubated for 30 min at 37°C with constant agitation, as was done for the standard assay. After the incubation, the experimental tubes contained a mixture of rosettes (CRL), free lymphocytes, and free EAC. The control tubes contained only free lymphocytes and E. Each suspension was carefully deposited on the top of a 5 ml cellulose nitrate tube containing 1.5 ml of BSA 33% overlaid with 1.5 ml of BSA 23%. The tubes were then placed in an SW39 head and centrifuged as above.

After the centrifugation, the red cells as well as the rosettes were found in the pellet, and the

free lymphocytes were collected from the upper layers. Cell counts and the standard assay for CRL were performed before and after the centrifugation.

Assay for Detection of Plaque-Forming Cells (PFC).—The direct hemolytic-plaque assay of Jerne as modified by Dresser (6) was used. The number of cells plated was corrected for the number of viable cells after a trypan blue exclusion test. The statistical analysis of the results was made as suggested by Dresser and the results were expressed as the geometric mean of the number of plaques observed.

Demonstration of Immunoglobulin (Ig) Determinants on Lymphocytes by Cytotoxic Tests. The antiserum used was prepared by immunizing rabbits with specifically purified mouse anti-dinitrophenyl antibodies. It contained precipitating antibodies directed against mouse immunoglobulin H chains (γ_1 [++], γ_{2A} [++], γ_{2B} [+], γ_A [±]), and L (K) chains (±) as shown by specific precipitation in agar gel. The gamma globulin fraction of this antiserum was separated by chromatography on DEAE-cellulose after equilibration with 0.02 M phosphate buffer, pH 7.6. The eluted gamma globulin fraction was then dialyzed against saline and kept frozen (-20°C) until used. The mouse spleen cells ($4 \times 10^6/\text{ml}$) used for these tests were always suspended in RPMI medium. Complement was absorbed prior to use with mouse spleen

TABLE I
Binding of EA after Interaction with Purified Human Complement Components to Human Peripheral Blood Lymphocytes

Red cell intermediate	Rosettes*
	%
EAC4	2.2 ± 0.6
EAC43	18.1 ± 6.6

* Mean of three separate experiments (\pm SE).

cells as follows. Fresh guinea pig serum was incubated for 30 min at room temperature with $\frac{1}{10}$ of its volume of washed spleen cells. The mixture was centrifuged in the cold at 600 g for 15 min and the supernatant used as a source of C on the same day. The cytotoxic test was performed exactly as indicated in reference 14. The results were expressed as percentage of cells taking up trypan blue in experimental tubes corrected for the percentage of stained cells in control tubes. The control tubes contained cells and complement but no antiserum and never showed more than 6% stained cells. Several preliminary experiments were performed in order to determine the optimum concentrations of the reagents employed in the test, as suggested in reference 14. The antiserum was always used in great excess.

RESULTS

Complement Components Involved in the Binding of EAC to Lymphoid Cells.—In order to determine the complement components necessary for the binding of EAC to lymphocytes, EAC4 and EAC43 were prepared (using human purified complement components) and incubated with lymphoid cells from human peripheral blood. After the usual incubation at 37°C for 30 min with agitation, the percentage of rosettes in the two preparations was determined. The results shown in Table I demonstrated that fixation of C3 to the sensitized red cell is necessary for its binding to the lymphocyte membrane. In addition, other ex-

periments² showed that the sensitizing activity for rosette-formation of normal mouse serum is completely destroyed by the addition of purified cobra-venom fraction which is known to inactivate C3.

In the experiments reported above, the lymphoid cells and the complement originated from the same species. However, EAC prepared with either human or mouse complement were found to bind also to heterologous lymphoid cells (Table II). It can be seen that EAC prepared with mouse serum as a source of complement adhered to lymphocytes from rat, rabbit, man, and mouse. Also, EAC43 prepared with human complement components adhered to rabbit, guinea pig, and human lymphocytes. Interestingly, although mouse complement

TABLE II
Binding of EAC Prepared with Human and Mouse Complement to Lymphocytes of Different Animal Species

Source of lymphocytes	Binding observed with complement from	
	Man*	Mouse†
Human peripheral blood	+	+
Rabbit lymph nodes	+	+
Guinea pig lymph nodes	+	+
Rat lymph nodes	n.d.	+
Mouse lymph nodes	—	+
Chicken spleen	n.d.	—

* EAC 43, prepared as described in the Materials and Methods section, was used. Controls incubated with EAC4 never showed any significant binding.

† EAC was prepared with fresh mouse serum as a source of complement. E and EA cells were used as controls. All experiments were performed at the same time with a single preparation of sensitized red cells and lymphocytes obtained from different animal species. The percentages of rosettes observed in the different groups varied from 10–25%.

sensitized EA to bind to human lymphocytes, the reverse was never found to be true.

Specific Elimination of CRL from a Population of Lymphoid Cells.—Lymphoid cells can be specifically depleted of CRL by centrifugation in an albumin gradient. The method used takes advantage of the higher density of rosettes (formed by CRL and red cells bound to their membranes) as compared to the density of free lymphocytes. The procedure for depletion is schematically represented in Fig. 1. After centrifugation, a fraction of the cells recovered from the supernatant was reincubated with EAC in order to assess the number of remaining CRL. Results of several experiments performed with lymphocytes from peripheral lymph nodes of mice are summarized in Figs. 2 and 3. Fig. 2 demonstrates that lymphoid cells treated with EAC and ultracentrifuged contain a

² Lay, W.H., and V. Nussenzweig. Unpublished observation.

significantly smaller proportion of CRL than the controls. Fig. 3 shows that, in addition, a large proportion (78%) of non-CRL in the experimental tubes are recovered after ultracentrifugation, while the majority of CRL are lost.

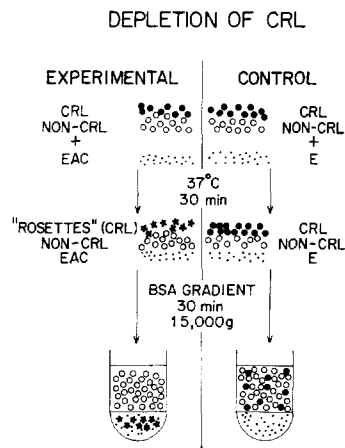


FIG. 1. Schematic representation of the method used for depletion of CRL (explanation in the text).

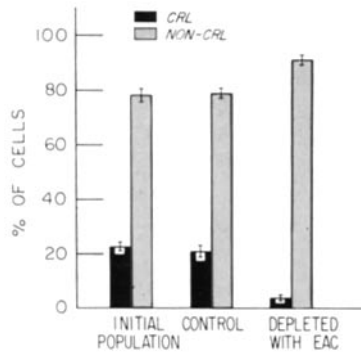


FIG. 2. Depletion of CRL from lymphocytes obtained from the peripheral lymph nodes of CF1 mice. The bars show the percentages of CRL and non-CRL before (initial population) and after ultracentrifugation in the presence of E (control) or EAC (experimental). Means of seven experiments \pm standard error are presented.

Properties of CRL and non-CRL.—CRL and non-CRL have other distinctive properties as will be shown in the experiments below.

Density: Mouse peripheral lymph node lymphocytes, which contained 16% CRL, were used in these studies. The distribution of CRL and non-CRL after flotation of these cells in a discontinuous BSA gradient is shown in Fig. 4.

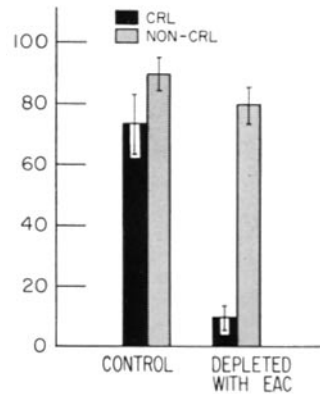


FIG. 3. Depletion of CRL from lymphocytes obtained from the peripheral lymph nodes of CF1 mice. The bars show the final recoveries of CRL and non-CRL after specific depletion. Means \pm standard error were calculated from seven experiments. The recoveries of CRL (and non-CRL) were calculated as follows:

$$\% \text{ of CRL recovered} = \frac{\text{number of CRL recovered}}{\text{number of CRL loaded}} \times 100$$

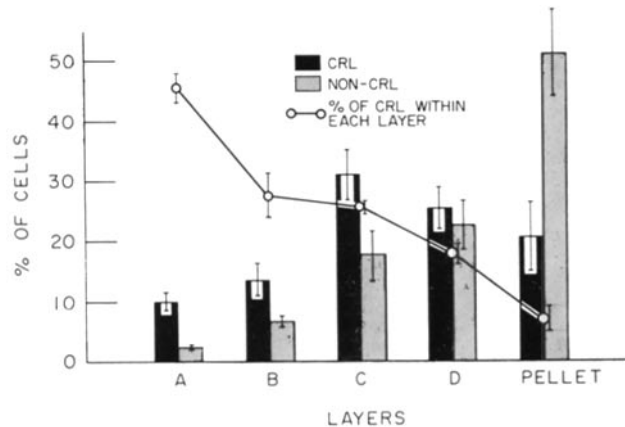


FIG. 4. BSA density gradient ultracentrifugation of cells from mouse peripheral lymph nodes. The proportion of CRL, determined by the standard assay before ultracentrifugation, was 16.1 ± 1.5 . After ultracentrifugation the standard assay was repeated with cells obtained from each layer. The percentage of CRL (or non-CRL) recovered in each layer was calculated as follows:

$$\% \text{ of CRL} = \frac{\text{number of CRL found in the layer}}{\text{number of CRL loaded}} \times 100$$

The values for the pellet were calculated by difference. Results of four experiments (\pm mean standard error) are represented by the bars. The percentage of cells within each layer of the gradient found to be CRL by the standard assay is represented by a continuous line (O—O). The concentration of BSA in each layer was: A-10%, B-23%, C-26%, D-29%, bottom-33%.

All layers of the gradient contained both CRL and non-CRL. In absolute numbers the majority of both CRL and non-CRL were recovered from the densest layers of the gradient. However, CRL were relatively enriched in the lighter layers of the gradient. In the top layer almost 50% of the cells were CRL. The possibility that this result was due to the presence of contaminating macrophages in the lighter layers could be excluded because: (a) almost all cells from this layer were morphologically undistinguishable from lymphocytes; (b) only 5% of the cells took up neutral red in vitro; (c) the number of CRL found in the

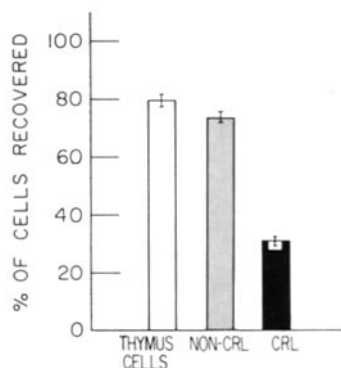


FIG. 5. Recoveries of mouse thymus cells and mouse lymph node cells after passage through nylon wool columns. The total numbers of CRL and non-CRL cells in the lymph node cell population were determined by the standard assay before and after passage through the columns. Percentages of recovery of CRL (or non-CRL) were obtained as follows:

$$\% \text{ of CRL recovered} = \frac{\text{number of CRL recovered}}{\text{number of CRL loaded}} \times 100$$

Bars represent the results of four experiments (means \pm standard error). The differences in recovery between thymus cells or non-CRL and CRL are statistically significant ($P < 0.05$). The difference in recovery between thymus cells and non-CRL is not significant.

top layers was not altered when the standard assay was performed in the presence of 0.01 M EDTA (the binding of EAC to polymorphonuclear leukocytes, monocytes, and macrophages takes place only in the presence of Mg^{++} ions (3, 11).

Adherence to nylon wool: The following experiments established that CRL and non-CRL differ widely in their capacity to bind to nylon wool. This was demonstrated by passing mouse lymphocytes through standard columns containing nylon wool and studying the recovery of each type of cell. The results (Fig. 5) can be summarized as follows: (a) While 28% of cells among mouse lymphocytes from peripheral lymph nodes were identified as CRL before filtration through nylon wool columns, only 6% were detected among cells in the

effluent. (b) A significantly lesser proportion of CRL ($30.7\% \pm 3.1$) than of non-CRL (73.9 ± 1.4) was recovered after passage through the columns. (c) Thymus cells, which do not have a receptor for EAC, behave as non-CRL when passed through similar columns. The recovery of thymus cells is significantly higher than the recovery of lymph node cells after filtration through nylon wool.

Presence of immunoglobulin determinants on the membrane of CRL: It has been recently reported (15, 16) that a certain proportion of mouse lymphoid cells display immunoglobulin (Ig) determinants on their membranes; this was determined by the use of immunofluorescence and immunoradioautography. The reported distribution of these cells in the various tissues of normal mice (15) coincides with the distribution of CRL (see results of following section).

TABLE III
*Simultaneous Loss from Mouse Spleen of CRL and of Cells Bearing Ig Determinants on their Membranes after Specific Depletion of CRL**

Experiment No.	Depleted			Control		
	Cells recovered	CRL	Cells bearing Ig	Cells recovered	CRL	Cells bearing Ig
	Cells loaded $\times 100$			Cells loaded $\times 100$		
		%	%		%	%
1	n.d.	3.4	9.5	n.d.	26.0	20.3
2	67.7	6.0	10.1	95.0	36.5	30.3
3	61.0	7.5	12.7	100.0	30.6	26.9
4	65.0	2.6	7.9	99.0	22.2	24.0
Mean	64.6	4.9	10.1	98.0	28.8	25.4
\pm SE	± 1.9	± 1.1	± 1.0	± 1.5	± 3.1	± 2.1

* The depleted population was preincubated with EAC while controls were preincubated with E.

The number of lymphocytes bearing Ig determinants on their surface is approximately 40% in the spleen, 20% in the peripheral lymph nodes, 15% in the thoracic duct; they are conspicuously absent in the thymus. For this reason we decided to determine whether the same subpopulation of lymphocytes actually bears both Ig determinants and a C3-dependent receptor on their membranes. The experimental approach used was to study in which way the specific depletion of CRL from a population of lymphocytes would affect the number of Ig-bearing cells (before and after depletion of CRL the number of cells bearing Ig determinants was measured by a cytotoxic test using a rabbit anti-mouse Ig antiserum). The results showed a simultaneous loss of CRL and of cells bearing Ig determinants on their membranes (Table III). Before depletion, the spleen-cell suspension contained 29% of CRL and 25% were killed by the anti-Ig antiserum; only 5% of CRL and 10% of Ig-containing cells were found among the

CRL-depleted population. After depletion, the recovery was 65% and the losses could be almost entirely accounted for by the elimination of CRL.

In control tubes, where lymphoid cells were treated with E instead of EAC, the remaining population showed no significant decrease in the number of CRL or Ig-containing cells, and the recovery from the gradient was almost total.

These results indicate that the CRL population must contain most if not all of the Ig-containing cells; the lack of an absolute coincidence between the numbers of cells which were shown to bear these two distinct markers might be due to different sensitivities of the detection assays.

Distribution of CRL and non-CRL among long- and short-lived lymphocytes:

It is known that lymphocytes can be divided into two populations according to their life span, and that they can be distinguished from each other after

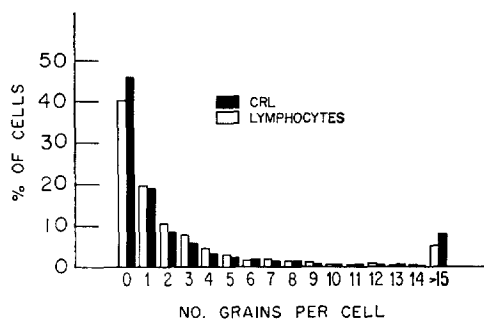


FIG. 6. Distribution of grains in CRL and in the total population of mouse lymph node lymphocytes after a series of ^3H -thymidine injections intended to selectively label long lived lymphocytes. Grains from a total of 5000 rosettes and 5000 lymphocytes were counted.

in vivo labeling with ^3H -thymidine (17). Studies were performed in order to determine whether CRL would be more frequently found among long-lived or short-lived cells. The distribution of grains in CRL and in the total population of lymph node lymphocytes of mice injected with ^3H -thymidine *in vivo* is shown in Figs. 6 and 7. No significant differences were found. Thus, CRL can be found among both short- and long-lived lymphocytes.

Studies on the Distribution of CRL Among the Lymphoid Organs of Mice.—

Several hundreds of standard assays for the detection of CRL were performed with lymphocytes obtained from different mouse organs. Our observations can be summarized as follows. (a) Thymus: No CRL were ever detected in thymus cell suspensions from young adult mice. (b) Lymph nodes and spleen: There is a great variability in the proportion of CRL (10–25%) found among lymphocytes from peripheral and mesenteric lymph nodes. It is not known whether this is due to some undetected cause of variation in the sensitivity of the standard assay or if it reflects physiological changes in individual animals.

More CRL are certainly found among nucleated spleen cells than among lymph-node lymphocytes. For example, in a series of simultaneous tests performed with lymph node and spleen cells from the same 10 mice, they contained respectively $13.9\% \pm 2.0$ and $22.2\% \pm 2.0$ of CRL. It is not unusual to find spleens with 40–50% of CRL. This higher frequency is not due to the binding of EAC to spleen macrophages or polymorphonuclear cells, because similar results are obtained when the standard assay is performed in the presence of 0.01 M EDTA. (c) Thoracic duct: 20% of CRL were found among lymphoid cells obtained from the thoracic duct of one mouse. The standard assay was performed on the pooled cells obtained after 24 hr of continuous drainage. Also, from 10–20% of CRL were detected among thoracic duct cells of several rats drained from 1 or 2 days. (d) Bone marrow: Mononuclear cells, morphologically indistinguishable from lymphocytes, and which bind EAC in the

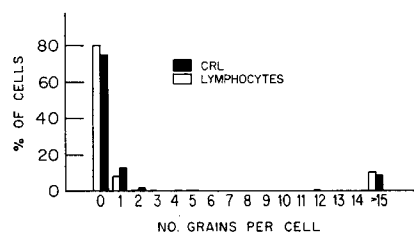


FIG. 7. Distribution of grains in CRL and in the total population of mouse lymph node lymphocytes after a series of ^3H -thymidine injections intended to selectively label short lived lymphocytes. Grains from a total of 2000 rosettes and 2000 lymphocytes were counted.

presence of 0.01 M EDTA, were found among mouse bone marrow cells. These cells comprise approximately 5–8% of the population of mononuclear cells of the bone marrow.

Absence of CRL Among Certain Antibody-Producing Cells.—The question of whether the membrane receptor for EAC is present on cells actively producing antibody was studied by comparing the number of antibody-forming cells among populations of spleen cells before and after specific depletion of CRL.

Mice were immunized by intravenous injection of 10^9 burro red cells. 3–4 days later they were killed, their spleen cells collected and washed with medium 199. Spleen cells from three donors were mixed with EAC (experimental) or E (controls), and specifically depleted of CRL. After the BSA gradient ultracentrifugation, the lymphoid cells recovered from the supernatant were washed, counted, and incubated in Jerne plates for the enumeration of cells producing 19S antibodies against burro red cells. The results are shown in Table IV.

In two out of three experiments there was a statistically significant increase in the proportion of plaque-forming cells after depletion of CRL. It is pertinent

to point out that in every case, the total number of plaque-forming cells recovered in experimental and control tubes was almost identical. Thus, the relative enrichment of PFC in the CRL-depleted population suggests the absence of receptors for EAC among the antibody-producing cells.

TABLE IV
Direct PFC Among CRL-Depleted and Nondepleted Populations of Spleen Cells of Mice Immunized with Burro Red Blood Cells

Experiment	CRL Depleted			Nondepleted		
	CRL	Mean PFC/10 ⁶	S ²	CRL	Mean PFC/10 ⁶	S ²
	%		log	%		log
1	6	918.0 ^(a)	0.005116	19.1	575.0 ^(b)	0.018706
2	6	832.7 ^(c)	0.007146	39.5	741.4 ^(d)	0.006167
3	5.8	734.3 ^(e)	0.009076	23.9	541.0 ^(f)	0.007485

Student's test: (a) × (b) significant $P < 0.05$; (c) × (d) nonsignificant $P > 0.05$; (e) × (f) significant $P < 0.02$.

DISCUSSION

The results obtained demonstrate that two distinct subpopulations of lymphoid cells can be identified and actually physically separated on the basis of differences in their membrane properties. The basic observation is that some lymphocytes (CRL) bind antigen-antibody-complement complexes (Ag-Ab-C) to their membrane, and others do not (non-CRL). Most of the experimental data have been obtained by measuring the interaction between lymphocytes and red cells sensitized with antibody and complement, which may lead to the formation of rosettes. However, other Ag-Ab-C complexes, of quite a different nature, bind to the same population of cells, strongly suggesting that the receptor on the lymphocyte membrane actually interacts with some component of the complex rather than with some secondary modification of the red cell membrane. For example, aggregated, γ -globulin after it has fixed complement, binds to CRL and inhibits the binding of EAC to the same cells.³ In addition Uhr and Phillips (1, 2) showed that some guinea pig lymphoid cells bind, to their membranes, complexes formed of flagellar antigens, antibodies, and complement.

The interaction between EAC and CRL only occurs after the binding of C3. The presence of C1 and C2 is not necessary, as EAC43 bind effectively to lymphocytes. Moreover, it appears that the fixation of complement components subsequent to C3 may be irrelevant. Fresh serum from strains of mice deficient in C5 or from rabbits deficient in C6 is as good as normal serum when used as a source of complement to sensitize EA for rosette formation (3). It would be

³ Bianco, C., and V. Nussenzweig. Manuscript in preparation.

of interest to determine whether the receptor on the lymphocyte membrane interacts with the modified C components and/or with modified antibody in the complex. The fact that the EAC-CRL interaction occurs as effectively in the presence of 20% normal serum as in culture medium 199 suggests that the normal serum and complement constituents as such do not compete effectively for the same sites.

Although CRL and non-CRL were defined operationally, there are a number of reasons to believe that they constitute distinct populations of lymphoid cells.

1. CRL bind preferentially to nylon wool in the presence of mouse serum at 37°C. When a mixture of lymph node cells, containing both CRL and non-CRL, is passed through a nylon wool column, the effluent population is depleted of CRL. In contrast, lymphocytes from the thymus, which do not contain CRL (3), behave as non-CRL when filtered through similar columns.

Although CRL share the property of binding to nylon wool with macrophages, these cells can be distinguished from each other because: (a) CRL have the morphologic characteristics of lymphocytes. The morphology of CRL can be studied by examining either cells at the center of rosettes, or populations of cells very much enriched in CRL, as for example the cells obtained in the lightest layer of a BSA density gradient ultracentrifugation (see Results). (b) CRL are found in very large numbers among cells from different lymphoid organs. For example, up to 50% of cells from the mouse spleen and 20% of cells from the thoracic duct of the mouse may be CRL. (c) CRL do not passively bind cytophilic antibody to their membrane as do macrophages and monocytes (18-20). (d) CRL do not take up neutral red in vitro. (e) Although macrophages, monocytes, and PMN share with CRL the property of binding EAC in vitro, in the case of CRL this binding may take place in the presence of EDTA. The interaction of EAC with the other cells is Mg^{++} -dependent, and completely inhibited by EDTA (3, 11).

2. CRL and non-CRL can also be distinguished by their relative distribution following ultracentrifugation in a discontinuous BSA density gradient. A significantly higher proportion of CRL are found among the lightest layers of the gradient.

3. As previously mentioned, a subpopulation of lymphocytes which bears Ig determinants on their membranes has been identified among cells from mouse lymphoid organs. It appears that these cells either coincide with the CRL subpopulation or overlap extensively with it because (a) of the simultaneous diminution of CRL and of Ig-bearing cells after specifically depleting a population of lymphoid cells of CRL, and (b) of the coincidence in the reported distributions of CRL and of Ig-bearing cells in lymphoid organs.

4. The distribution of CRL and non-CRL is quite different among lymphocytes obtained from various lymphoid organs and tissues. While CRL are not

found in the thymus of 6-8 wk-old mice, they constitute 20-50% of the cells obtained from their spleens. A smaller proportion of CRL are found among lymphocytes obtained from the peripheral lymph nodes (10-25%) or from the thoracic duct (10-20%).

5. The membrane receptor for EAC was not found among plaque-forming cells from the spleen of mice immunized with heterologous red cells. This would imply that either (a) CRL are not the precursors of 19S antibody-secreting cells, or (b) that the C3-dependent receptor is lost during differentiation of lymphocytes into plasmocytes. It is pertinent to point out that cells actively producing immunoglobulins lack several membrane markers present on other lymphoid cells. For instance, Ig determinants were not demonstrated on the membranes of cells from plasmacytomas (16). Also, Takahashi et al. (21) recently showed that mouse spleen cells actively producing antibody lack surface antigens θ , Ly-A, and Ly-B, which are present in different proportions among lymphocytes. It certainly appears possible that some of these markers are lost when lymphocytes transform into cells specialized in the production of immunoglobulins.

6. Finally, we have recently found that the pattern of localization of CRL in whole lymphoid organs is not random.⁴ CRL, conspicuously absent from the thymus, can be identified in circumscribed regions of the spleen, lymph nodes, and Peyer's patches of the mouse. In lymph nodes, for example, they are found only within the cortical follicles, but not in the paracortical area. In the spleen, CRL are confined to the follicular regions of the white pulp and the surrounding marginal zone, but are not found in the periarteriolar lymphocyte sheaths.

In summary, the data presented demonstrate that CRL and non-CRL are separate subpopulations of lymphocytes. The relationship between these two types of cells and the so-called thymus- and bone marrow-derived lymphocytes (22-25) is presently under investigation. It appears possible that CRL actually constitute the bone marrow-derived thymus independent lymphocytes for the following reasons: (a) CRL are absent from the thymus and constitute only 10-20% of thoracic duct lymphocytes. (b) CRL are found in lymphoid organs in areas which Parrot et al. (26) showed to contain most of the thymus independent lymphocytes.

It is reasonable to assume that the presence of a complement-dependent receptor on the surface of some lymphocytes is related to their function. The ability of these cells to bind Ag-Ab-C onto their membrane suggests that they may play a role in antigen localization, which is currently considered to be an exclusive function of macrophages or reticular dendritic cells. It is interesting in this respect that CRL, as well as macrophages, bind to nylon wool, and it

⁴ Dukor, P., C. Bianco, and V. Nussenzweig. *Proc. Nat. Acad. Sci. U.S.A.* In press.

may well be that some of the functions ascribed to surface adherent cell populations are actually performed by CRL.

While most investigators agree that antigen localization *in vivo* can take place in phagocytic cells, attention has been focused more recently on the accumulation of antigen within certain structures of the lymphoid organs, *i.e.*, the lymphoid follicles. Three cell types are mainly found within the follicle: macrophages, lymphocytes, and reticular cells, some of which are usually described as dendritic. Antigen can undoubtedly bind *in vivo* to the membrane of reticular cells (27-29). In addition, Ada et al. (29) and Nossal et al. (28) mention that antigen can frequently be seen between densely packed lymphocytes and that no separating cell process can be resolved. The accumulation of antigen in the follicular areas of lymph nodes and spleen is antibody dependent (29-31). Passively administered antibody markedly increases follicular localization, and in tolerant animals completely unresponsive to an antigen it does not occur (32). The mechanism of localization of complexes in these areas, which also contain CRL, is unknown and it seems possible that complement is involved. It has been reported, for example, that the fragment of the antibody molecule which mediates C-fixation (F[c]) is also necessary for the retention of Ag-Ab complexes in the follicles (29). In addition, complement components have been detected within the germinal centers which contain Ag-Ab complexes (33). It remains to be determined whether the deposition of Ag-Ab-C complexes in these areas takes place through the C3-dependent receptors found on the membranes of CRL, macrophages, and possibly reticular cells.

In addition, another possible function of CRL should be considered. The presence of C3-dependent receptors on membranes of these cells would allow for their accumulation in sites where Ag-Ab-C complexes have been deposited. Indeed, it has recently been shown that, at least in some situations, complement factors may be of importance in mediating cell cytotoxicity *in vitro*. For example, mononuclear cells from human peripheral blood are capable of destroying EAC1423 but not EAC142 and, moreover, the effector cells bind to glass (34).

Finally, it is obvious that the demonstration of a population of lymphocytes which shares membrane properties with monocytes and macrophages raises several questions in relation to the real nature of the cells which appear to cooperate during the immune response.

SUMMARY

A population of lymphoid cells from several animal species, including man, was identified through a membrane receptor which binds sheep red blood cells treated with antibody and complement. When cells from different lymphoid organs were incubated with EAC at 37°C, only part of the lymphocytes (named CRL) bound EAC and formed rosettes, and this interaction was shown to be C3-dependent.

Mouse lymphoid cells could be specifically depleted of CRL by allowing them first to interact with EAC and then submitting the mixture to ultracentrifugation in a gradient of BSA. After ultracentrifugation, a population of cells containing 95% or more of non-CRL were recovered from the upper layers of the gradient.

In addition to their different abilities to bind EAC, CRL and non-CRL from mouse lymphoid organs could be distinguished by the following properties: (a) CRL adhered preferentially to nylon wool at 37°C in the presence of mouse serum. (b) After differential flotation in a gradient of BSA, a significantly higher proportion of CRL were recovered from the upper layers of the gradient. (c) The population of CRL contained most of the lymphocytes bearing immunoglobulin determinants on their membranes. (d) The distribution of CRL was quite different among lymphocytes obtained from various lymphoid organs, and they were never found in the thymus. (e) The membrane receptor for EAC was not detected in plaque-forming cells of mice which had been previously immunized with burro red cells.

CRL and non-CRL could not be distinguished by their life span, as they were found in similar proportions among long-lived and short-lived lymphocytes from mouse peripheral lymph nodes.

The function of this receptor on the membrane of certain lymphoid cells may be related to (a) the trapping and localization of antigen in lymphoid organs or (b) the localization of lymphoid cells in inflammatory sites.

We wish to thank Dr. J. Phillips-Quagliata for performing some thoracic-duct cannulations, Dr. I. Lepow for his help in preparing the purified human C components, Dr. Fred Miller for determining the specificity of antibodies in rabbit antisera against mouse immunoglobulins, and Dr. Carmen Merryman for her generous supply of antisera. We thank Dr. P. Dukor for correcting this manuscript and for many enlightening discussions. We also acknowledge the skillful technical help of Miss Judith Gibian.

BIBLIOGRAPHY

1. Uhr, J. W. 1965. Passive sensitization of lymphocytes and macrophages by antigen-antibody complexes. *Proc. Nat. Acad. Sci. U.S.A.* **54**:1599.
2. Uhr, J. W., and J. M. Phillips. 1966. In vitro sensitization of phagocytes and lymphocytes by antigen-antibody complexes. *Ann. N.Y. Acad. Sci.* **129**:793.
3. Lay, W. H., and V. Nussenzweig. 1968. Receptors for complement on leukocytes. *J. Exp. Med.* **128**:991.
4. Nussenzweig, V., W. H. Lay, and P. A. Miesher. 1969. IgG- and C'-dependent receptor sites on leukocytes. *In Cellular Recognition*. R. T. Smith and R. A. Good, editors. Appleton-Century-Crofts Inc. N.Y. 317.
5. Lay, W. H., and V. Nussenzweig. 1969. Ca⁺⁺-dependent binding of antigen-19S antibody complexes to macrophages. *J. Immunol.* **102**:1172.
6. Dresser, D. W., and H. H. Wortis. 1967. Localized haemolysis in gel. *In Handbook of Experimental Immunology*. D. M. Weir, editor. F. A. Davis Co. Philadelphia, Pa. 1054.

7. Lepow, I. H., G. B. Naff, E. W. Todd, J. Peasky, and C. F. Hinz, Jr. 1963. Chromatographic resolution of the first component of human complement into three activities. *J. Exp. Med.* **117**:983.
8. Borsos, T., and H. J. Rapp. 1967. Immune hemolysis: A simplified method for the preparation of EAC'4 with guinea pig or with human serum. *J. Immunol.* **99**:263.
9. Dias Da Silva, W., J. W. Eisele, and I. H. Lepow. 1967. Complement as a mediator of inflammation. III. Purification of the activity with anaphylotoxin properties generated by interaction of the first four components of complement and its identification as a cleavage product of C'3. *J. Exp. Med.* **126**:1028.
10. Brittinger, G., R. Hirschhorn, S. D. Douglas, and G. Weissmann. 1968. Studies on lysosomes. XI. Characterization of a hydrolase-rich fraction from human lymphocytes. *J. Cell Biol.* **37**:394.
11. Huber, H., and S. D. Douglas. 1970. Receptor sites on human monocytes for complement: binding of red cells sensitized by cold autoantibodies. *Fed. Proc.* **29**:621.
12. Tyler, R. W., N. B. Everett, and M. R. Schwarz. 1969. Effect of antilymphocytic serum on rat lymphocytes. *J. Immunol.* **102**:179.
13. Raidt, D. J., R. I. Mishell, and R. W. Dutton. 1968. Cellular events in the immune response. Analysis and in vitro response of mouse spleen cell populations separated by differential flotation in albumin gradient. *J. Exp. Med.* **128**:681.
14. Boyse, E. A., L. J. Old, and I. Chouroulinkov. 1964. Cytotoxic test for demonstration of mouse antibody. *Methods Med. Res.* **10**:39.
15. Raff, M. C., M. Sternberg, and R. B. Taylor. 1970. Immunoglobulin determinants on the surface of mouse lymphoid cells. *Nature (London)*. **225**:553.
16. Paraskevas, F., S. T. Lee, and L. G. Israels. 1970. Cell surface receptors γ -globulins: a new technique for their detection on normal lymphoid mouse and human cells and their lack from neoplastic plasma cells. *Fed. Proc.* **29**:697.
17. Everett, N. B., R. W. Caffrey, and W. O. Rieke. 1964. Recirculation of lymphocytes. *Ann. N.Y. Acad. Sci.* **113**:887.
18. Boyden, S. V., and E. Sorkin. 1960. The adsorption of antigen by spleen cells previously treated with antiserum in vitro. *Immunology*. **3**:272.
19. Boyden, S. V., and E. Sorkin. 1961. The adsorption of antibody and antigen by spleen cells in vitro. Some further experiments. *Immunology*. **4**:244.
20. Berken, A., and B. Benacerraf. 1966. Properties of antibodies cytophilic for macrophages. *J. Exp. Med.* **123**:119.
21. Takahashi, T., L. J. Old, and E. A. Boyse. 1970. Surface alloantigens of plasma cells. *J. Exp. Med.* **131**:1325.
22. Miller, J. F. A. P., and G. F. Mitchell. 1969. Thymus and antigen-reactive cells. *Transplant. Rev.* **1**:3.
23. Davies, A. J. S. 1969. The thymus and the cellular basis of immunity. *Transplant. Rev.* **1**:43.
24. Claman, H. N., and E. A. Chaperon. 1969. Immunologic complementation between thymus and marrow cells—a model for the two-cell theory of immunocompetence. *Transplant. Rev.* **1**:92.
25. Taylor, R. B. 1969. Cellular cooperation in the antibody response of mice to two serum albumins: specific function of thymus cells. *Transplant. Rev.* **1**:114.
26. Parrott, D. M. V., M. A. B. de Souza, and J. East. 1966. Thymus-dependent areas

- in the lymphoid organs of neonatally thymectomized mice. *J. Exp. Med.* **123**:91.
27. Mitchell, J., and A. Abbot. 1965. Ultrastructure of the antigen-retaining reticulum of lymph node follicles as shown by electron-microscopic autoradiography. *Nature (London)*. **208**:50.
 28. Nossal, G. J. V., A. Abbot, J. Mitchell, and Z. Lummus. 1967. Antigens in immunity. XV. Ultrastructural features of antigen capture in primary and secondary lymphoid follicles. *J. Exp. Med.* **127**:277.
 29. Ada, G. L., C. R. Parish, G. J. V. Nossal, and A. Abbot. 1967. The tissue localization, immunogenic, and tolerance-inducing properties of antigens and antigen-fragments. *Cold Spring Harbor Symp. on Quant. Biol.* **32**:381.
 30. Nossal, G. J. V., G. L. Ada, C. M. Austin, and J. Pye. 1965. Antigens in immunity. VIII. Localization of ¹²⁵I-labelled antigens in the secondary response. *Immunology*. **9**:349.
 31. Cohen, S., P. Vassalli, B. Benacerraf, and R. T. McCluskey. 1966. The distribution of antigenic and nonantigenic compounds within draining lymph nodes. *Lab. Invest.* **18**:1143.
 32. McDevitt, H. O., B. A. Askonas, J. H. Humphrey, I. Schechter, and M. Sela. 1966. The localization of antigen in relation to specific antibody-producing cells. *Immunology*. **11**:337.
 33. Gajl-Peczalska, K. J., A. J. Fish, H. J. Meuwissen, D. Frommel, and R. A. Good. 1969. Localization of immunological complexes fixing β_{1c} (C3) in germinal centers of lymph nodes. *J. Exp. Med.* **130**:1367.
 34. Pearlmann, P., H. Pearlmann, H. J. Müller-Eberhard, and J. A. Manni. 1969. Cytotoxic effects of leukocytes triggered by complement bound to target cells. *Science (Washington)*. **163**:937.