STUDIES ON ANTIBODY-PRODUCING CELLS

I. Ultrastructure of 19S and 7S Antibody-Producing Cells*

By FRED G. GUDAT,‡ M.D., T. N. HARRIS, M.D., SUSANNA HARRIS, Ph.D.,
AND KLAUS HUMMELER, M.D.

*(From the Children's Hospital of Philadelphia, and the Department of
Pediatrics, School of Medicine, University of Pennsylvania,
Philadelphia, Pennsylvania 19146)*

Recent developments in the detection of individual antibody-forming cells by hemolytic antibody plaque production (1, 2) or by adherence of antigen-bearing red blood cells to antibody-forming cells (3, 4) have made possible the electron microscopic study of cells involved in antibody production. More of the cells examined in this way have been those detected by the adherence technique (rosette formation), but it has also been possible to study a small number of hemolytic antibody plaque-forming cells by electron microscopy Such studies of plaque-forming cells (PFC)[1] by Harris et al. (5), Hummeler et al. (6), and Neher and Siegel (7), and of rosette-forming cells (RFC) by Cunningham et al. (8), and Storb et al. (9, 10) have revealed a considerable morphological heterogeneity among such cells. Cells of both the lymphocytic and plasmacytic categories, in various stages of differentiation, were found among both PFC and RFC. In addition, phagocytic cells were identified among RFC of the spleen.

In our earlier studies of plaque-producing lymph node cells (5), the number of cells available by the method employed was not sufficient to yield meaningful data on the distribution of cells between these categories, or on the relation between the two morphologic categories of antibody-forming cells. Subsequently, a method was developed for collecting rosette-forming cells by micropipette, and since then it has been possible to adapt this technique to the collection of plaque-forming cells in substantially greater numbers. The present paper gives electron microscopic descriptions of RFC and PFC obtained under various experimental conditions. In antibody-producing cells detected by rosette formation, which is a substantially more sensitive method than plaque formation, examination of several hundred such cells showed these to be in both the

lymphocytic and plasmacytic categories, as we had found in plaque-forming cells of the rabbit lymph node (5), but with the great majority in the lymphocytic category, and a few cells which showed morphologic indications of a transition between the two groups. Plaque-producing cells, both direct and facilitated by the use of anti-IgG (19S and 7S, respectively), were again found to be in both categories: in this case with a substantial majority in the plasmacytic group.

Materials and Methods

Animals and Immunization.—Female BALB/c or C57BL/6 mice (The Jackson Laboratory, Bar Harbor, Maine) were injected intraperitoneally with 1 ml of 2% washed sheep red blood cells (SRBC) to stimulate the spleens, or 0.1 ml of a 20% suspension into both forelegs to stimulate the brachial lymph nodes. Rabbits were injected with 0.5 ml of 50% SRBC into each hind footpad. To obtain 19S antibody-producing cells from the mouse spleen or rabbit popliteal lymph node, the animals were sacrificed on the 4th or 5th day after primary injection. Cells producing 7S antibody were studied 10 or 18 days after primary injection, or 5 days after a second injection, with an interval of at least 4 wk between the two injections.

Rosette Formation.—Spleens or lymph nodes of two or three animals were pooled for each experiment on selected days after primary or secondary injection of SRBC, teased in cold Eagle's Minimum Essential Medium (MEM) with 10% fetal calf serum (FCS), and passed through an 80 mesh wire screen into a pointed centrifuge tube. Larger particles were allowed to settle out for 5 min. The cells in the supernatant were washed once and used for rosette formation as follows: triplicate 0.9 ml portions of the cell suspension, containing 20×10^6 leukocytes, were mixed with 0.1 ml of 20% washed SRBC (5% for lymph node cells) in a 100×13 mm test tube and incubated for 1 hr at 37°C without motion.

Production of 7S Rosettes.—Rosettes were obtained by incubating 20×10^6 cells of spleen or lymph node in 1 ml of Eagle's MEM, containing 10% FCS, with 2% SRBC for 1 hr at 37°C. Two methods were employed to select 7S rosettes based on their resistance to the hemolytic action of complement. (a) Freshly prepared individual rosettes were collected and placed in plastic embedding capsules (see below). Guinea pig serum 1:10 was added to the capsule for incubation at 37°C for 45 min, followed by fixation and embedding in Epon 812. This resulted in rosettes with "ghost" red cells attached to the central cell (19S rosette). Rosettes which retained intact red cells were considered to be due to 7S antibody. (b) Guinea pig complement was added to the rosette preparation at a final concentration of 1:10 at the beginning of the 37°C incubation. The complement–resistant rosettes were then collected, fixed, and embedded for electron microscopy.

Prefixation.—The rosette preparations were suspended in glutaraldehyde, 1% final concentration, for 30 min or overnight. The rosettes were then washed and separated from the majority of free cells by two centrifugations in 6 ml of buffered saline at 60 g for 5 min. The resulting pellet, rich in rosettes, was suspended in 4–6 ml of saline.

Rosette Collection for Electron Microscopy.—"Braking" micropipets were prepared in the following way. A piece of 1.5 mm capillary glass tubing, which had been passed through a rubber vial stopper, was drawn out at both ends, using a minute flame, by gas allowed to flow through a hyperdermic needle. The "front" end was so fine as to just admit the largest rosettes (OD approximately 0.02 mm), and the other end fine enough to slow the rate at which air could pass through that end of the tube. The rubber stopper was then fitted into one end of a piece of glass tubing (8 mm), the other end being connected to a rubber tube leading to a mouthpiece. The rate of inflow at the tip was thus determined by the rate of air passage at the outlets.

Plastic embedding capsules for electron microscopy (Beem capsules, Size 00) were obtained from Better Equipment for Electron Microscopy, Inc., New York. Depressions of a trapezoidal cross-section measuring approximately 0.30 × 0.25 × 0.30 (depth) mm were made in the tapered end of the capsules with a punch machined for this purpose, and were filled with a drop of saline.

To collect the rosettes, portions of the enriched and prefixed rosette suspension were placed in a depression slide. Rosettes were randomly picked up, 10–20 at a time, with the braking micropipet, under a Leitz (E. Leitz, Inc., New York) dissecting microscope, using an 8 × objective and a 12.5 × ocular magnification. Using the microscope at the same enlargement, we transferred the rosettes to the embedding capsule. While the rosettes were settling to the bottom, most of the free cells still present at this stage could be removed from the collection. Pellets of 100–150 rosettes could be collected in this way within an hour. Later, it was possible to collect a single layer of approximately 50 rosettes on the bottom of the hole or on the flat bottom of the capsule without disturbing them during the procedures which followed.

Plaque Formation.—For the collection of individual plaque-forming cells, it was desirable to have the hemolytic plaques produced in as thin a layer of agar as possible. Thin layers of plaque-forming cells in agar were prepared by mixing 0.6 ml of washed spleen or lymph node cells at concentrations of 5–10 × 10^6/ml with 1 ml of a 0.9% solution of agarose (L'Industrie Biologique Francaise S.A., Gennevilliers, France) in Earle's solution containing 12.5% SRBC, and maintained at 46°C. (For the most uniform distribution of red blood cells in these plates it was found preferable to add the salts and buffer to a distilled water solution of agar shortly before use.) The mixture was poured into plastic Petri dishes 5 cm in diameter (Falcon Plastics, B-D Laboratories, Inc., Los Angeles, Calif.) over a hardened layer of 1.2% agarose. After rapid spreading, any excess mixture was discarded with a rapid flip of the wrist. The remaining thin layer was allowed to harden in a slightly inclined position. Plaques produced by 19S antibody were obtained by addition of 1 ml of 1:10 guinea pig serum and incubation for 60 min at 37°C. For the collection of 7S plaques, this first incubation was extended to 90 min. At that time the 19S plaques were marked with the point of a needle. Plaques due to 7S antibody were then produced by further incubation of the plates at 37°C with an appropriate dilution of a goat anti-mouse IgG serum in 1:10 guinea pig serum (11, 12) which had been twice absorbed with SRBC. Control plates, to indicate that all 19S plaques had been identified by the marking, were further incubated with complement alone.

For the isolation of plaque-forming cells, micropipets with an extremely fine tip were used that made it possible to aspirate the central cell of a plaque under a Leitz stereo microscope, using a magnification of 100. Successful isolation was checked on a slide and then the individual cells were gathered in an embedding capsule as described above, and prefixed *in situ* for 30 min in 1% glutaraldehyde.

Postfixation, Embedding, and Electron Microscopy.—Postfixation and dehydration were carried out at room temperature by stepwise replacement of the fluids with fine pipettes under low-power microscopic observation. Osmic acid, 1%, was applied for 10 min. This was then replaced by a preliminary wash with a mixture of equal parts of 1% osmic acid and 70% alcohol, followed by several washings with 70% alcohol. The alcohol concentration was then increased stepwise, ending with three changes of 100% alcohol, at intervals of 10 min. The alcohol was removed by repeated changes of Epon 812 (13) and the cells left standing overnight at 37°C. After 3 changes of Epon containing 2% accelerator, polymerization was allowed to proceed for at least 2 days at 60°C. Sections were cut on a Porter-Blum MT-2 microtome (Ivan Sorvall Inc., Norwalk, Conn.) with a diamond knife which was adjusted to be parallel to the layer of rosettes. Double counting of rosettes in the sections was avoided by using only one representative section when a single layer of rosettes had been embedded. When rosettes within a trapezoidal pellet were cut, thick sections totaling 5 μ were discarded between representative ultrathin sections. These were stained with 0.01% lead citrate and 1% uranyl

acetate for 3–5 min in each solution. The grids were viewed and photographed in a Siemens Elmiskop I electron microscope (Siemens America, Inc., New York) at screen magnifications between 5,000 and 10,500.

RESULTS

Rosette-Forming Cells of Mouse and Rabbit.—

Rosettes of SRBC adherent to antibody-producing cells were prepared from cells of mouse and rabbit lymph nodes and spleens obtained 0–10 days after a

TABLE I

Mean Diameters of Mouse and Rabbit Rosette-Forming Cells in Ultrathin Sections

|	Mouse				Rabbit		
	No. of cells measured	Cell diameter		Nuclear diameter	No. of cells measured	Cell diameter		Nuclear diameter
		Mean	Range			Mean	Range	
		μ	μ	μ		μ	μ	μ
Small to medium lympho-cytes	34	6.9	5.2–8.5	5.1				
Large lym-phocytes	40	10.0	7.5–13.3	6.9	14	10.0	7.9–12.9	6.8
Plasma-blasts	3	9.7	8.4–11.3	6.5	8	8.9	8.2–11.0	6.4
Mature plasma-cytes	6	8.5	8.3–10.2	5.9	6	8.2	7.6–9.4	5.6

single injection of SRBC. The individual rosettes were collected as described above and they were prepared for study by electron microscopy. Examination of lymph node cells forming rosettes showed these to be almost entirely of the lymphocytic or plasmacytic series. In cells obtained from mouse spleens, macrophages also appeared, in variable numbers; their numbers increased in later days after immunization.

The classification of cells into the lymphocytic or plasmacytic group was determined by the state of the endoplasmic reticulum (ER) according to the descriptions below. The mean diameters of representative cells so classified are listed in Table I.

There were no major morphologic differences between cells of the mouse and the rabbit, within each of the categories, so that the following descriptions apply to both species unless otherwise stated.

Lymphocytic Cells.—

Small to medium lymphocytes: Cells typical of small, inactive lymphocytes
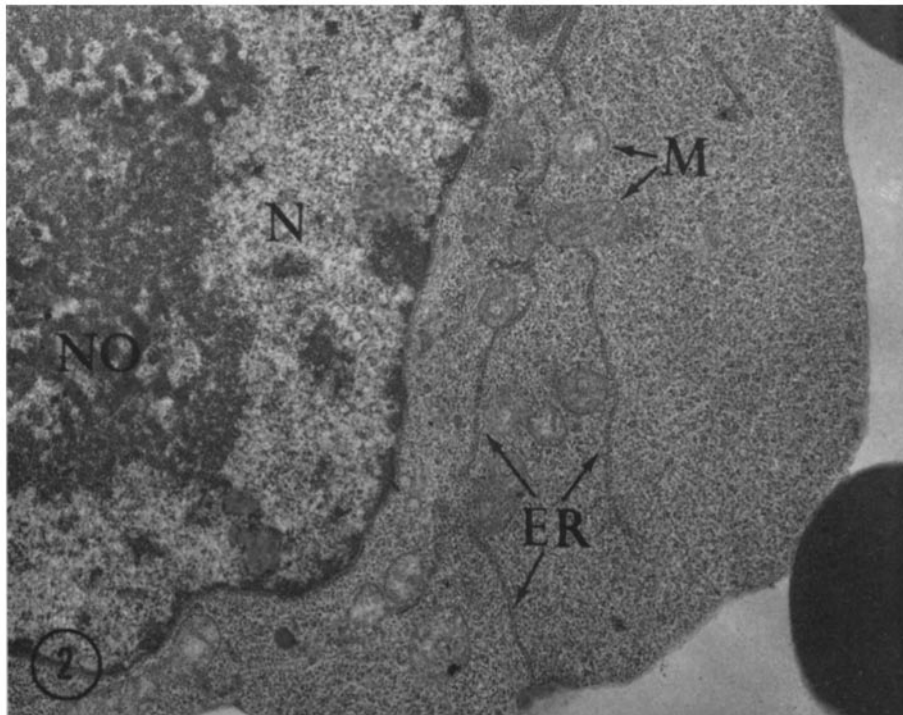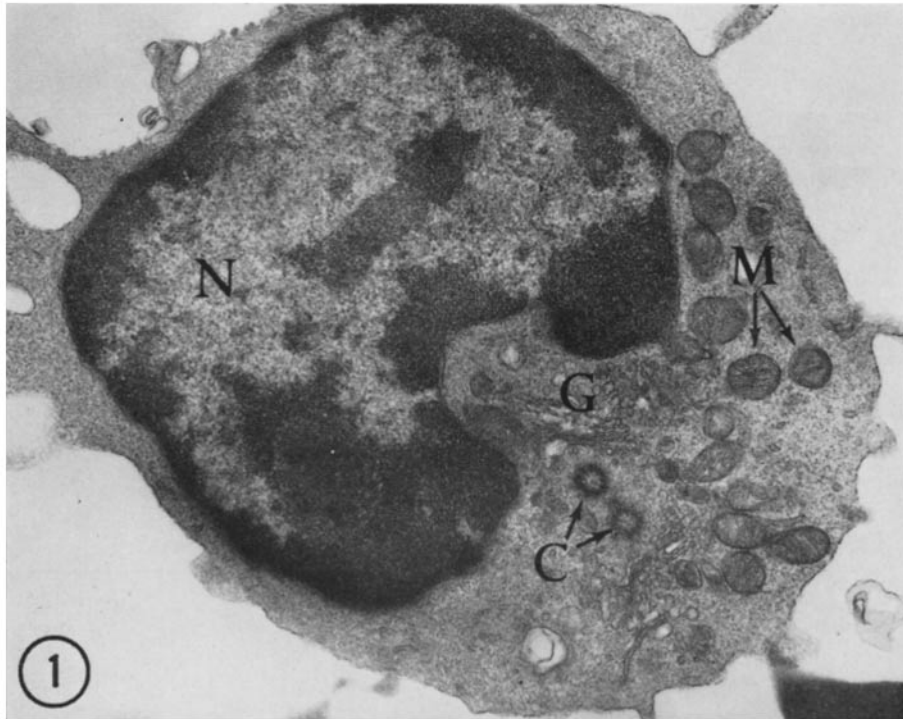
(14) occurred only in rosettes of uninjected animals. These contained an indented, electron-opaque nucleus, a small Golgi area, and a narrow rim of cytoplasm with few mitochondria and rare narrow profiles of ER. A few of these background rosette-forming lymphocytes, and those of SRBC-injected animals, were larger, with a higher degree of cytoplasmic differentiation of the type shown in Fig. 1. The slightly larger nucleus of these cells showed a loosening of the central chromatin, leaving a shell of condensed chromatin along the nuclear membrane, with chromatin projections into the interior of the nucleus. Nucleoli were prominent in many of these cells. The cytoplasm was moderately broadened, and the ribosomes appeared less densely packed. In varying degrees there were increases in Golgi sacs and vesicles, and in the number of mitochondria opposite the nuclear indentation. Single pinocytotic vesicles were regularly found, and frequently one or two centrioles. Most of these cells had single narrow channels of rough ER as well as partly distinct perinuclear spaces.

Large lymphocytes: Rosette-forming cells of this group had the same cytoplasmic components, differing only in quantitative aspects. Typical examples from rabbit and mouse are shown in Figs. 2–5. The enlargement of the cells (Table I) was due somewhat to an increase in the size of the nucleus but primarily to the increased cytoplasmic volume. With increasing size, the nuclei were more irregular in shape, but the nuclear indentation usually remained visible. The condensed chromatin was reduced to irregular patches along the nuclear membrane and to the periphery of the enlarged nucleoli, the central euchromatin becoming very light with increasing size. The ample cytoplasm was studded with free ribosomes which either appeared to be randomly distributed or were clustered in polyribosomes. Thus, the largest cells had a typical blastoid appearance similar to that of the cells with "large, pale nuclei" of Storb et al., (10). An example of a large lymphocyte with an extremely light nucleus, and a large, honeycomblike nucleolus in a rabbit RFC is shown in Fig. 2.

Some ER was demonstrable in almost every cell, in one of the following variations. In medium and large lymphocytes, there were channels with a constant, narrow distance between the rows of ribosome-bearing membrane, and no evidence of protein storage. Channels of this type occurred singly and were usually short, although some could be traced over a long distance. In other

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Abbreviations:

N, nucleus   ER, endoplasmic reticulum
M, mitochondrion   G, Golgi body
NO, nucleolus

FIG. 1. Rosette-forming cell (RFC). Medium sized lymphocyte. This cell shows several mitochondria and a well developed Golgi area including 2 centrioles (c). (BALB mouse, spleen, primary injection, 7 days). × 16,720.

FIG. 2. Large lymphocyte. Rabbit RFC with very light nucleus and prominent honeycomblike nucleolus. Increase in number of mitochondria and of narrow empty profiles of ER. (Rabbit, lymph node, primary injection, 5 days). × 13,200.
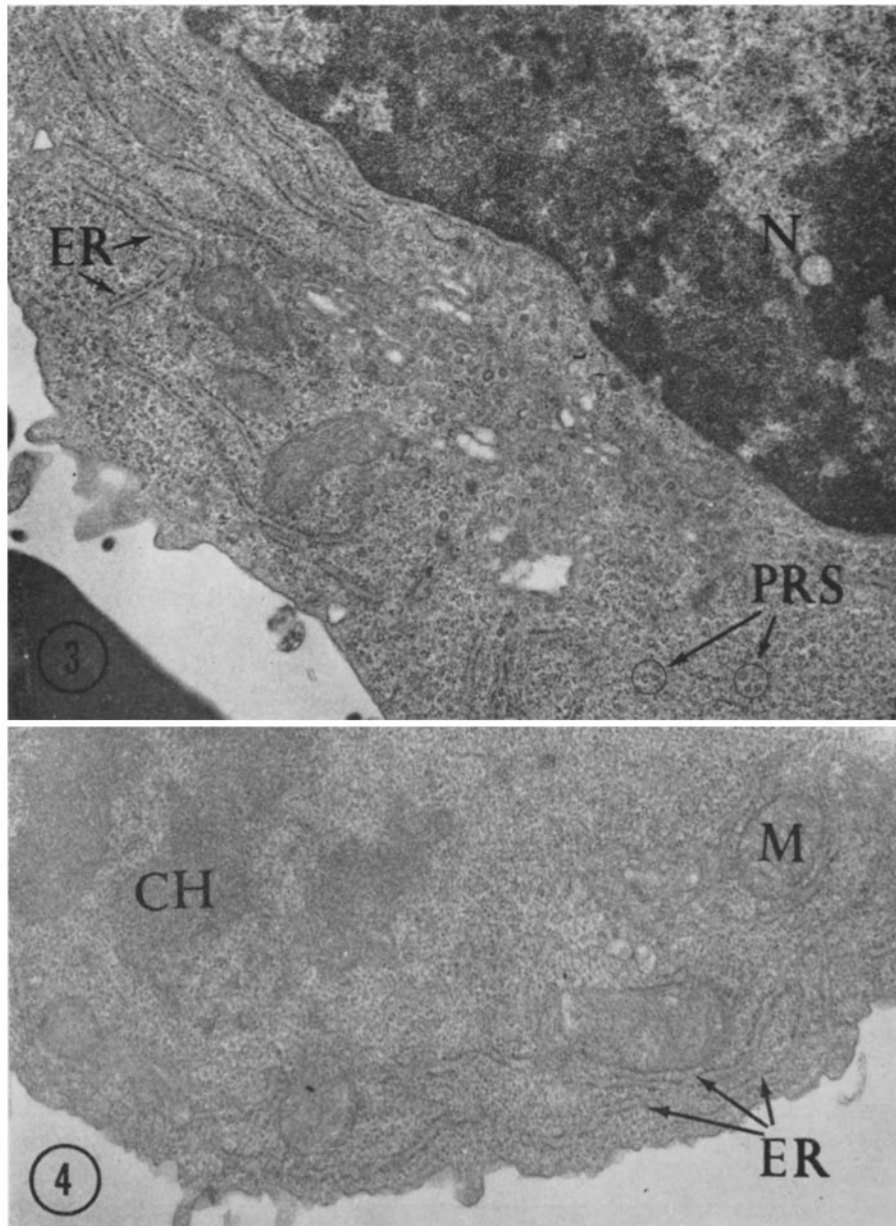
Fig. 3. RFC of the mouse in more advanced stages of differentiation. Large lymphocytic cell, possibly in transition to plasmablast. Conspicuous rough ER channels in parallel orientation, distinctly wider than those in Fig. 2 and filled with grayish material. Prominent polyribosomes (PRS). (BALB mouse lymph node, primary 4 days). $\times$ 17,000.

Fig. 4. RFC in mitosis. The cell shows an advanced stage of differentiation with up to three layers of moderately wide, filled, ER channels as seen in the probable transition forms to plasmablasts. Chromosomes (CH). (BALB mouse spleen, primary, 4 days). $\times$ 21,000.

Fig. 5. Detail of large lymphocytic cell. Prominent Golgi body with two stacks of Golgi lamellae (GL) and Golgi vesicles (GV) in the vicinity. Between these there is a peripheral section of a centriole (c) with several fine tubules (TB) pointing towards it. Rough-surfaced channels of ER with deposition of some gray material. Pinocytotic vesicles at various distances from the infolded cell membrane (PV). Note also a stack of wavy fibrils close to the cell surface (F). (BALB mouse lymph node, secondary, 5 days). $\times$ 31,600.

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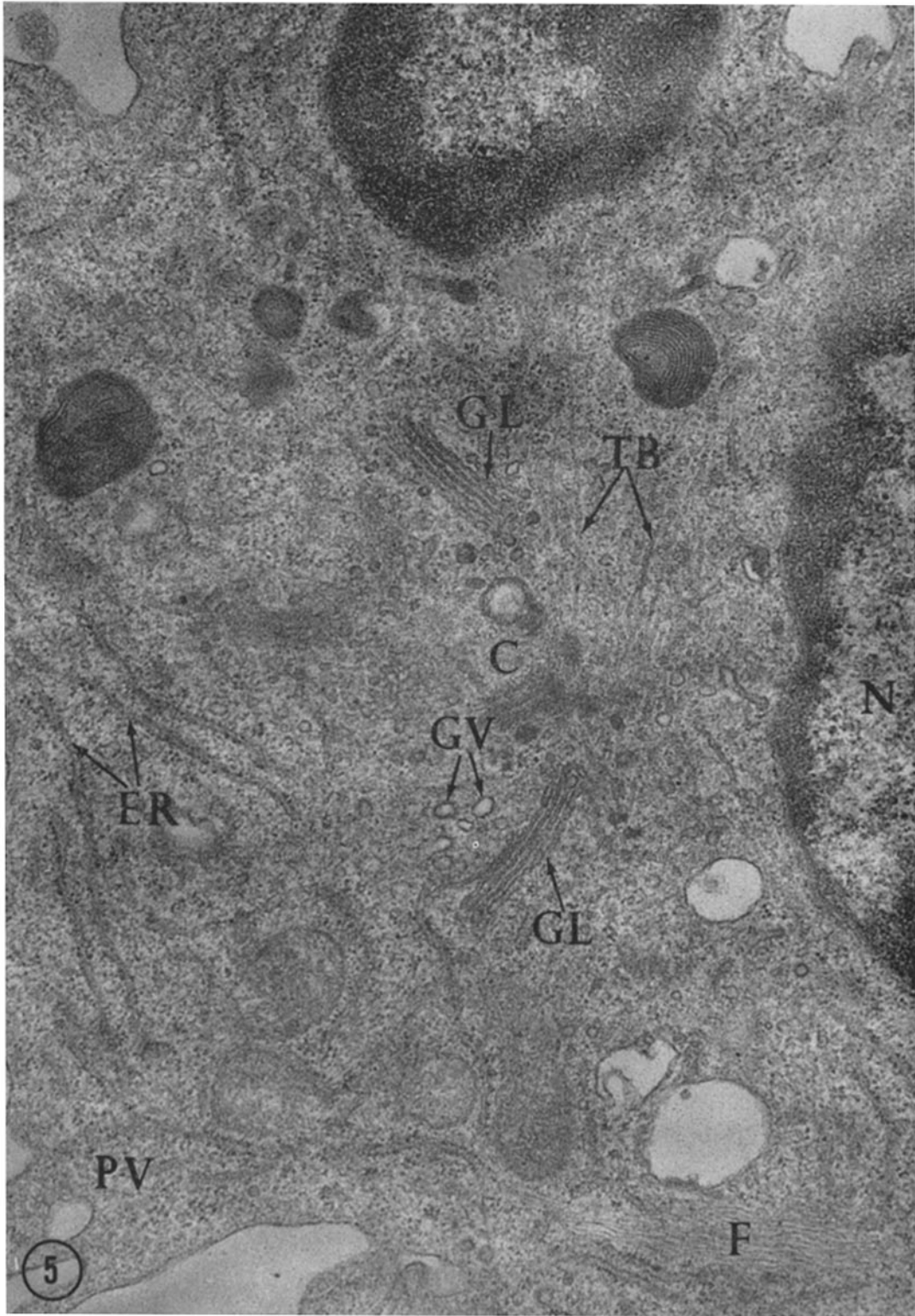
lymphocytes, especially in those which were rich in mitochondria, multiple short narrow pieces were detectable; they characteristically had some contact with a mitochondrion or even partially encircled it (Fig. 2). Usually these channels were not oriented with respect to each other, but occasionally some parallel layering was seen, as at the top of Fig. 2.

In some of the large lymphocytes, a difference in the ER was noted in that the channels were slightly and variably distended, and appeared to have deposits of protein-like material (Fig. 3). In addition, a more nearly parallel orientation of the channels became apparent. These cells, which still had a predominantly ribosomal cytoplasm, were regarded as transitional forms between the lymphocytes described in the previous paragraphs and the early plasmablasts. Regardless of the form or amount of ER, the Golgi bodies were extensive; they consisted of stacks of lamellae and many small vesicles which seemed to infiltrate into the periphery, obscuring the boundary of the Golgi area.

The cytoplasm of the large lymphocytes showed pinocytotic vesicles and centrioles with the same frequency as the medium sized lymphocytes. Occasionally, fine microtubules were visible, converging toward the centrioles. The mitochondria showed further hypertrophy and hyperplasia. Sometimes small electron-opaque bodies, probably lysosomes, were observed. These often showed a striking resemblance to pieces of red blood cell (RBC) which were also attached to the cell surface (e.g. Figs. 2, 3, 5). A good representation of all the cytoplasmic elements found in the large lymphocytes is shown in Fig. 5, which also shows the parallel-layered wavy fibrils described by Zucker-Franklin (15).

A few RFC were found in mitosis. They showed a range in the degree of cytoplasmic differentiation. Of 10 such cells, 3 showed no appreciable ER formation, and 5 showed solitary narrow channels, as in Fig. 2. The most advanced degree of cytoplasmic differentiation found among cells in mitosis involved two to three channels of the slightly wider ER, as in the cell of Fig. 3. Of the 10 cells in mitosis, 2 showed this level of differentiation. One of these is shown in Fig. 4.

Plasmacytic Cells.—

Plasmablasts: These cells were characterized by further increase in number, length, and width of the channels of ER, with evidence of the deposition of more protein within them. With increasing volume of the ER there was a corresponding reduction in the volume occupied by free ribosomes, which were either in random distribution (Fig. 6) or in clusters. There was also a reduction in the number of mitochondria. The Golgi area, however, maintained its size and differentiated organization, as did the nucleus.

Mature plasma cells: Cells with regular circular lamellae of ER channels, and relatively few free ribosomes between them, were considered mature plasma cells. The Golgi area, between layers of the ER, was unusually large and complex. Even at this stage the nuclei could show remarkably loose chromatin. In-
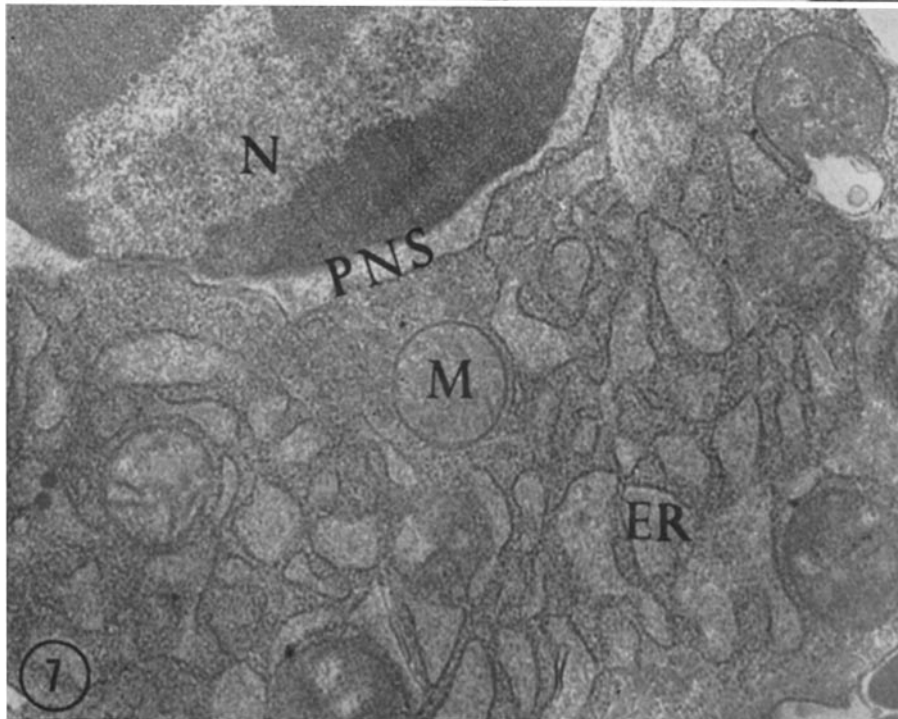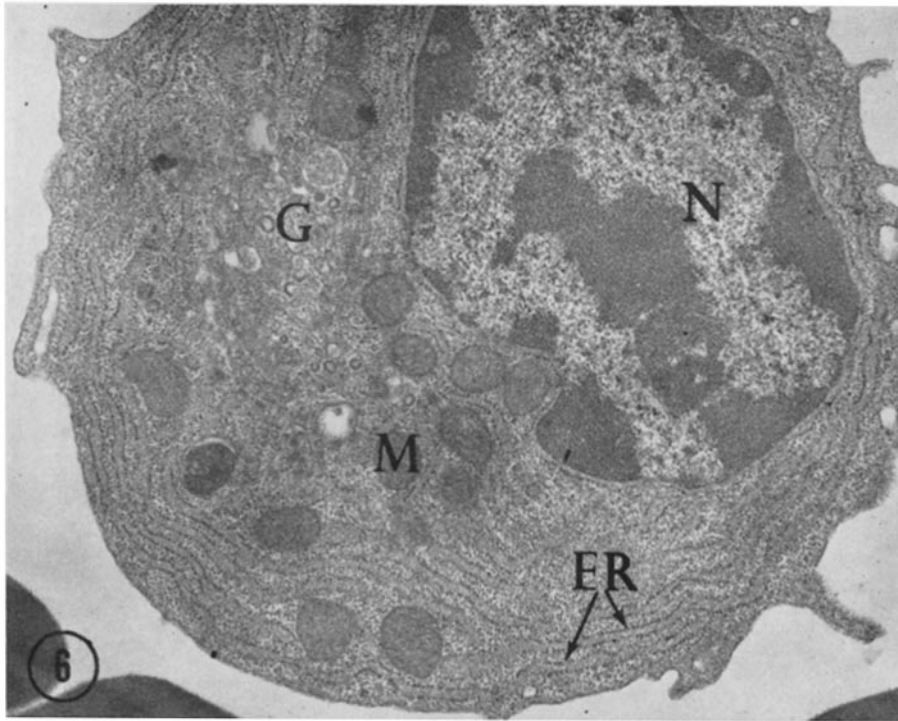
FIG. 6. Mature plasma cell with stretched circular *ER* channels and a reduction of the area of free ribosomes. A large Golgi apparatus consists mostly of small vesicles. (BALB mouse spleen, primary, 4 days). × 14,000.

FIG. 7. Mature plasma cell with dilatation of the perinuclear space (*PNS*) and advanced condensation of chromatin. (BALB mouse spleen, primary, 4 days). × 25,500.

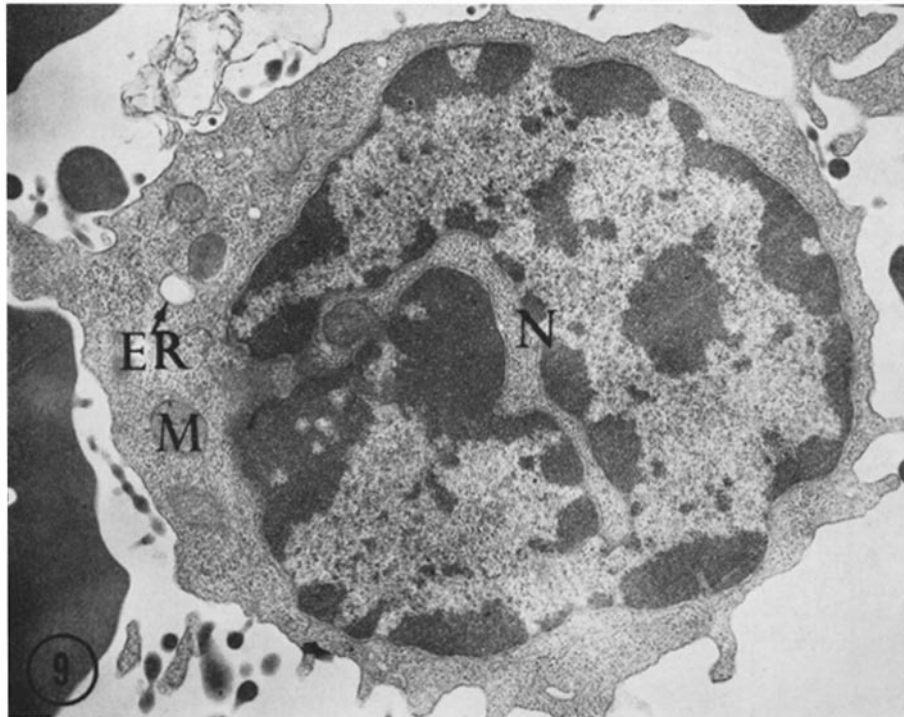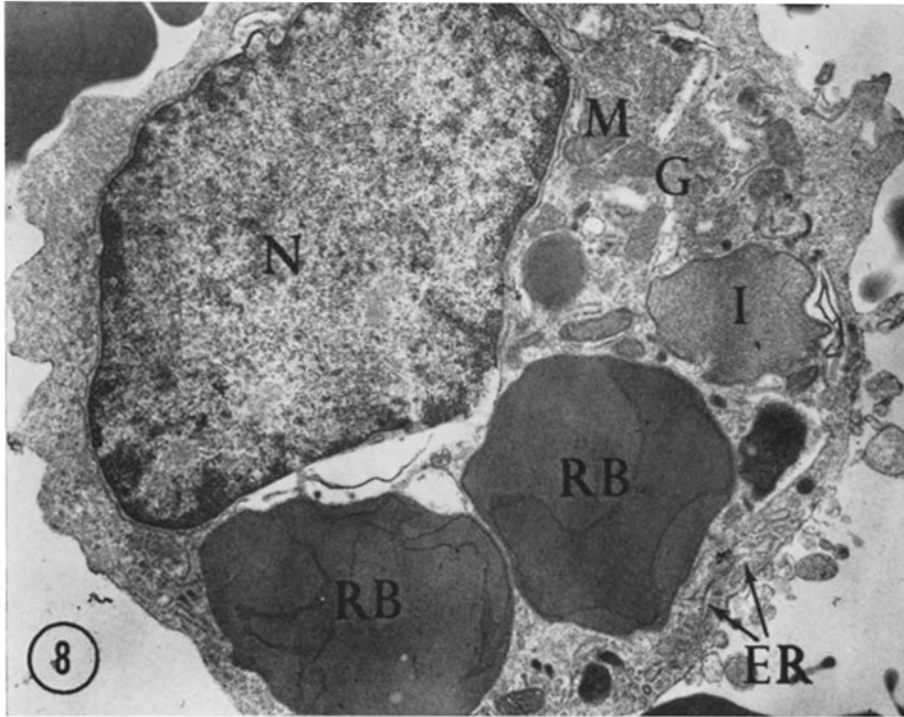FIG. 8. Small macrophage, containing two red cells (*RB*) and amorphous material (*I*) of different electron density. Golgi vesicles, some *ER* channels and some mitochondria are visible in the cytoplasm. (BALB mouse spleen, primary, 2 days). × 10,800.

FIG. 9. RFC of the mouse, 7S. Small lymphocyte with deep nuclear indentation and poorly differentiated cytoplasm. (BALB, primary, 11 days, complement resistant). × 14,200.

creasing condensation along the nuclear membrane was evident in some of these cells, especially in those which developed dilatation of the ER to irregular sacs. These two features are seen in Fig. 7.

Macrophages: Rosette-forming macrophages were found in spleen-cell preparations, with increasing frequency after the 5th day. They were recognized by their ingested red cells and other amorphous material, and varied considerably in size. The smaller cells (Fig. 8), between 7.8 and 10.4 μ in diameter, had eccentric nuclei which, in contrast to lymphocytes of this size, had a rather uniform loose chromatin with only small areas of condensed chromatin attached to the nuclear membrane. The cytoplasm contained one or two red cells and electron-dense amorphous bodies in various stages of breakdown. The cells contained a fair number of mitochondria, a small to medium Golgi area, and short, irregularly distended ER channels. Rather large parts of the cytoplasm consisted of evenly distributed ribosomes. Larger cells, up to 15 μ in diameter, contained many red cells or parts of RBC, up to eight per section. Their cytoplasm was of a lighter electron-opacity.

7S rosette-forming cells: The rosette-forming cells described thus far were examined without distinguishing whether they were produced by 19S or 7S antibody. Since either species of antibody could cause rosette formation, rosettes produced by cells obtained 10–12 days after primary injection were selected on the basis of the resistance of the RBC to lysis by complement. Of cells producing complement-resistant rosettes, eight were examined. All of them were found to contain lymphocytes, with only slight degrees of differentiation. Of 11 such cells obtained at the peak of the secondary response, 8 were small to large lymphocytes (Fig. 9), and 3 were plasma cells. Of 7S rabbit RFC, 41 of the 42 complement-resistant rosettes contained lymphocytes quite similar to the majority of rabbit RFC.

19S rosette-forming cells: The addition of complement to rosettes produced by cells obtained on the 3rd day after primary immunization causes hemolysis of the RBC in the substantial majority of the rosettes formed, presumably because these rosettes are due to 19S antibody (16 and footnote 2). For a preparation largely composed of 19S RFC, rabbit lymph node cells were obtained on the 4th or 5th day following a primary injection of sheep RBC. Rosettes were prepared and incubated with normal guinea pig serum (1:10) as a source of complement for 30 min at 37°C, before embedding. In the subsequent electron microscope examination the RFC surrounded by ghosts of RBC were taken as the 19S RFC. The cells thus examined were few in number, but showed the range of morphologic character which had been found in the entire group of rabbit RFC. One such cell, seen in Fig. 10, is a mature plasmacyte in a late

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[2] Moav, N. 1970. Studies of rosette forming cells as antibody synthesizing cells, and their relation to other populations of antibody producing cells. Unpublished Ph.D. thesis, University of Pennsylvania.

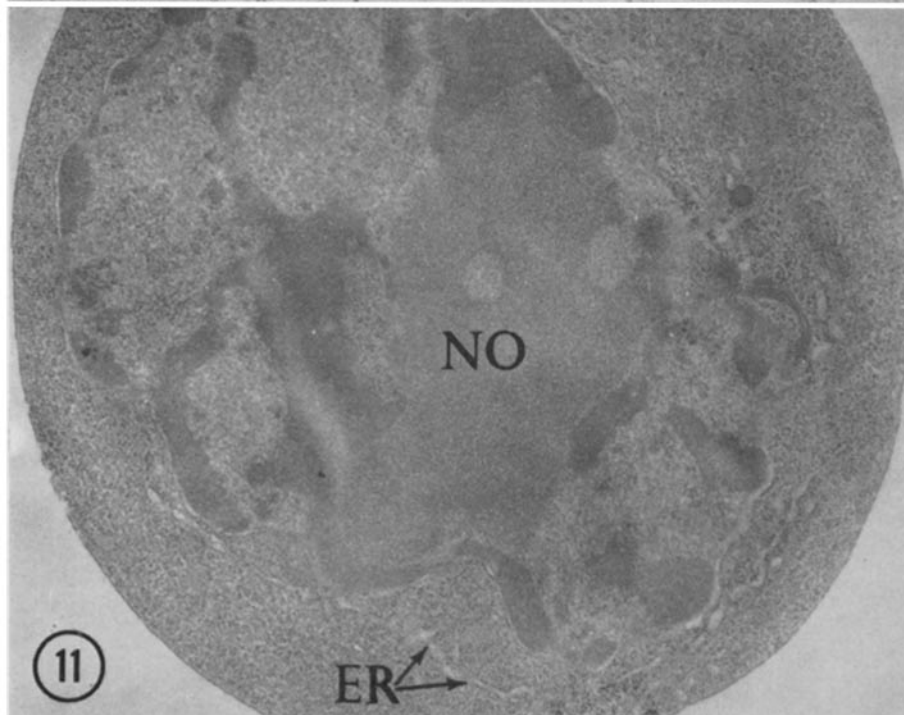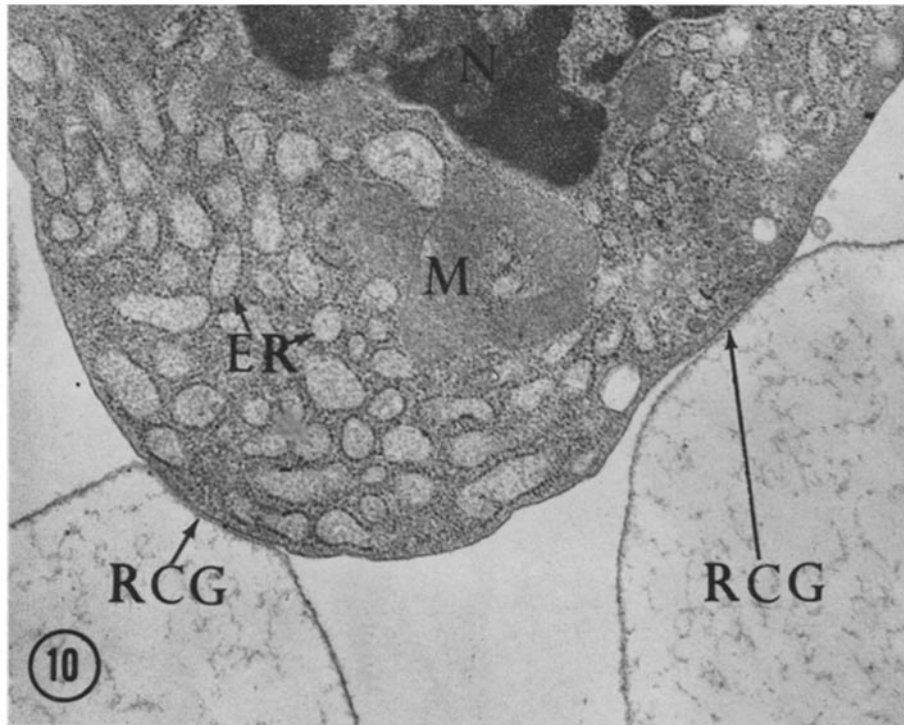Fig. 10. Rosette-forming cell (RFC), 19S. Mature plasma cell. Broad and intimate contact (arrows) of bound red cell ghosts, (RCG). (Rabbit lymph node, primary, 5 days, complement treatment in vitro). × 23,800.

Fig. 11. Plaque-forming cell (PFC) of the mouse, 19S. Large lymphocyte with clustered ribosomes, few solitary *ER* channels and large nucleolus. (BALB mouse, primary injection, 4 days). × 14,600.

stage of differentiation, with vesicular ER and some rounded sacs. The relation between the membrane of this cell and the membrane of the RFC ghost is shown in this figure.

Morpho-Kinetics of Rosette-Forming Cells from Mouse Spleens.—The classification described above was derived from a study of 220 electron micrographs, and was then found suitable for the grouping of 320 additional RFC which were examined on the screen of the microscope.

TABLE II

Distribution of Rosette-Forming Cells within the Lymphocytic and Plasmacytic Categories in Lymph Node and Spleen of Mouse and Rabbit in Relation to Time after Injection of Antigen

Source of cells	Injection	Day	Lymphocytic cells			Plasmacytic cells		
			Per cent of total RFC	No. of cells		Per cent of total RFC	No. of cells	
				Small to medium	Large		Plasma-blast	Mature plasma-cyte
BALB	None	0	100	4	0	0		
spleen	1st	2	72	3	2	29	1	1
	"	4	68	3	20	32	6	5
	"	7–10	100	4	15	0		
	2nd	4	88	3	5	12	0	1
BALB	1st	3	100	3	21	0		
lymph	"	5	87	0	13	13	1	1
node	2nd	5	100	2	10	0		
C57BL6	1st	2	100	3	11	0		
spleen	"	3	100	12	9	0		
	"	4	91	8	22	7	3	0
	"	7–11	100	8	1	0		
	2nd	4–5	68	3	14	32	5	3
Rabbit	1st	5	83	0	15	17	2	1
lymph node	2nd	5	100	15	17	0		

The distribution of the cell types encountered during the course of the primary and secondary response to SRBC in mice is summarized in Table II.

In the spleens of uninjected BALB mice, all of the four RFC examined belonged to the category of small lymphocytes. The predominance of this cell type was confirmed by light microscopy; of 103 such background rosettes classified, 94% fell into this group.[3] On the 2nd day after primary injection medium size lymphocytes were remarkably rich in mitochondria and contained several narrow ER channels. In addition, large lymphocytes were found, some with clustering of ribosomes. One plasmablast and one mature plasma cell were found at this stage.

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[3] Gudat, F. Data to be published.

During the 3rd and 4th day the total percentage of lymphocytic cells ranged between 70 in BALB mice and 100 in C57BL, and of these 45–90% were large lymphocytes. All morphologic stages described for this group were found during this period. More or less confined to this period were the very large blastoid lymphocytes (with light nucleus) and the transitional forms referred to above. Many of the large lymphocytes contained well-defined polyribosomes, which were less pronounced on the 4th day. Plasmacytic cells were less frequent and less differentiated in spleens of C57BL than of BALB mice.

Between the 7th and 11th day RFC were found to be exclusively of the lymphocytic category. These late cells were generally less differentiated. As in the case of C57BL mice, medium

TABLE III

Electron Microscopic Classification of Rosette-Forming Cells after Injection of Various Doses of SRBC

Dose of SRBC	Animal	RFC/10⁶	Lymphocytic cells		Plasmacytic cells	
			No.	Per cent	No.	Per cent
50%, 0.5 ml (3 injections)	Rabbit, individual	950	18	95	1	5
		465	22	85	4	15
		270	28	88	4	12
50%, 0.5 ml	Rabbit, pooled	780	14	70	6	30
		870	14	70	6	30
1%, 0.5 ml	Rabbit, individual	1770	21	81	5	19
		290	19	73	7	27
20%, 0.1 ml (3 injections)	Mouse, pooled	1420	32	89	4	11
20%, 0.1 ml		1770	13	87	2	13
5%, 0.1 ml		263	16	100	—	—
1%, 0.1 ml		153	19	100	—	—
0.1%, 0.1 ml		—	5	100	—	—

Rabbit: day 5.
Mouse: day 4.

sized lymphocytes were the most common type. BALB mice showed a higher proportion of large lymphocytes. However, the largest blastoid cells, the transitional forms, and plasma cells were absent. The large lymphocytes found had substantially fewer mitochondria, relatively small Golgi areas, and fewer ER channels.

The distribution of cell types during the 4th and 5th day of the secondary response did not differ significantly from that of the primary. About two-thirds of the RFC were lymphocytic cells and of these about 60–80% were large lymphocytes. Plasmablasts and mature plasma cells constituted one-third of the RFC population.

Rosette-Forming Cells of Lymph Nodes of Mice and Rabbits.—In RFC from lymph nodes the frequency of cells in the lymphocytic category was even higher than in RFC from spleen cells. This is reflected in Table II by the low frequency of plasma cells in the primary response, and the absence of plasma cells

in the secondary. In addition, there was a rapid appearance of large lympho-
cytes (day 3) which showed some ER at that time, as well as on day 5. Typical
cells are shown in Fig. 5 for mice, and in Fig. 2 for rabbits. Another feature
consistently observed was the absence of macrophages in the RFC from mouse
lymph nodes.

The Effect of Antigen Dose on the RFC Population of Lymph Nodes.—Experiments were
done to study the influence of increased or decreased doses of antigen on the distribution of
cells between the lymphocytic and plasmacytic categories. The results are summarized in
Table III. In rabbits, repeated injections of SRBC did not cause a further increase in the

TABLE IV

Electron Microscopic Classification of 19S and 7S Antibody-Producing Cells of Mouse and Rabbit

Cells examined			Lymphocytic cells		Plasmacytic cells					
							Mature plasmacytes, with			
			No.	Per cent	No.	Plasma-blasts	La-mellar ER	Dis-tended ER	Vesic-ular ER	Per cent
Mouse	19S	Plaque	4	13	27	13	10	1	3	87
	7S	"	2	6	33	5	16	11	1	94
	7S	Rosette	16	84	3	—	2	—	1	16
Rabbit	19S	Plaque	1	4	25	2	21	—	2	96
	19S*	"	—	—	15	1	14	—	—	100
	19S‡	"	2	6	29	7	22	—	—	94
	7S	"	—	—	24	1	17	3	3	100
	7S	Rosette	41	98	1	—	1	—	—	2

Usual antigen injection for the rabbit: 50% SRBC, 0.5 ml in each hind foot pad.
* High dose of antigen: 50%, 0.5 ml, 3 injections.
‡ Low dose of antigen: 1%, 0.5 ml.

rosette number, but there was a higher percentage of lymphocytic cells (85–95%) than after
primary injection (70%). With a 50-fold lower dose the percentage of lymphocytes did not
differ from that obtained with the usual dose of antigen.

In mice three injections of the usual dose did not significantly change the rosette number
or cell distribution; lymphocytes comprised 89%. At lower levels of antigen dose, only lympho-
cytes were found within the limited numbers of RFC studied. These results indicated that
within this range of dosage there was no consistent difference in the cell types of the RFC.

Plaque-Producing Cells of the Mouse and Rabbit.—Plaque-producing cells were
classified according to the nomenclature used above for rosette-forming cells.
They were called lymphocytic when free ribosomes were the main constituent
of the cytoplasm, with only a few solitary, unoriented channels of endoplasmic
reticulum, very narrow and of constant width throughout the typically short
pieces seen. These could, again, be classified as small, medium, or large. In a
few of the cells which were otherwise typical lymphocytes, usually large, the

few channels of ER were slightly wider and showed signs of protein deposition. These fell into the category of transitional lymphocytes, described above.

In the plasmacytic series, cells were classified as plasmablasts if the ER was oriented in more or less parallel lamellae and showed definite indications of protein deposition in widened channels, but occupied less of the cytoplasm than did the area of free ribosomes. Within the group of mature plasma cells, the ER occupied the greater part of the cytoplasm, being distributed either as parallel channels of uniform medium width or as irregularly distended vesicles. A transition from lamellar to vesicular ER is shown in Fig. 13. An additional group of plasma cells with rounded sacs of ER was recognized (5). This type was regularly combined with a homogenization of the nuclear contents (Fig. 16) or a separation of amorphous heterochromatin, usually into a semilunar area. This nuclear alteration was also observed in a few cells with parallel lamellae or vesicular ER (Fig. 15). Table IV summarizes the distribution of cell types in mice and rabbits during 19S and 7S antibody production, the cells having been detected by formation of plaques or rosettes.

19S PFC of the mouse: Of 31 such cells examined, 4 were large lymphocytes (13%), ranging in diameter between 7.5 and 7.9 μ. One of them, Fig. 11, shows the predominance of free ribosomes, with a substantial degree of clustering, and the scarcity of the ER. The majority of 19S PFC were plasmacytic, about one-half being plasmablasts, 7.4 to 10.1 μ in diameter. A fine gradation towards mature plasma cells (Fig. 12) was seen, and 3 cells showed rounded sacs of ER. This population, then, showed a broad, finely graduated range of antibody-producing cells from lymphocytes to mature plasma cells.

19S PFC of the rabbit: In the case of the rabbit PFC only one large lymphocyte, 8.2 μ in diameter, was found among 26 cells. All other cells (96%) of this group were plasmacytic, two of them being plasmablasts. Most of the cells were mature plasmacytes with moderately distended, concentric layers of ER (Fig. 13). Only two cells had partial or complete vesiculation of the ER.

To see whether the small differences found between the two species might be due to a difference in the dose of antigen, rabbits were injected with antigen at a lower dose (1/50), or at a higher dose, by repeating the usual injection after 8 and 16 hr. The lower dose yielded a somewhat higher number of plasmablasts and lymphocytes, but the higher dose did not cause any further increase in the percentage of mature plasma cells. Thus, the 19S PFC of rabbit lymph nodes appeared to constitute a more homogeneous population of well differentiated mature plasma cells than did the mouse spleen cells.

7S PFC of the mouse: This group of cells showed substantial similarity in distribution of cell types to the 19S cells of this species. Of the 35 cells in this category, 2 were lymphocytes. One of these was a typical large lymphocyte with a light nucleus and single pieces of narrow ER. The other, a transitional lymphocyte, is presented in Fig. 14. This cell has lymphocytic features such as the condensation of the nucleus and the narrowness of the cytoplasm, which is
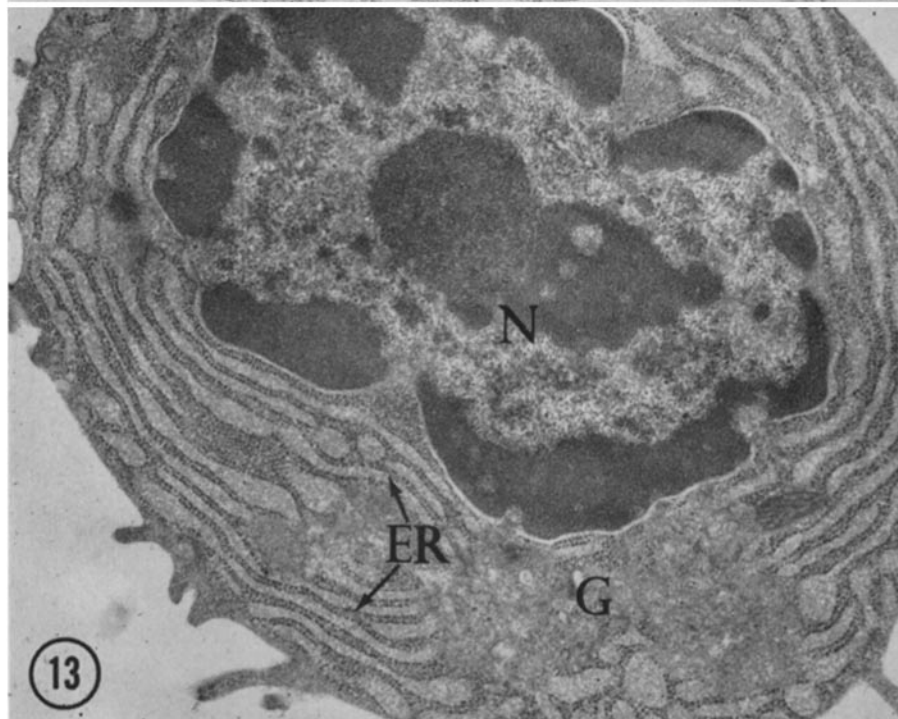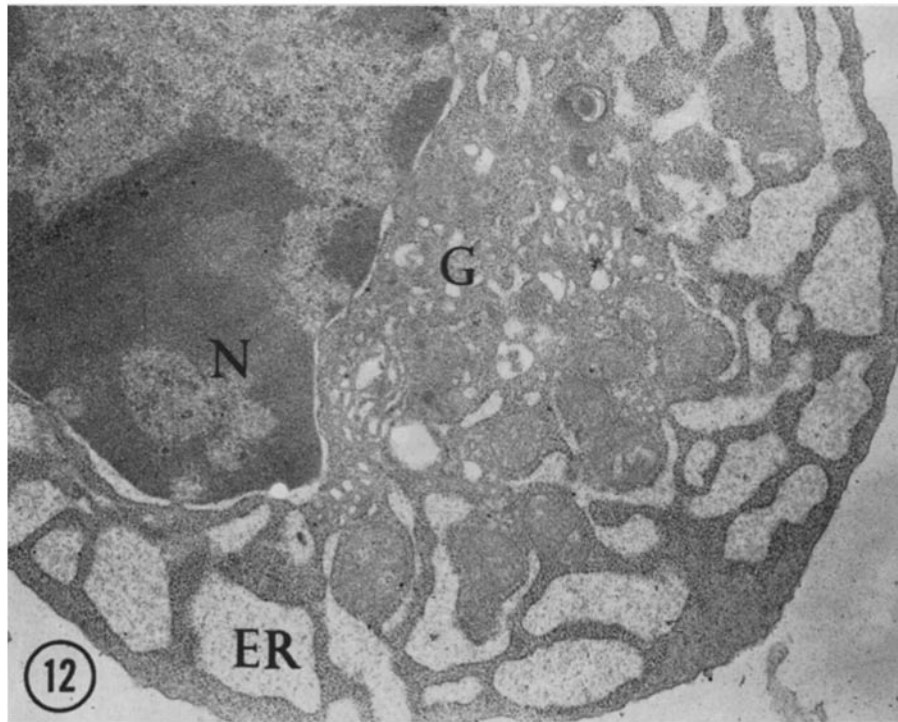
Fig. 12. PFC of the mouse, 19S. Plasma cell with distended perinuclear space and vesicular *ER*. (BALB mouse, primary, 4 days). × 17,800.

Fig. 13. PFC of the rabbit, 19S. Mature plasma cell with broad circular cisternae of the *ER*. Note small, but well demarcated Golgi area. (Lymph node, primary, 5 days). × 17,800.
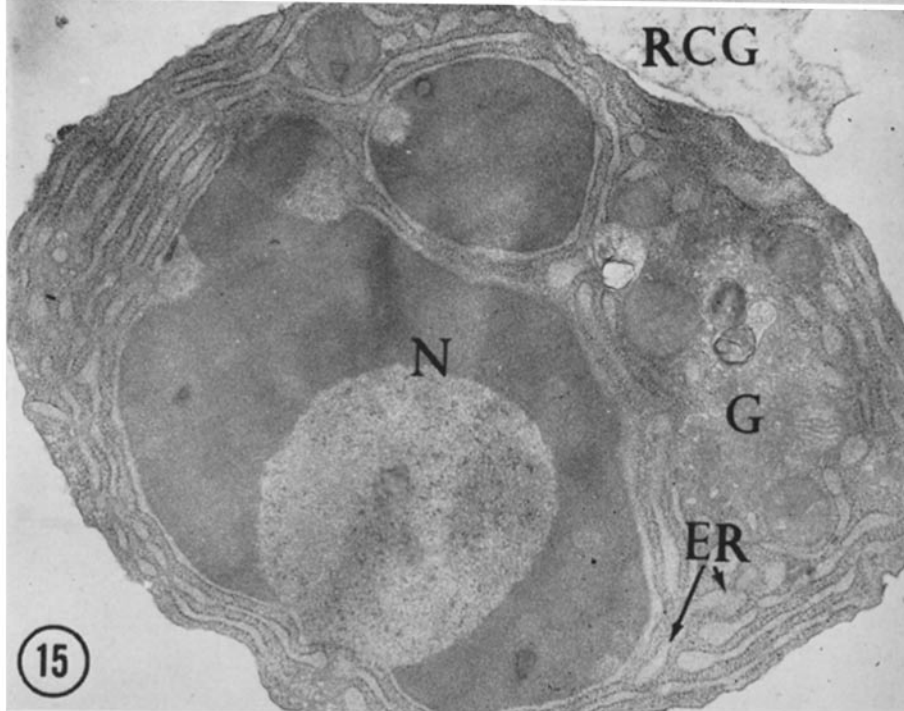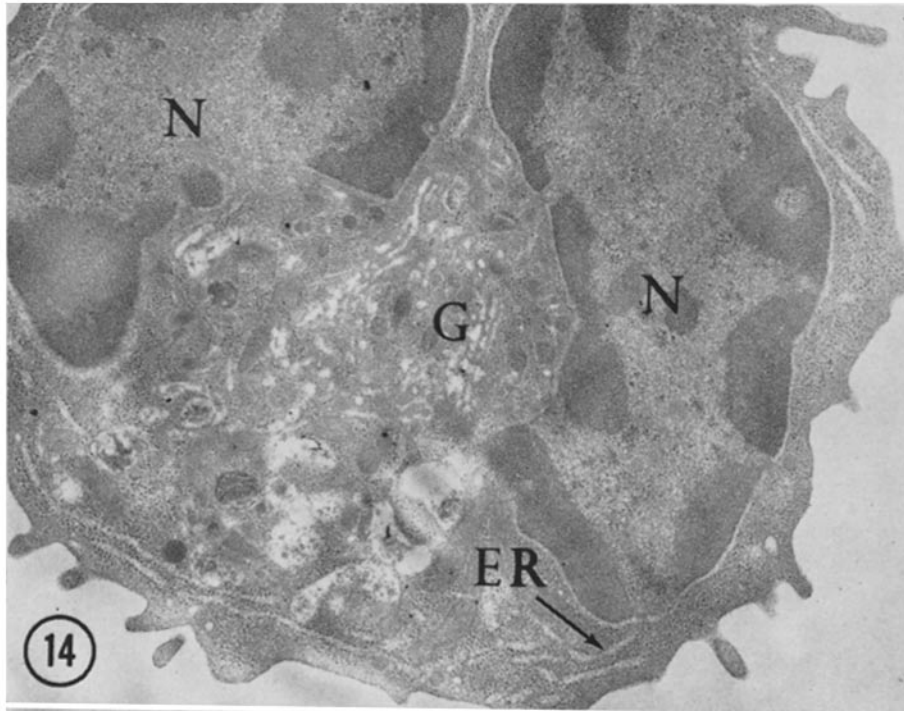
FIG. 14. PFC of the mouse, 7S. Transitional cell showing large Golgi body, a few channels of *ER*, slightly widened, and an irregular nucleus with condensed chromatin. (C57BL mouse, secondary, 5 days). × 15,800.

FIG. 15. PFC of the mouse, 7S. Mature plasma cell showing parallel bundles of rough *ER* of medium width and a small Golgi field. The nucleus shows a homogenization of the heterochromatin in a semilunar area. Note attached red cell ghost (*RCG*). (C57BL mouse, secondary, 5 days). × 18,000.
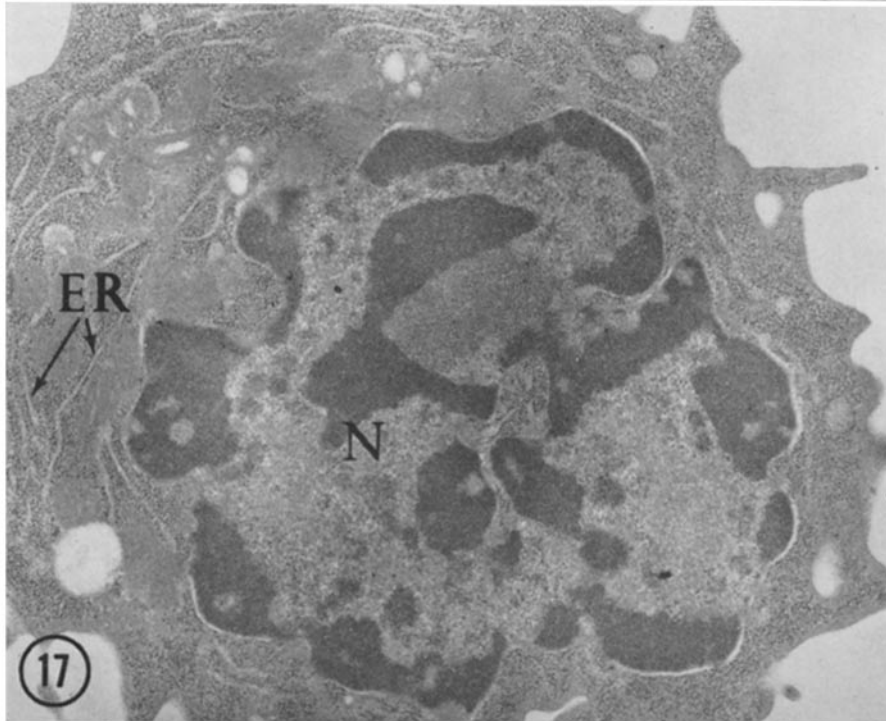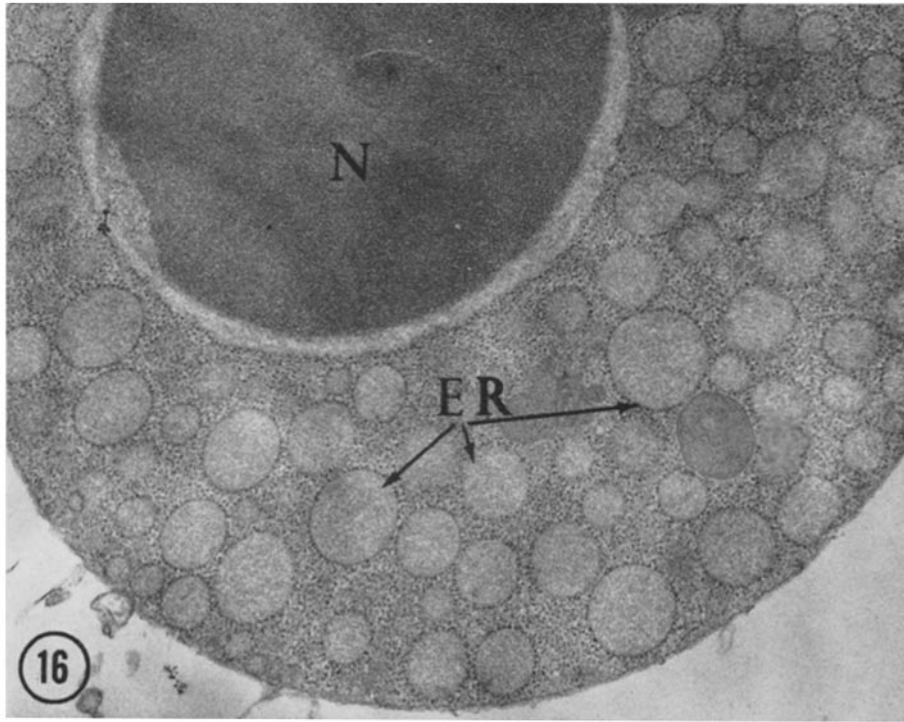
FIG. 16. PFC of the mouse, 7S. Plasma cell with homogenized nucleus and rounded sacs of rough *ER*. (C57BL mouse, secondary, 5 days). × 21,000.

FIG. 17. PFC of the rabbit, 7S. Large plasmablast with long, single *ER* channels in the ribosome-rich cytoplasm. (Lymph node, secondary, 5 days). × 18,000.

rich in ribosomes. However, its few channels of ER are, as in plasmablasts, slightly distended, and are of lengths (7.9 μ) unusual for lymphocytes of this size. It also has a notably large Golgi area.

Of the 33 mouse 7S PFC in the plasmacytic category, 5 were plasmablasts. The remaining, mature plasmacytes were approximately equally distributed
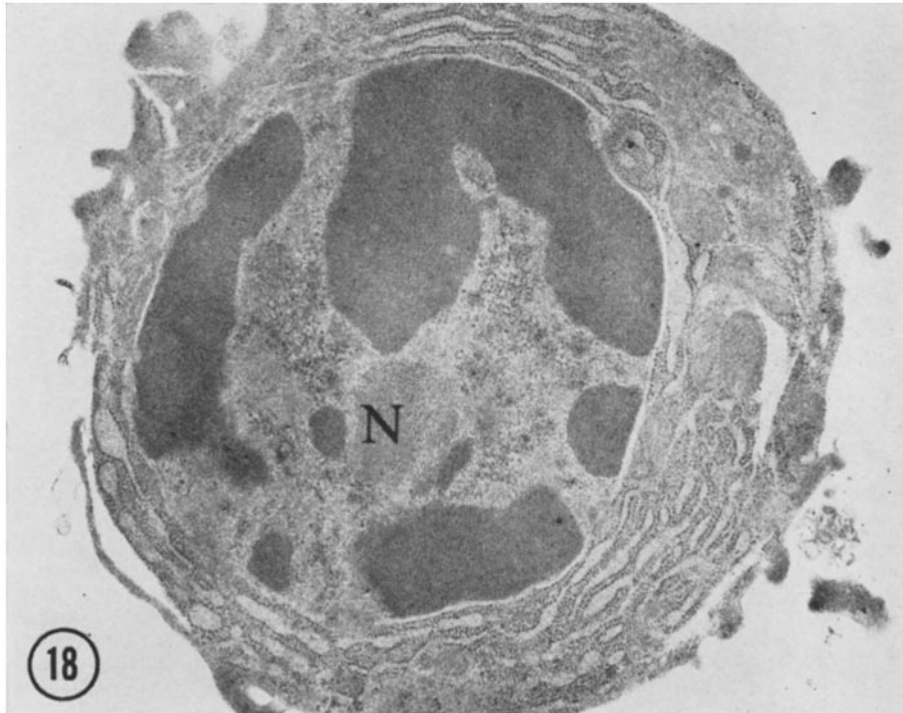

FIG. 18. PFC of the rabbit, 7S. Typical mature plasma cell with advanced nuclear chromatin condensation. (Rabbit lymph node, secondary, 5 days). × 19,000.

between those with parallel lamellae of ER (Fig. 15) and those with vesicular ER (Fig. 16).

7S PFC of the rabbit: Among the 24 cells examined in this group, no lymphocytes were found, and there was only one plasmablast (Fig. 17) that showed some resemblance to the transitional cell of Fig. 14 in that the channels of ER were comparably elongated, but rather narrow. The majority of these 7S rabbit PFC were well differentiated, typical plasma cells with concentric layers of rough ER of medium width (Fig. 18). These cells usually had only three to four parallel lamellae of ER in a rather narrow ring of cytoplasm, with a mean cell diameter of 6.6 μ (range 5.4–7.9 μ). Plasma cells with distended ER were

less frequent than in mouse 7S PFC, with only three of these showing vesicular ER.

The most general conclusions emerging from the results presented here are the following. First, antibody-producing cells detected by both methods, rosette formation and hemolytic-plaque production, were found in both the lymphocytic and plasmacytic categories. Second, a wide range of pleomorphism was found within each of these categories. Third, cells of the lymphocytic series constituted the great majority of the RFC and, in contrast, the plasmacytic category constituted the great majority of the PFC. Fourth, in comparisons of 19S- and 7S-producing cells among both RFC and PFC, no essential differences were found. Fifth, among both the RFC and PFC, cells were encountered which could only be classified as transitional. These were typical of the more differentiated large lymphocytes with some aspects of plasmablastic character in their ER.

The Rosette-Forming Cells.—It is generally assumed that RFC are antibody-producing cells. The most direct evidence for the involvement of antibody in rosette formation comes from the observation that this can be suppressed by specific antiglobulin (16–18). Further, it has been shown by several kinds of evidence that an active process is involved in the formation of rosettes by antibody-producing lymphoid cells.[2]

In the case of the macrophage, on the other hand, it was shown by Storb and Weiser (19) that antibody adsorbed to the surface of this cell could cause rosette formation. Rosette formation by macrophages was confirmed in this study. It is consistent with this mechanism of rosette formation by the macrophage that such rosettes were found with much higher frequency in spleens, with their rich blood supply, than in lymph nodes. Further, as will be shown in a subsequent study, the rosette-forming macrophages in the spleen increase substantially after day 4, when the serum antibody level begins to rise. In contrast, it has been shown that lymphoid cells cannot be coated passively with antibody to the point of rosette formation (8, 9, 19, 20).

The fact that even small and medium lymphocytes were found to produce rosettes would indicate that antibody production starts before the development of large lymphocytes or blast forms. However, it would appear that to reach the full rate of antibody secretion, cells would generally require morphologic adaptations from the inactive stage. Varying degrees of this adaptation or differentiation could account for the heterogeneity of the rosette-forming population.

The Plaque-Forming Cells.—The marked contrast between the predominance of lymphocytic cells among RFC and of plasmacytic cells among PFC is of interest in conjunction with the numbers of cells in a given suspension which can

make rosettes or plaques. By the techniques used in these studies the number of RFC in a given suspension has been found to be between 10 and 12 times as high as the number of PFC. This has also been shown by Zaalberg et al. (16). The findings that the plasmacytic cells constitute about 10% of the RFC but about 90% of the PFC, and that the number of RFC in our suspensions have been roughly 10 times the number of PFC, suggest that the plasmacytic cells are among the approximate 10% of the antibody-producing cells of a lymph node or spleen which have the highest rate of synthesis in a given response. This interpretation is also consistent with the consideration that the rate of antibody synthesis required to produce a hemolytic plaque would be substantially higher than the rate required to produce a rosette, even excluding the fact that cells might be producing rosettes on the basis of previously synthesized antibody. It is of interest in this connection that Cunningham et al. (8) found that plaque-producing cells which they had isolated could produce rosettes, but not all RFC could produce plaques.

The interpretation that the plasma cells could constitute a subpopulation of lymph node cells with the greatest rate of synthesis of antibody was supported by the finding of antibody-forming cells which were considered the most differentiated cells of the lymphocytic category—the transitional cells found among RFC (Figs. 3 and 4) and PFC (Fig. 14)—which had some points of similarity to the plasmablasts. These transitional forms also suggest, with the limited implications of morphologic evidence, a relation between lymphocytes and plasma cells, in which the plasmacytic cells are derived from the immunologically active lymphocytes of a given immune response, and are among the most differentiated and most actively synthesizing members of this group of responding cells.

The Detection of Antibody-Synthesizing Cells.—The observation that lymphocytic cells produce antibody, especially among the RFC, is consistent with our earlier findings (5, 6) that lymph node lymphocytes equipped with prominent nucleoli, Golgi bodies, and mitochondria, but lacking a substantial amount of ER, could produce antibody plaques, and were therefore judged to be continuously secreting antibody. On the other hand, in recent studies of antibody-forming cells based on the detection of antibodies within cells, even by the sensitive methods of electron microscopy, antiferritin (21) and antiperoxidase (22) have been demonstrated in the ER only of plasma cells, plasmablasts, and immature blast cells. It was considered, in those studies, that the plasma cells were derived from these blast cells and that antibody production starts at this stage, since no evidence of antibody was found in small lymphocytes, which had very scanty ER. The disagreement between these studies and ours, with respect to antibody formation by the lymphocytic cells, might be explained in part by quantitative differences in antibody production and in the sensitivity of the methods involved. Another difference between studies involving detection

of antibody within cells, referred to above, and those like the rosette and plaque studies, is that the former detect only the cells which contain antibody in sufficient concentration to be detectable by the technique employed, (each method, of course, having its own threshold of detection). The rosette and plaque tests, however, detect antibody-producing cells which can be secreting most of the antibody which they are synthesizing. Thus a cell could be producing and secreting enough antibody to produce a rosette or plaque without containing, at a given time, enough completed antibody to be detectable even by the sensitive electron microscopic anti-ferritin method.

The Fine Degrees of Differentiation in Antibody-Producing Cells.—This study gave evidence of fine gradations from small to large lymphocytes and blast forms. Fine degrees of differentiation between large lymphocytes and plasmablasts were also found; we have referred to these cells as transitional lymphocytes. We consider this nomenclature justified, although it rests solely on morphologic observations, because of the similarity of this type of cell to the large lymphocyte (especially the blast form). There is similarity in every respect except for one organelle, the ER, in which the cell shows fine differences in the direction of the ER of the plasmablast. Thus, if there is in fact a transition from the lymphocytic to the plasmacytic cell, this is probably via blast cell formation. Also, on the assumption of such a transformation, it would appear that only some of the large lymphocytes develop into plasma cells, while the others remain within the lymphocytic group, with considerably expanded cytoplasm.

Whether the cellular differentiation is in the direction of a large lymphocyte or a plasma cell, however, the majority of the active cells acquire a considerable volume of cytoplasm, while the increase in size of the nucleus is substantially less. This addition of lower-density cytoplasmic material causes a decrease in mean density of the cell as a whole, and accounts for the appearance of active cells, on differential centrifugation in solutions of bovine plasma albumin, in lower density portions of the tube. This has been noted in both discontinuous layers (23) and continuous gradients (24) of albumin solutions.

The finding of mitotic cells with morphology ranging from cells with no ER to cells with the slightly distended ER of the transitional lymphocytes indicates that cells with this degree of differentiation are within the proliferating population of antibody-producing cells. In a subsequent paper[4] it will be shown by in vitro pulse labeling with ^{3}H-thymidine that proliferating RFC include plasmablasts as well as all types of lymphocytes.

19S and 7S Producing Cells.—The population of cells producing 19S and 7S antibody were essentially similar. There were only minor differences in the rates of occurrence of cells of each category; both had the same fine gradations, and occasional transitional cells. Such

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[4] Gudat, F., T. N. Harris, S. Harris, and K. Hummeler. Data in preparation.

differences as were found were, in the case of both the mouse and the rabbit, a somewhat higher frequency of occurrence of mature plasmacytes than of plasmablasts in the 7S PFC than the 19S. Similarly, among the plasmacytes themselves, the 7S PFC showed a higher rate of occurrence of the cells with the most advanced levels of differentiation, i.e., those with the vesicular ER or the rounded sacs. In view of the very low efficiency of 7S antibody for heholysis, a greater amount of 7S than of 19S antibody could be required for plaque production, even with the use of anti-IgG. The 7S plaques might require especially high rates of synthesis by PFC, and therefore might favor the frequency of cells which, by the abundance of their ER, give other evidence of the highest rate of synthesis. Alternatively, the greater amounts of antibody required to produce a 7S plaque could be made available by the more highly differentiated cells by "leakage" of antibody from the cell, since we have often found degenerating cell membranes in the more highly differentiated plasma cells.

The observations made in this study are of interest in relation to the question of whether 7S antibody-producing cells are derived by conversion from 19S producing cells, a development suggested by Nossal et al. (25), and by Möller et al. (26). Our data would suggest that 7S antibody-producing cells can arise independently and need not develop from 19S producing cells. First, among PFC of the mouse, cells in early stages of differentiation could be found among the 7S PFC, suggesting direct development from their precursors. Further evidence of this is provided by the data obtained with RFC. In the total population of RFC it was seen that the same proportion of undifferentiated cells found early in the primary response was found in the late stages of the primary response as well as during the secondary response when 7S producing cells were to be expected. With the additional aspect of complement treatment of the rosettes to select out the 7S antibody RFC, it was found that, against the background of the high percentage of lymphocytes in the total population of RFC, the 7S RFC showed no shift toward an increase in plasma cell percentage, as one would expect if the 7S antibody-producing cells represented late stages in the maturation of cells originally producing 19S antibody.

SUMMARY

Antibody-bearing cells of spleen and lymph node of the mouse and rabbit detected by rosette formation with the antigenic red blood cells were collected by micropipet and studied by electron microscopy. More than 300 such cells were examined. In the lymph nodes, rosette-forming cells were all in the lymphocytic and plasmacytic categories. In cells of the mouse spleen, macrophages were also found among the RFC, especially in the later days after immunization.

The great majority of the RFC, 70–100%, were of the lymphocytic category. These included small, medium, and large lymphocytes with fine gradations of differentiation, and blast forms with little heterochromatin. The endoplasmic reticulum of these cells occurred in short, very narrow pieces, usually in contact with a mitochondrion. The cells of the plasmacytic category also showed fine gradations from plasmablasts to typical mature plasma cells.

Plaque-forming cells of mouse and rabbit were also collected by micropipet. Of 162 such cells, fine gradations were also found throughout the lymphocytic and plasmacytic categories, but in this case the great majority were in the plasmacytic group, and more plasma cells showed amorphous nuclear chromatin.

Among antibody-forming cells detected by both reactions, some of the more highly differentiated large lymphocytes contained ER which differed from that in the other large lymphocytes in that the channels were slightly and variably distended, with deposition of some precipitate, and with some tendency to a more nearly parallel orientation of the few channels seen. These were considered transitional cells.

Of 10 RFC found in mitosis, all were in the lymphocytic category, in various stages of differentiation, the most advanced of which (in 2 of the 10 cells) was that of the transitional lymphocyte described here.

Cells producing plaques facilitated by antisera vs. IgG of the mouse or rabbit (7S) showed the same distribution between cell categories and the same fine gradations as the direct (19S) PFC.

Cells producing rosettes which were resistant to lysis in the presence of complement, and were thus presumably producing 7S antibody, showed a distribution similar to that found generally with rosette-forming cells, approximately 80–90% in the lymphocytic category.

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