STUDIES ON ANTIBODY-PRODUCING CELLS

II. Appearance of ^{3}H-Thymidine–Labeled Rosette-Forming 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, Philadelphia, Pennsylvania 19146, and the Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104)

(Received for publication 18 August 1970)

A preceding paper described the ultrastructure of antibody-forming cells of mice and rabbits studied in considerable numbers (1). In confirmation of previous studies from this and other laboratories, it was shown that lymphocytes and plasmacytes in various stages of differentiation contributed to the heterogeneity of the antibody-producing cells (2–8). Morphological forms suggesting transition between cells of the lymphocytic and of the plasmacytic series were also described (1). A comparison of cells forming rosettes with those producing plaques showed that the rosette-forming cells (RFC)[1] were largely in the lymphocytic series, whereas the plaque-forming cells (PFC) were largely plasmacytic (1).

For further study of antibody-producing cells, especially in the early stages of proliferation and antibody release, RFC were chosen, since the number of rosettes produced by a given suspension of antibody-producing cells is many times the number of plaques, so that rosette formation would clearly provide the more sensitive technic. In the present study the kinetics of antibody-forming cells was studied both in terms of the numbers of RFC and of uptake of radioactive label. Cells were obtained from antibody-producing spleens of mice and lymph nodes of rabbits, at various intervals after a first or second injection of antigen, for determination of frequency of RFC and of RFC which could be

* This study was supported by Grants HE 04598 AI, AI 09657, and AI 04911 of the National Institutes of Health, U.S. Public Health Service, and Grant T526B of the American Cancer Society.

‡ Present address: Pathologisches Institut, University of Basel, Basel, Switzerland.

§ Reprint requests should be sent to Dr. T. N. Harris at the Children's Hospital of Philadelphia, 1740 Bainbridge St., Philadelphia, Pa. 19146.

[1] *Abbreviations used in this paper:* ER, endoplasmic reticulum; MEM, minimal essential medium; PFC, plaque-forming cell; RBC, red blood cell; RFC, rosette-forming cell; SRBC, sheep red blood cell; 1°, primary injection of antigen; 2°, secondary injection of antigen.

labeled with tritiated thymidine in the total spleen cell population, in the nonadherent cell population, and among the large lymphocytes. A time relation was shown between the labeling index of RFC and the numbers of lymphoid cells producing rosettes by 19S and 7S antibody. It was also found that during the period of rapid increase of rosettes, labeled RFC were found to range in morphology from lymphocytes to well differentiated plasmablasts.

Materials and Methods

Animals and Immunization.—Female BALB/c and C57BL/6 mice (the Jackson Laboratory, Bar Harbor, Maine) were given intraperitoneal injections of 1 ml of 2% (BALB) or 10% (C57BL) washed sheep red blood cells (SRBC). The animals were pooled in groups of two to three and sacrificed 2–10 days after the primary or secondary injection. Rabbits received 0.5 ml of 50% SRBC suspension in each hind footpad and were sacrificed 4–5 days later.

Rosette Formation.—As described earlier (1), 1 ml of mouse spleen cells or rabbit lymph node cells, 20×10^6/ml, were incubated with SRBC at a final concentration of 2% for mouse cells and 0.5% for rabbit cells. After 1 hr of incubation at 37°C, counts were made in a hemocytometer and the number of rosettes was calculated per 10^6 leukocytes.

In Vitro Labeling with ^{3}H-Thymidine.—Pellets of $20–40 \times 10^6$ leukocytes were suspended in Eagle's minimal essential medium (MEM) containing tritiated thymidine (Schwartz Bioresearch, New York, specific activity 6.0 Ci/mmole, or New England Nuclear Corp., Boston, Mass., specific activity 15 Ci/mmole) in concentrations of 10 μCi/ml for light microscopy, or 20 μCi/ml if electron microscopy was to be done. After an incubation of 1 hr at 37°C the cells were washed twice and suspended at 20×10^6/ml for rosette formation. Alternatively, the isotope could be added to the rosette preparation, thus avoiding the preincubation time.

Embedding of Rosettes for Light and Electron Microscopy.—The method for collecting rosettes and embedding for electron microscopy has been described in detail previously (1). For light microscopy the rosettes were distributed with micropipettes over the bottom of plastic embedding capsules, as for electron microscopy, and embedded in Epon 812. For each experiment at least two capsules were prepared, with 50–100 rosettes per capsule. After hardening, the blocks were sectioned without further trimming on a Porter-Blum MT-2 microtome (Ivan Sorvall Inc, Norwalk, Conn.). The knife was adjusted at such an angle that the block face containing the monolayer of rosettes was hit in a parallel plane. The first 1 μ sections were transferred to a drop of distilled water on a microscope slide. The sections were flattened and dried to the glass over a small flame.

Radioautographic Methods.—Ultrathin sections were covered with Ilford L4 emulsion (Ilford Limited, Ilford, England) diluted 1:3 by using the loop method of Caro and van Tubergen (9). After an exposure time of 4–6 wk the sections were developed in Microdol X (Eastman Kodak Co., Rochester, N. Y.) for 5 min, passed through 2% acetic acid, and fixed in Kodak rapid fixer for 5 min. The sections were stained for 20–30 min in 0.01% lead citrate and 10 min in 1% uranyl acetate, and were viewed in a Siemens Elmiskop I electron microscope.

Slides with semithick sections were dipped into Kodak NTB3 emulsion and exposed for 4 days. They were then developed in Kodak D11 (3 min, 20°C), fixed in Kodak rapid fixer (2 min), rinsed, and stained for 3–5 min in 0.1% toluidine blue as described by Trump et al. (10). The cytological classification was carried out under oil immersion, using the section which showed most of the rosettes in an optimal plane.

RESULTS

Kinetics of Rosette-Forming Cells.—Rosette counts yielded by mouse spleen cells at various intervals after injection of SRBC are shown in Table I, and the curve of the mean values is shown in Fig. 1. After primary injection of the antigen, the number of rosettes began to rise above background level (300/10^6 spleen cells) after a lag of approximately 3 days, followed by an exponential rise from the 3rd to 5th day, when a first peak of about 6000 RFC/10^6 spleen cells was reached. After a reduction in rosette count at about the 7th day, a second peak was reached on day 10. Thereafter, the number of rosettes decreased slowly.

After a repeated injection of the primary dose into BALB/c or C57BL/6

TABLE I

Rosette-Forming Cells and Percentage of ^3H Labeling in Mouse Spleen Cells Obtained after Injection of SRBC

Day after SRBC injection	Rosette-forming cells per 10^6 spleen cells									Per cent of ^3H labeling in RFC					
	BALB (1°)			BALB (2°)			C57BL (2°)			BALB (1°)		BALB (2°)		C57BL (2°)	
	No. of Exps.	Mean	Range	No. of Exps.	Mean	Range	No. of Exps.	Mean	Range	No. of. Exps	Mean	No. of Exps.	Mean	No. of Exps.	Mean
0	6	330	40–790	5	840	590–1370	9	940	850–1370	3	0.5	2	5.7	2	13.0
2	7	380	260–590	2	1570	1500–1650	3	1530	1300–2000	3	24.5	1	13.4		
3	5	700	410–1630	2	2550	2260–2850	5	3400	1700–7300	2	44.3	2	22.0	3	48.4
4	21	3430	1250–6150	6	4290	2500–6800	6	8570	2330–13,660	11	43.9	1	54.0	2	45.7
5	10	5760	1850–124,000	6	4250	1260–6600	5	9800	2040–17,660	5	37.9	2	44.7	1	43.5
6	6	3750	2090–4300							2	42.8				
7	11	3550	1180–9860				6	7100	1880–14,100	8	49.0			3	27.7
10	7	5500	3100–6000	5	1690	1260–2030	4	3000	2300–2030	4	34.2	1	5.9	3	12.4
18	6	4110	2480–5000												

mice, the number of rosettes began to increase, from a slightly higher background level, on the 2nd day, and reached a maximum on the 4th or the 5th day in the respective strains. The number of rosettes produced by C57BL/6 cells was twice that of BALB/c cells. The curves of both strains, however, were similar in that they were monophasic and that they returned to background level more rapidly than during the primary response. On the 10th day of the secondary response the rosette count was lower than on the corresponding day of the primary response.

Light-Microscopic Radioautography.—In radioautographic slides examined by light microscopy the lymphoid cells were classified as small to medium, and large, lymphocytes (including blastoid forms), according to their size, cytoplas-

mic volume, and basophilia of the nucleus. Labeled macrophages were extremely rare and were not included in this tabulation.

In preliminary experiments with 4-day cells (Table II) the optimal procedure for the in vitro labeling of lymph node or spleen cells was found to be a maximal exposure time of 60 min with either 10 or 20 μCi of tritiated thymidine. This

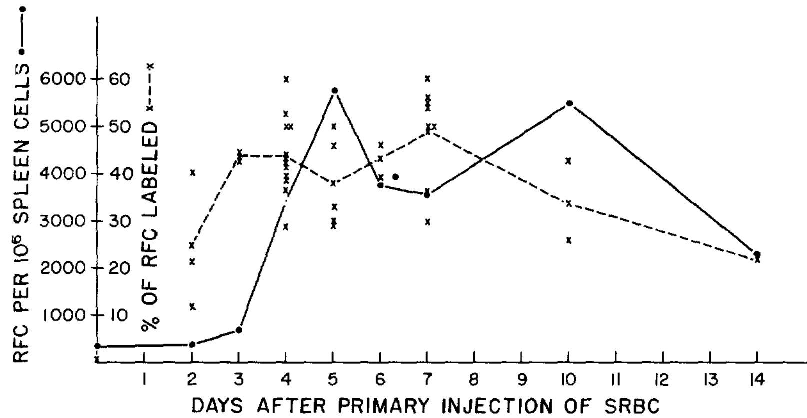

FIG. 1 *a*. Mean values of RFC/10^6 spleen cells (●——●), and percentage of RFC labeled with tritiated thymidine (X----X), at various days after primary injection of SRBC into BALB/c mice. Individual values (X), as well as the mean, are shown for the per cent of RFC abeled.

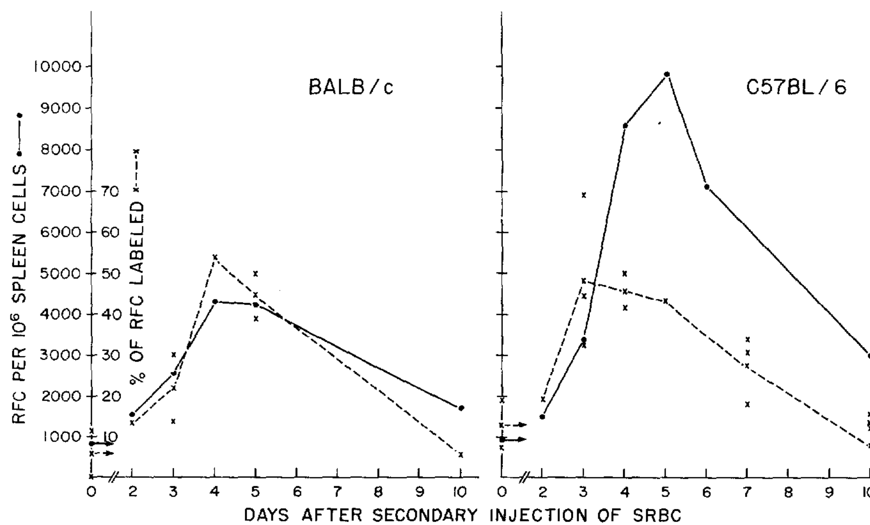

FIG. 1 *b*. RFC/10^6 spleen cells, and percentage of RFC labeled with tritiated thymidine, after secondary injection of SRBC into BALB/c or C57BL/6 mice. Notations as for Fig. 1 *a*.

amount of the isotope had no inhibitory effect on rosette formation. However, the extension of the preincubation to 2 hr led to a reduction in the rosette count. Table II also shows that the ^3H index of rosette-forming cells was significantly higher than that of the original spleen cell population. This level of 40–50% labeled lymphoid cells was also found in rosettes from lymph node cells of mouse and rabbit. In comparative experiments on in vivo labeling, SRBC-injected mice were given 0.15-0.30 mCi over a period of 5 hr, and then were sacrificed an hour later. In spleen cells of such mice, tritium labeling was found in 40–65% of the lymphoid cells. The similarity between the in vitro and in vivo uptake indicated that the conditions of in vitro pulse labeling used here reflected the in vivo pattern of cell replication.

The labeling index of rosette-forming lymphoid cells is shown in Fig. 1 in relation to the kinetics of the rosette formation. From a background of 1 labeled

TABLE II

In Vitro Labeling with ^3H-Thymidine of Rosette-Forming Cells of Mouse Spleen

Exposure to ^3H	1 μCi/ml			20 μCi/ml			Control
	RFC per 10^6 cells	Per cent ^3H labeling		RFC per 10^6 cells	Per cent ^3H labeling		RFC per 10^6 cells
		RFC	Total cells		RFC	Total cells	
(min)							
30	7850	7.6		6750	51.5	9.6	6150
60	4750	37.0	10.3	5750			6260
120	4100	37.8	11.2	4100	55.5	6.8	4000
240	1500	49.0	4.0	1600	48.0	5.4	1600

cell among 103 rosettes from uninjected animals, the percentage of labeled cells in the primary response (Fig. 1 *a*) rose on the 2nd day, at a time when an increase in the number of rosettes had not yet been observed. Maximal labeling was reached on the 3rd day. After a decreased level on days 5 and 6, a second peak was found on the 7th day. Thus, the maximal thymidine incorporation into rosette-forming cells was reached 2–3 days before the peaks of the total rosette counts.

In the secondary response the level of thymidine incorporation in the background population was slightly higher than in the primary response. The full rate of labeling was reached on the 3rd day in C57BL/6 and on the 4th day in BALB/c mice, as can be seen in Fig. 1 *b*. The degree of labeling showed a monophasic curve, as did the rosette counts.

The data in Fig. 1 *a* show, in the latter part of the primary response, a second rise in rosette numbers, which followed the second rise in labeling index by approximately 2 days. However, since macrophages among the RFC are increasing to considerable percentages during this period (5–10 days), and since the macro-

phages are labeled only extremely infrequently, as will be noted below, it was necessary to evaluate the contribution of macrophages to the total RFC during this period in order to study the relation of the replicating population of antibody-producing cells to rosette formation. In order to examine this point with more appropriate data, the interval of 6–10 days after primary injection of SRBC was reexamined, including rosette counts not only of the total suspension but also of the cells in the suspensions which were not adherent to plastic, to

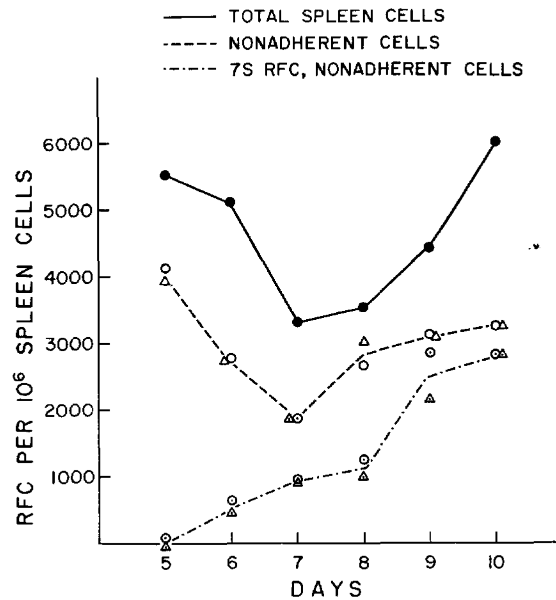

FIG. 2. RFC/10^6 spleen cells following primary injection of SRBC into BALB/c mice. Numbers are given for the total spleen cell suspension, for cells of this suspension which did not adhere to plastic, and, of the nonadherent cells, RFC with red blood cells resistant to lysis by complement (7S RFC). Nonadherent cells were collected after incubation of cells in plastic dishes on an oscillating surface (○) or on a rotating surface (△).

eliminate macrophages from the population examined. When this was done a rise of rosette-forming cells between the 7th and 9th day due to nonadherent cells was observed. Finally, in order to test one possible source for the new wave of RFC, nonadherent cells were examined for rosette formation by a method which had been found to eliminate rosettes due to 19S antibody, and thus presumably select out rosettes due to 7S producing cells, by including complement in the rosette preparation. The data of such experiments are shown in the bottom curve of Fig. 2, where it can be seen that the number of rosettes presumably due to 7S antibody rose from very nearly zero on the 5th day to almost 3000/10^6 cells on the 10th day after primary injection of SRBC, the greatest rate of increase being between days 8 and 9.

Contribution of Large Lymphocytes to RFC.—Cytologic examination showed that there was a marked increase in the number of large lymphocytes among the RFC during the first 3 days after immunization. As shown in Fig. 3 (solid line), from a very low percentage of large lymphocytes among the background RFC, this percentage rose to 97 by the 3rd day in the primary response and to about 90 on the 4th day in the secondary. The percentage of large lymphocytes decreased more slowly in the primary than in the secondary response, as did the rosette counts. The two broken lines in this figure show the percentage of

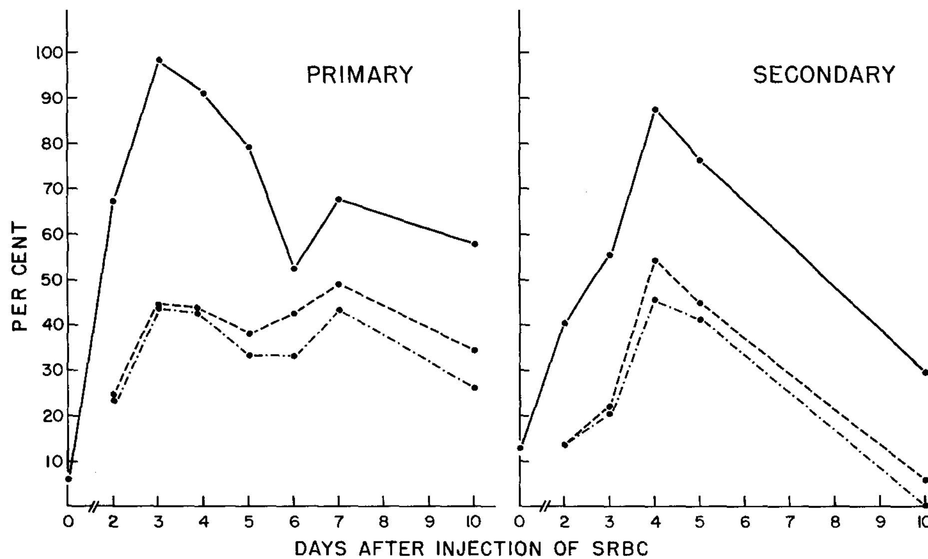

FIG. 3. Large lymphocytes and labeled lymphocytes among RFC. Percentage of RFC which are large lymphocytes (●——●); percentage of RFC which are labeled (●-----●). percentage of RFC which are labeled large lymphocytes (●-·-·●).

RFC which were labeled lymphoid cells and large lymphoid cells respectively. It can be seen that the majority of the labeled cells were in the large lymphocyte population. There was only a small contribution of small to medium sized lymphoid cells to the labeled RFC, represented by the differences between the broken curves in Fig. 3.

Electron Microscopy.—The terminology used in the following descriptions is that of a previous report (1). In brief, within the lymphocytic category, cells were classified as small to medium, and large lymphocytes, the latter including also blastoid lymphocytes and the transitional cells which we have found among RFC and PFC. The transitional cells, more fully described elsewhere (1), are typical large lymphocytes except for channels of endoplasmic reticulum (ER),

slightly and variably distended, with deposition of protein-like material and more nearly parallel orientation of the channels.

The results of the classification of pictures of individual RFC taken from various experimental conditions is summarized in Table III. Extensive observations of such cells showed that most of the labeled cells were large lymphocytes, but that incorporation of thymidine also took place in plasmablasts of considerable differentiation. The silver grains were usually located at the borderline of condensed and loose chromatin, in typical cases (Fig. 4) as a corona along the nuclear membrane.

The earliest cells examined were RFC of mouse lymph nodes on the 3rd day after primary immunization (Figs. 4 and 5). These were lymphocytes, mostly large with a relatively narrow unorganized cytoplasm (Fig. 4) containing dense ribosomes, occasionally in clusters. The majority of the cells showed an increase of mitochondria and short collapsed profiles of ER (Fig. 5). The nuclei of labeled

TABLE III

Classification of ^{3}H-Labeled Rosette-Forming Cells by Electron Microscopy

Cell source	No. of lymphocytes		No. of plasmacytes	
	Medium	Large	Plasmablast	Plasmacyte
Mouse Lymph node 1° — day 3	—	6	—	—
Spleen 1° — day 4–5	3	19	2	—
Spleen 1° — day 7–10	—	4	—	—
Spleen 2° — day 4–5	6	18	2	—
Rabbit Lymph node 1° — day 5	—	4	2	—
Lymph node 2° — day 5–6	—	13	1	—

cells showed an advanced loosening of the chromatin and only marginal patches of condensed chromatin. The nucleoli were prominent in many of these cells.

In lymphocytes of mouse spleens of the 4th and 5th day more advanced differentiation of the cytoplasm was seen. An increase of mitochondria, cytoplasmic volume, and numbers of polyribosomes was seen in 6 out of 22 cells. In 5 others there was, in addition, a marked formation of rough ER, generally narrow to medium but occasionally wide, suggesting morphologic transition towards plasmablasts. Such cells were also seen in the secondary response, but more of the RFC were undifferentiated lymphocytes. Occasionally, the transitional lymphocytes described above were seen with markedly elongated very narrow ER. Some plasmablasts with dilated ER were also found, like the rabbit plasmablast of Fig. 7.

A similar range of differentiation was also encountered in RFC of the rabbit lymph node. Again the majority of the cells, especially in the secondary response, was in the category of large lymphocytes with rudimentary ER, as in the mouse cells of Figs. 4 and 5. Plasmablasts of different developmental stages were some-

what more frequent, ranging in degree of differentiation from cells such as that shown in Fig. 6, to a rare cell with the most advanced level of differentiation, shown in Fig. 7.

The Role of Macrophages in Rosette Formation.—From the examination by light microscopy it was possible to obtain data concerning the numbers of macrophages involved in rosette formation, and the percentage of phagocytic cells among RFC was found to vary among experiments. Of the low number of

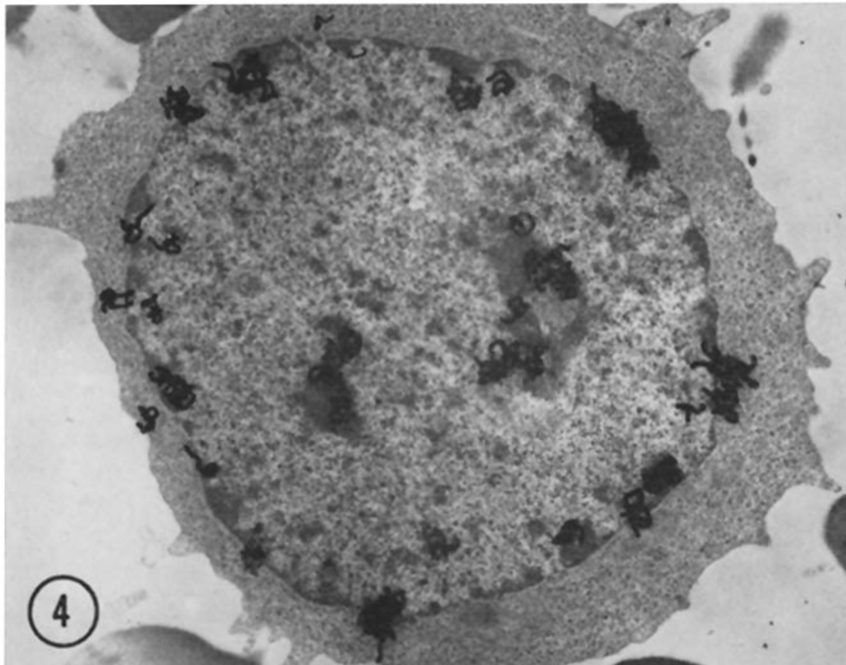

FIG. 4. ^{3}H-thymidine-labeled rosette-forming cell from mouse lymph node, 3 day primary. Lymphocyte with narrow, undeveloped cytoplasm containing polyribosomes. × 11,760.

RFC in spleen cells of uninjected mice (background RFC), the macrophages constituted about 20%. As the replicating lymphocytes began to contribute to the population of RFC the percentage of macrophages fell to about 10% on day 4. In the latter days of the primary response (after the 5th day) the percentage of macrophages among RFC began to rise, occasionally reaching levels as high as 60%. After the secondary injection of antigen the percentage of macrophages began to increase earlier. Labeling with tritiated thymidine was not observed in any of 1156 rosettes formed by phagocytic cells of the BALB/c primary response. In the secondary response the tritium label was found in 2 of 454 macrophage RFC (in BALB/c cells), and in 11 out of 801 rosettes (C57BL/6).

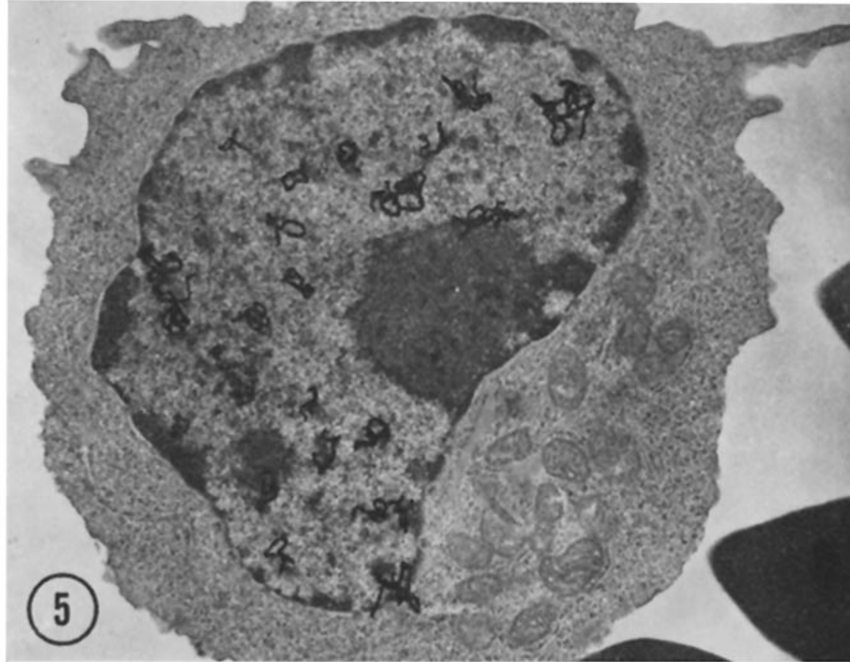

FIG. 5. Same as Fig. 4. Cytoplasm is enlarged, containing several mitochondria at one cell pole and short, narrow channels of ER, in some cases in close proximity to mitochondria. × 12,920.

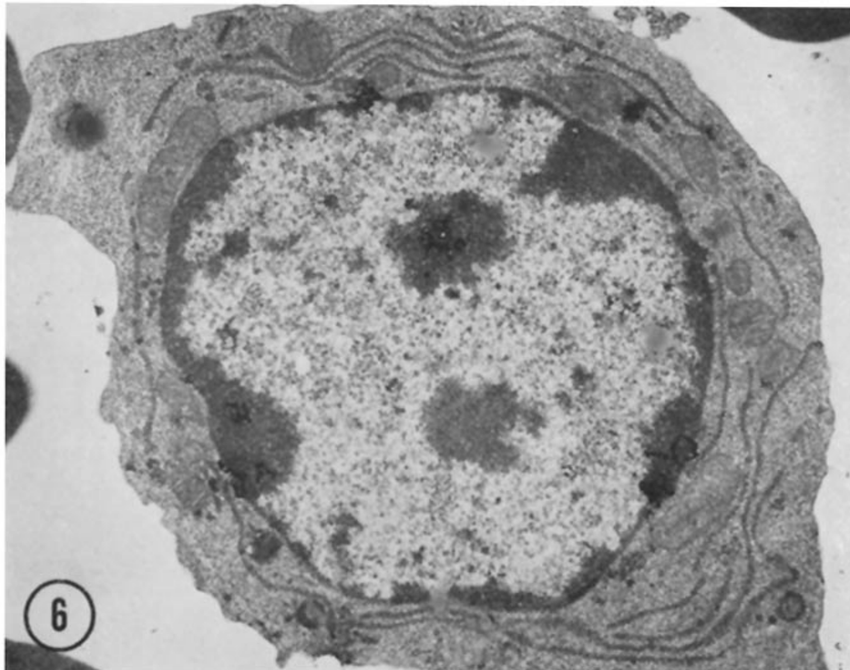

FIG. 6. ^{3}H-thymidine-labeled RFC from rabbit lymph node, 6 day secondary. Small plasmablast with long, narrow channels of ER and several dispersed mitochondria. × 10,830.

A frequent observation was the attachment of lymphocytes, mostly small to medium sized, to rosette-forming macrophages. These "mixed" rosettes were more frequent in early days than in the late stage of the immune response. The labeling index of such macrophage-associated lymphocytes at various days of

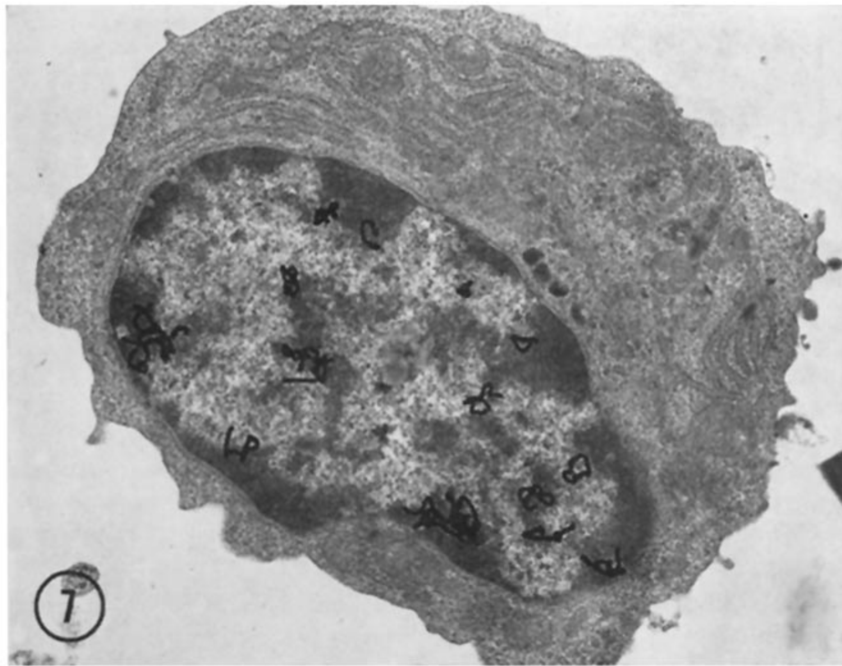

FIG. 7. Same as Fig. 6. 5 day primary. Numerous widened channels of ER are visible. × 12,600.

TABLE IV

Lymphoid Cells Attached to Rosette-Forming Macrophages, Numbers and Per Cent Labeled with ^{3}H-Thymidine

Day after SRBC injection	No. of unlabeled lymphoid cells	No. of labeled lymphoid cells	Per cent of attached lymphoid cells which were labeled
Day 0	27	2	6.9
1° – day 2	68	11	13.9
1° – day 3	21	15	41.6
1° – day 4	56	29	34.2
1° – day 10	57	24	29.6
2° – day 0	80	4	4.6
2° – day 2	98	24	20.0
2° – day 3	65	43	39.7
2° – day 4	13	8	38.0
2° – day 10	26	3	10.3

primary and secondary response, shown in Table IV, was considerably higher than that of the lymphocytes of the total spleen cell population (Table I) and was, in fact, quite similar to that of the lymphocytes involved in rosette formation (Figs. 1 *a* and 3). In a few radioautographs of ultrathin sections such cells could be examined. They were small lymphocytes with only moderate levels of differentiation.

DISCUSSION

The number of RFC in an antibody-producing mouse spleen, plotted in relation to time after injection of antigen, showed two peaks, as seen in Fig. 1 *a*, the first at day 5 and the second at about day 10. When the percentages of RFC which were labeled with tritiated thymidine in vitro were similarly plotted, it was found that the labeling index also showed two peaks, one peak being attained on day 3 and the other on day 7, each about 2 days before the respective peak frequencies of RFC among the spleen cells. The interpretation of the first peak of RFC and of tritium-labeled cells appears to be clear in the light of recent knowledge that a population of replicating cells is involved in the immune response after injection of an antigen (11–13). However, to interpret correctly the second peak of RFC, it was necessary to take into account the contribution to the total RFC of macrophages, which have a secondary role in producing rosettes, by adsorbed antibody (14, 15). This consideration was particularly relevant here since the macrophages, reflecting their passive role of adsorption in the production of rosettes, increase in number substantially with the increase in serum titer of antibody from day 5 to day 10, a curve which follows by about 2 days the second curve of increasing tritiated thymidine uptake by the replicating RFC of the lymphatic system. To eliminate this source of misleading or irrelevant data by macrophage RFC, the numbers of RFC in this period were reexamined in suspensions of cells from which the macrophages had been very largely removed by their property of adherence to plastic surfaces (16). A comparison of rosette formation by suspensions of spleen cells which were largely free of macrophages is shown in Fig. 2, where again there was a decline in the number of RFC per million spleen cells from day 5 to day 7, and then an increase, especially to day 9. This could now be attributed to the lymphoid cell population. Since this second rise in numbers of RFC, with associated replication of lymphoid cells, occurred at a time when 7S antibody shows its main rate of increase, it was of special interest to see whether RFC due to 7S antibody could be shown to account for a substantial increase in the thymidine-labeled RFC at this time. The curve of 7S RFC, obtained by the use of complement to exclude rosettes due to 19S antibody, showed an increase which, in fact, had the same time relation to the second rise of labeled RFC as that of the earlier peak of RFC to the first peak of thymidine-labeled cells.

The fact that the second increase in labeled cells slightly precedes the time of

greatest increase of 7S-producing cells suggests that at least a substantial part of the 7S-producing cells are a replicating population rather than a result of differentiation or development from cells producing 19S antibody of that specificity, as suggested by Nossal et al. (17) and by Möller et al. (18).

It is consistent with the interpretation that the second rise in RFC of the primary response is due to replicating cells producing 7S antibody, that in the secondary response the curves of both the RFC and the percentage of labeled RFC were monophasic, since in the secondary response the appearance of 7S antibody-producing cells is so early in the response that it could well not be possible to demonstrate a separation in time from the 19S part of that response.

The new population of lymphocytes involved in a beginning immune response is characterized by increased size,[2] due largely to an increase in cytoplasmic volume, in comparison with the resting lymphocyte. Use was made of this property of lymphocytes newly involved in antibody synthesis to refine our observations, by determining the frequency of large lymphocytes in the RFC and comparing the time relations of the frequency of these with the percentage of labeled cells. Fig. 3 shows the sharp increases of large lymphocytes among the RFC in the early days of the immune response, such cells comprising almost all of the RFC on day 3. As in the data on RFC of the total spleen cell suspension of Fig. 1, there were also among the large lymphocytes two peaks of frequency of RFC and two peaks of labeling index, related in time. In this case, however, the time relation was different. Since the observations of Fig. 3 were on the population of newly involved cells, the peak frequency of large-lymphocyte RFC occurred on the same day that the peak frequency of labeled cells was attained, rather than 2 days later. The difference in the secondary immune response, where only a monophasic curve appeared for both the frequency among RFC of the large lymphocytes and their labeling index, is again for presumably the same reason, that the 7S response is so early in appearing that it is not demonstrably separated in time from the peak due to the 19S antibody-producing cells.

It is of interest that in the early days of both the primary and secondary response almost all of the labeled cells were accounted for by the large lymphocytes. The increasing gap between the frequency of labeling of the lymphoid cell population as a whole and of the large lymphocytes, in the latter days, represents cells which are differentiating or maturing, decreasing in size while still retaining some of the tritium label.

The electron micrographs of cells among the RFC labeled with tritium showed a distribution of cells not unlike that which we have recently described for

---

[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.

RFC generally (1). Since the radioactive label was applied in vitro and was therefore taken up by cells "preparing" for mitosis and developing no further differentiation until the cytologic examination, it is noteworthy that these labeled cells included some in the plasmablast category; this indicated that cells with this degree of differentiation could still be replicating. These observations were made in cells from both the mouse and the rabbit, the rabbit again showing a somewhat higher frequency of plasmablasts among the labeled cells, in keeping with the higher frequency of plasmablasts and plasmacytes among RFC in the rabbit than in the mouse, as previously observed (1).

The very low index of labeling among the macrophages is consistent with the evidence that macrophages are involved in rosette formation not by synthesis of antibody but by adsorption of antibody produced elsewhere, as has been shown by experiments involving washing of spleen cells or actual adsorption by macrophages of antibody from serum (14, 15 and footnote 2).

An interesting group of macrophage RFC were those found in immediate contiguity with lymphocytes. The lymphocytes of these pairs of cells could well have represented a sampling of the lymphocytes engaged in antibody production, since the labeling index of the macrophage-associated lymphocytes was found to follow the labeling index of the RFC among the total spleen cell population on the successive days as shown in Figs. 1 *a* and 3. Whatever the nature of the macrophage–lymphocyte association, and regardless of the kind of material, if any, transferred between the cells, the preferential rosette formation by such macrophages may be due to their immediate proximity to active antibody-producing cells, with a resulting high concentration of antibody in their immediate environment.

Alternatively, this association may reflect a binding by receptors on the macrophage. Since the macrophages must have receptors for IgG and IgM molecules on their surface, and since rosette-forming cells of the lymphatic system almost certainly have antibody molecules still attached to their surface, the possibility must be considered that the preferential binding to macrophages of labeled lymphoid cells, among all the lymphoid cells present, reflects the availability of portions of antibody molecules on the surface of these cells to receptors on the macrophages.

SUMMARY

A study of the kinetics of antibody-producing cells has been carried out by the use of rosette formation for detection of individual antibody-producing cells, and labeling with tritiated thymidine, in cells obtained from mouse spleens at intervals after injection of SRBC. Following a primary injection of the antigen, the number of RFC per million cells was found to increase to a peak at 5 days, then, after a decrease, to a second peak at about the 10th day. The curve of tritium labeling of RFC was also biphasic, with peaks on the 3rd and 7th day.

The second increase in rosette-forming cells could be shown to involve, especially between the 7th and 9th day, a second increase in lymphoid cell RFC and, among these, 7S antibody–producing cells. When the population examined was restricted to large lymphocytes, two peaks of RFC per million cells and two peaks of labeling were again found. In this case, however, the peaks of RFC and of labeling were reached on the same day in each instance, rather than with the 2 day difference found in the entire spleen cell suspension or the entire lymphoid cell population.

Electron microscopic examination of labeled rosette-forming cells showed these to be largely lymphocytes, but to include rather well differentiated plasmablasts as well. No macrophages were found among labeled RFC in the primary response. A substantial number of labeled lymphocytes were found in close contiguity with rosette-forming macrophages. The percentage of labeling in such lymphocytes was as high, on the respective days, as the percentage of labeled cells among the RFC of the entire suspension.

BIBLIOGRAPHY

1. Gudat, F. G., T. N. Harris, S. Harris, K. Hummeler. 1970. Studies on antibody-producing cells. I. Ultrastructure of 19S and 7S antibody-producing cells. *J. Exp. Med.* **132:**448.
2. Harris, T. N., K. Hummeler, and S. Harris. 1966. Electron microscopic observations on antibody-producing lymph node cells. *J. Exp. Med.* **123:**161.
3. Hummeler, K., T. N. Harris, N. Tomassini, M. Hechtel, and M. B. Farber. 1966. Electron microscopic observations on antibody-producing cells in lymph and blood. *J. Exp. Med.* **124:**255.
4. Neher, G. H., and B. V. Siegel. 1969. Ultrastructure and development of plaque forming cells from Balb/c mice. *Fed. Proc.* **28:**3092.
5. Cunningham, A. J., J. B. Smith, and E. H. Mercer. 1966. Antibody formation by single cells from lymph nodes and efferent lymph of sheep. *J. Exp. Med.* **124:**701.
6. Storb, U., V. Chambers, R. Storb, and R. S. Weiser. 1967. Antibody-carrying cells in the immune response. II. Ultrastructure of "rosette"-forming cells. *J. Reticuloendothel. Soc.* **4:**69.
7. Storb, U., W. Bauer, R. Storb, T. M. Fliedner, and R. S. Weiser. 1969. Ultrastructure of rosette forming cells in the mouse during the antibody response. *J. Immunol.* **102:**1474.
8. Avrameas, S., and E. H. Leduc. 1970. Detection of simultaneous antibody synthesis in plasma cells and specialized lymphocytes in rabbit lymph nodes. *J. Exp. Med.* **131:**1137.
9. Caro, L. C., and R. P. van Tubergen. 1962. High resolution autoradiography. I. Methods. *J. Cell. Biol.* **15:**173.
10. Trump, B. F., E. A. Smuckler, and E. P. Benditt. 1961. A method for staining epoxy sections for light microscopy. *J. Ultrastruct. Res.* **5:**343.
11. Urso, P., and T. Makinodan. 1963. The roles of cellular division and maturation in the formation of precipitating antibody. *J. Immunol.* **90:**897.

12. Dutton, R. W., and R. I. Mishell. 1967. Cell populations and cell proliferation in the in vitro response of normal mouse spleen to heterologous erythrocytes. *J. Exp. Med.* **126**:443.
13. Koros, A. M. C., J. M. Mazur, and M. J. Mowery. 1968. Radioautographic studies of plaque-forming cells. I. Antigen-stimulated proliferation of plaque-forming cells. *J. Exp. Med.* **128**:235.
14. Storb, U., and R. S. Weiser. 1967. Antibody-carrying cells in the immune response. I. Identification of "rosette"-forming cells by light microscopy. *J. Reticuloendothel. Soc.* **4**:51.
15. Storb, U., and R. S. Weiser. 1968. Kinetics of mouse spleen cell populations during the immune response. *J. Reticuloendothel. Soc.* **5**:81.
16. Mosier, D. E. 1967. A requirement for two cell types for antibody formation in vitro. *Science (Washington).* **158**:1573.
17. Nossal, G. J. V., A. Szenberg, G. L. Ada, and C. M. Austin. 1964. Single cell studies on 19S antibody production. *J. Exp. Med.* **119**:485.
18. Möller, E., S. Britton, and G. Möller. 1968. Homeostatic mechanisms in cellular antibody synthesis and cell-mediated immune response. *In* Regulation of the Antibody Response. B. Cinader, editor. Charles C Thomas, Publisher, Springfield, Ill. 141.