

## STUDIES ON ANTIBODY-PRODUCING CELLS

### IV. ULTRASTRUCTURE OF PLAQUE-FORMING CELLS OF RABBIT LYMPH\*

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In a recent study, a method was described for isolating plaque-forming cells (PFC)<sup>1</sup> in considerable numbers for electron microscope examination (1). In earlier studies involving small numbers of PFC obtained from an active rabbit lymph node (2), and from lymph emerging from such a node (3), all the PFC isolated from the lymph had been found to be in the lymphocytic category. Since this was in contrast to PFC of the lymph node, which included both lymphocytes and plasma cells, a study of PFC of efferent lymph was undertaken, using the method referred to for isolation of PFC in numbers sufficient to justify a picture of the population of cells involved. This question was of particular interest since in our recent study of some hundreds of rosette-forming cells (RFC) and PFC of lymph nodes and spleens, the plasma cells constituted the great majority of the PFC (1).

#### *Materials and Methods*

*Collection of Lymph.*—4 days after a single injection of 0.5 ml of 50% sheep red blood cells (SRBC) in each hind footpad, rabbits were anesthetized, the skin of the inner aspect of the knee was incised, the semitendinosus and semimembranosus muscles were cut, and each popliteal lymph node was exposed. A ligature was placed, but not tied, under the exposed efferent lymphatic vessel, and the leg was exercised in a prescribed manner. The ligature was then tied and 12 min later, following an exact repetition of the exercise, the lymphatic vessel was entered distal to the ligature with a 27 gauge needle. The lymph was collected in a syringe moistened with heparin solution, 1000 USP units/ml, and centrifuged at 1200 rpm for 5 min. The cell sediment was resuspended in Earle's balanced salt solution containing 5% rabbit serum albumin.

*Hemolytic Antibody Plaques.*—In order to obtain PFC in considerable numbers, a thin-layer agar plating method was used, which was an adaptation of a method recently described (4). Briefly, preliminary plating was done to approximate the number of PFC per milliliter of the suspension, and then a plating with an appropriately higher concentration of lymph

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<sup>1</sup> *Abbreviations used in this paper:* ER, endoplasmic reticulum; LN, lymph node; PBS, phosphate-buffered saline; PFC, plaque-forming cell; RFC, rosette-forming cell; SRBC, sheep red blood cells.

node cells and sheep RBC (SRBC) was used to allow for the loss of cells incurred in preparing the thin layer. For thin plating, immediately after the spreading of the mixture of lymph cells and SRBC, the bulk of the suspension was poured out of the plate with a few flips of the wrist, leaving a thin layer of agar in which the plaques developed on incubation at 37°C for 1 hr under guinea pig serum diluted 1:20. The plates were then exposed to 1% glutaraldehyde for 1 hr at room temperature and washed twice in PO<sub>4</sub>-buffered saline (PBS). Plaques with only one cell in the center were then picked directly with a braking pipet, working under a dissecting microscope at × 100 (1).

*Fixation and Embedding.*—The PFC picked as described above were blown out of the braking pipet into a B.E.E.M. capsule, size 00 (Better Equipment for Electron Microscopy, Inc., Bronx, New York), containing one drop of PBS. Osmium tetroxide, 1%, was added to the capsule, approximately half filling it. After 10 min the remainder of the capsule was filled with

TABLE I  
*Plaque-Forming Cells in Rabbit Popliteal Lymph Node and Efferent Lymph 4 Days after Injection of Sheep RBC*

Exp. No.	Lymph node (LN)			Lymph			PFC/10 <sup>6</sup> cells, lymph LN
	PFC/10 <sup>6</sup>	Cells/LN × 10 <sup>6</sup>	PFC/LN	PFC/10 <sup>6</sup>	Cells in 12 min collection × 10 <sup>6</sup>	PFC in 12 min collection	
11A1	225	340	77,000	365	14.4	5,400	1.6
13B2	230	1000	230,000	640	27	17,200	2.8
11B1	176	300	53,000	145	13.5	1,890	0.8
10A1	128	525	68,000	210	96	20,200	1.6
9C1	300	560	168,000	405	39	15,600	1.3
9B2	110	480	53,000	105	14.5	1,530	0.95
8B1	300	550	165,000	370	40	14,800	1.2
8B2	150	560	84,000	980	7.5	7,400	6.6
7A1	240	400	96,000	735	9.5	7,000	3.1

a mixture of equal parts of 1% OsO<sub>4</sub> and 70% ethanol, to avoid dislodging and scattering the cells by the turbulence which occurs when the ethanol is added directly to the OsO<sub>4</sub>. Half of the mixture in the capsule was then removed and replaced with another addition of the OsO<sub>4</sub>-ethanol mixture. After an additional 1 or 2 min, half of the fluid in the capsule was replaced with 70% ethanol. After one repetition of this, all the fluid in the capsule was removed except for the last drop, and the capsule was refilled with 70% ethanol. After 10 or 15 min, the dehydration proceeded with a succession of changes to 80%, then 95% ethanol, and, finally, two changes of absolute ethanol, all of these for 10 min. Embedding then continued as described previously (1).

#### RESULTS

*PFC in Lymph and Lymph Nodes.*—The PFC examined by electron microscopy were obtained from nine experiments. The 12 min timed collection of efferent lymph and cell suspensions obtained by teasing the entire popliteal lymph node were examined first by the usual plating technique. Table I shows the PFC per 10<sup>6</sup> cells of the lymph node and the lymph, the numbers of cells and of PFC in the entire popliteal lymph node, and the numbers of cells and

of PFC in the 12 min collection of efferent lymph. It can be seen that the PFC/ $10^6$  cells was generally higher in the lymph than in the lymph node, the ratio of lymph to lymph node being in the range of 0.8–3.1, except for one instance in which this ratio was greater than 6. The number of PFC in the entire lymph node was of course many times that obtained in the lymph in the timed 12 min collection.

To determine the extent to which cells from the lymphatic circulation could be contributing to the PFC found in this efferent lymphatic vessel, a comparison was made of the number of PFC in the local lymphatic system with the number to be found elsewhere in the lymphatic circulation. A number of rabbits were injected with the same antigen, but in one foot only. 4 days later the following were collected: the draining popliteal lymph node, the contralateral

TABLE II  
*Rabbits Injected in One Foot: Plaque-Forming Cells in the Draining and Contralateral Popliteal Lymph Nodes and in Efferent Lymph from Those Nodes*

Rabbit No.	PFC in lymph nodes			PFC in 12 min collection of efferent lymph from:	
	Draining popliteal	Contra-lateral	Axillary	Draining popliteal	Contralateral
474	71,000	100	9		
475	87,000	275	16		
476	324,000	164	48, 48	27,200	128
477	211,000	160	44, 27	25,000	32
478	116,000	240	51, 9	10,200	63
479	620,000	430	72, 50	14,800	36

node, and one or both axillary lymph nodes. In addition, a 12 min collection of lymph was made from the efferent lymphatic of the popliteal lymph nodes of both the injected and the opposite leg, where these could be obtained. The results are shown in Table II. It can be seen that the PFC against SRBC in the contralateral popliteal lymph node, which, except for the inevitable background PFC, are presumably the result of systemic circulation of lymphocytes, were extremely few in comparison with the PFC in the draining lymph node, the ratio being between 1:300 and 1:2000. In the axillary nodes, also distant from the active lymph node, the number of PFC was even smaller, probably reflecting the smaller size of an axillary rather than a popliteal node.

A comparison of lymph collected from the injected and the opposite leg gave the most direct measure of flow of PFC in the lymphatic circulation at this time which could be contributing to the total of PFC in the efferent lymph. Here, again, lymph from the opposite leg showed very few PFC in comparison with those obtained in a similar period of time from the efferent lymphatic of the active lymph node, the numbers always being substantially less than 1% of those from the injected leg (Table II).

*Electron Microscope Observations.*—It was possible to isolate for electron microscope examination 71 PFC of the lymph. Of these 71 PFC 66, or 93%, were lymphocytes. Of the 66 lymphocytes 52, or 79%, showed signs of physiologic activity, with many finger-like projections of cytoplasm from the generally rounded periphery of the cells. The nuclei of these cells showed typical



FIG. 1. Plaque-forming cell from efferent lymph of the rabbit; lymphocyte with sparse ER and extensive microvilli.  $\times 14,000$ .

well-marked condensations of chromatin, including many which bordered on the nuclear membrane. The cytoplasm of these lymphocytes showed moderate numbers of mitochondria, and was otherwise filled with free ribosomes, including the finger-like projections of the cytoplasm. The 52 lymphocytes included 37 in which the endoplasmic reticulum (ER) was barely detectable, as a few short, very narrow channels in an entire section of a cell. Such cells are shown in Figs. 1 and 2. In the remaining 15 the ER, although still in short

narrow channels, was somewhat better developed, so that in a few parts of the cell a radial cut would intersect two or three channels of ER. Examples of such cells are shown in Figs. 3 and 4. Many of these cells showed, in close proxim-

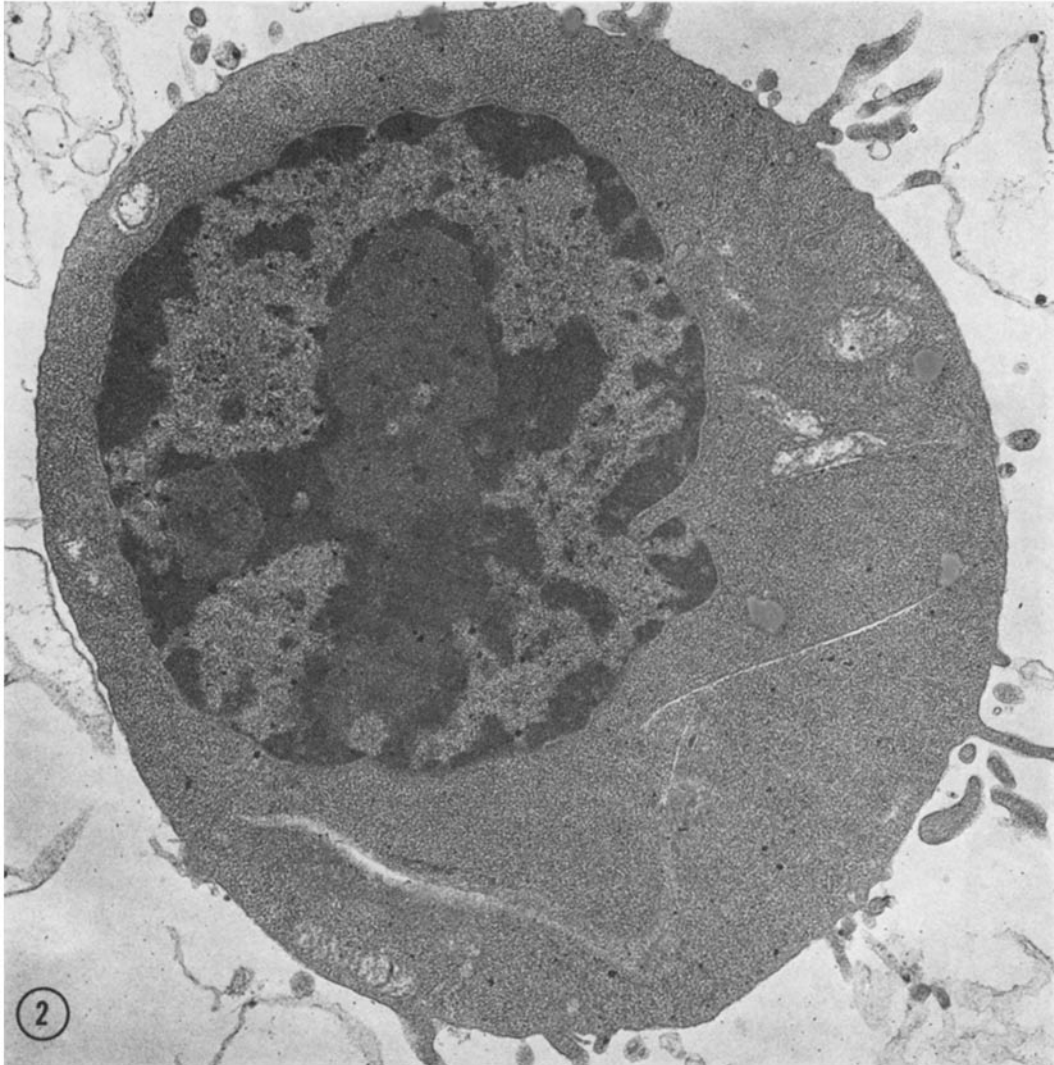


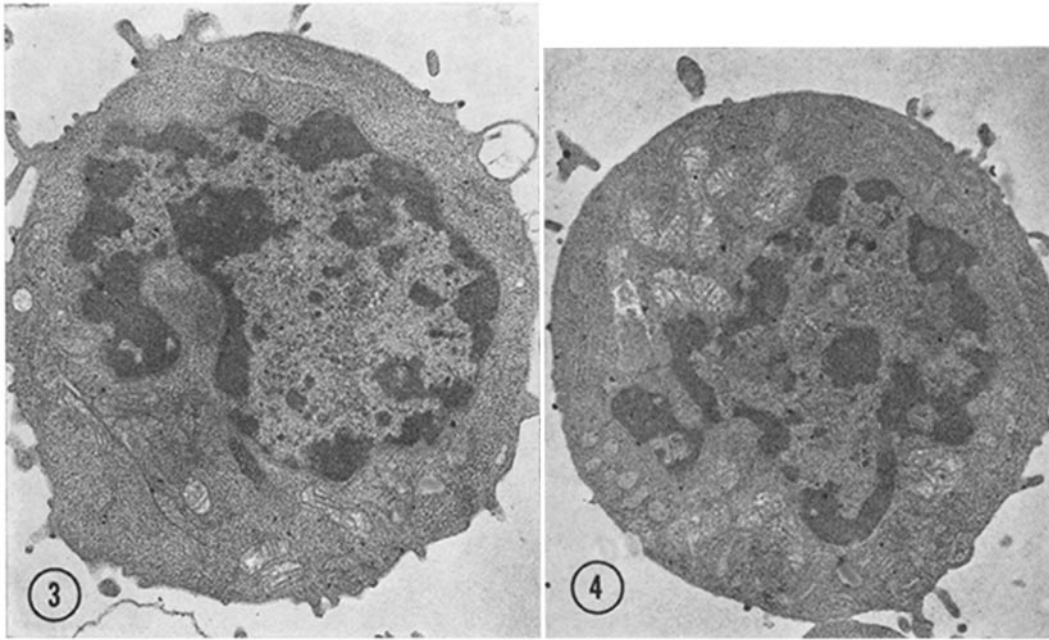
FIG. 2.—Same type of cell as in Fig. 1; cell is surrounded by lysed SRBC.  $\times 15,500$ .

ity, ghosts of RBC which were presumably bound by the PFC before the hardening of the agarose.

The remaining 14 lymphocytes (21%) presented an appearance which we

had not seen in lymphocytic PFC of the lymph node or spleen. These were obviously lymphocytes, but clearly senescent, with almost complete rounding of the cell, no cytoplasmic projections, a loss of definition of mitochondrial structure and of plasmalemma, amorphous nuclear chromatin, and a widened perinuclear space. A cell typical of this group is shown in Fig. 5.

The remaining five cells, or 7% of the PFC of lymph, showed an unusual combination of features. They resembled typical lymphocytes in that they were small and round, with the nucleus occupying the greater part of the area



FIGS. 3 and 4. Plaque-forming cells from efferent rabbit lymph; lymphocytes with more extensive ER and polyribosomes.  $\times 11,000$ .

of the cell and a narrow ring of cytoplasm around it. What was not characteristic of the lymphocyte, even among PFC, was the presence of several channels of ER in the cytoplasm, wide enough to see precipitated protein in them, and of considerable length. Such cells had been seen before, among PFC isolated from the blood of the rabbit (3). One of these cells is shown in Fig. 6.

#### DISCUSSION

The chief observation of this study was that in lymph collected from the efferent lymphatic vessel of an antibody-producing popliteal lymph node of a rabbit, almost all of the PFC were lymphocytes. This was in striking contrast

to the finding among PFC of lymph nodes and spleens of rabbits and mice injected with this antigen, where only 13% of PFC were in the lymphocytic category, the great majority being plasma cells (1). This difference in distribution indicates that the PFC emerging from the lymph node into the lymph are not a sampling or overflow of the antibody-producing cells from the lymphatic organ but represent a special category of the PFC produced in that node. The data obtained here, in conjunction with the earlier observations in

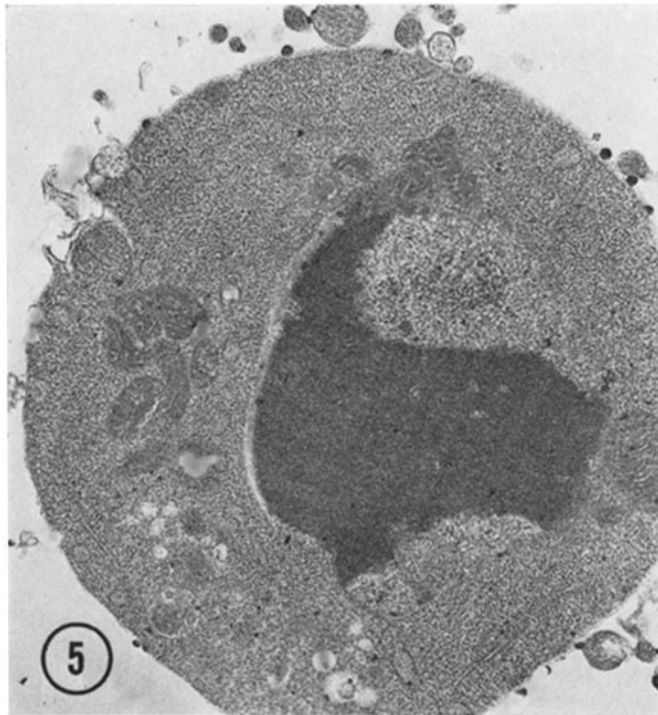


FIG. 5. Senescent plaque-forming lymphocyte with prominent perinuclear space and minimal development of microvilli.  $\times 15,000$ .

the lymph node, also suggest that the plasma cell is a morphologic form of the antibody-producing cell which is associated with organized lymphatic tissue. This association, which was also suggested by our earlier examination of PFC of lymph (3), is consistent with the observations of Reinhardt and Yoffey (5), who found that mature plasma cells do not leave the lymph node, of Cunningham et al. (6), who found plasma cells among PFC of the lymph node, but not of the lymph, and of Hall et al. (7), who found no cells with ultrastructural features of plasma cells in the lymph emerging from an antibody-producing lymph node of a sheep.

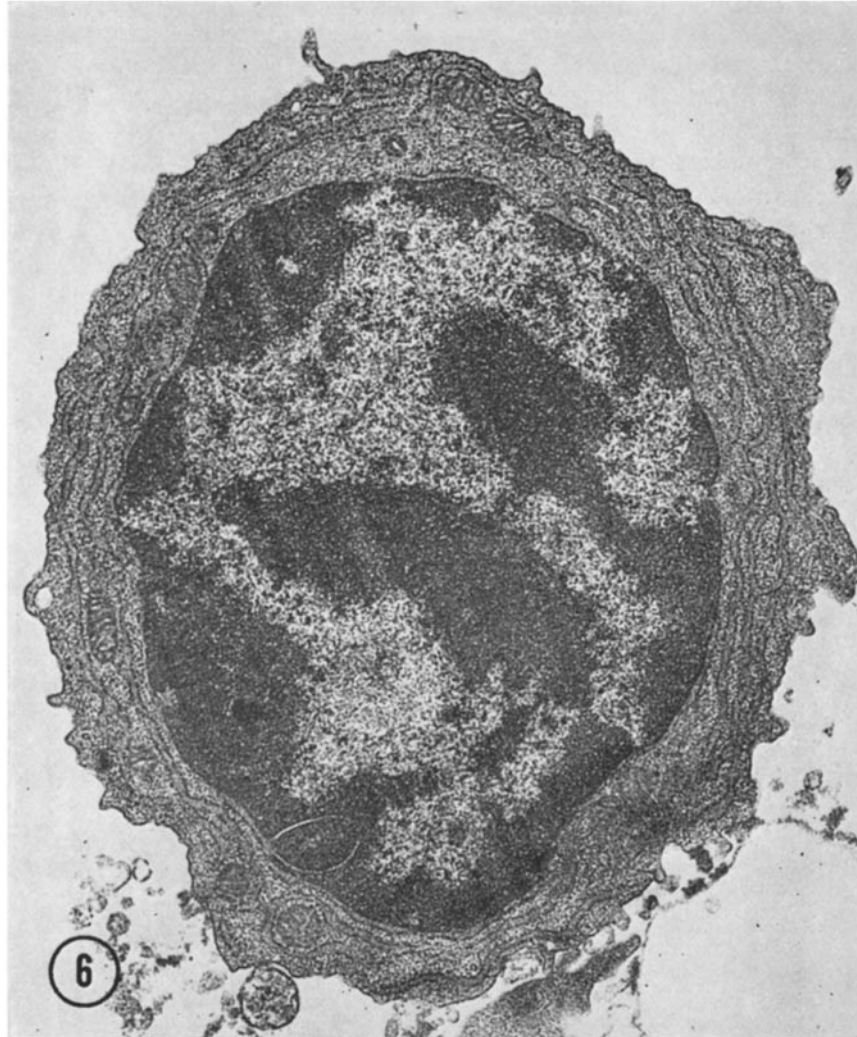


FIG. 6. Plaque-forming cell from rabbit lymph, one of the 7% found with features of both lymphocytes and plasma cells. This is a small round cell, with cytoplasm which is in a narrow ring but contains well-developed parallel channels of ER.  $\times 15,000$ .

The morphologic character of the PFC of lymph, and the contrast with the population of PFC in the lymph node from which those cells have emerged, raise a number of points of discussion in conjunction with other recent studies in these laboratories. For this discussion it will be assumed that, against the background of the well-known circulation of cells through the lymphatic



system, the popliteal lymph node is the source of the great majority of PFC found in its efferent lymph at the height of the immune response to antigen injected into the footpad.

This assumption is based on the data shown in Table II, that in comparison with the number of PFC in the draining lymph node, and in its efferent lymphatic, there were very few PFC in the contralateral popliteal lymph node and in the opposite lymphatic vessel at that time, indicating little circulation as yet of PFC produced in the immune response being studied. It is also supported by the finding in earlier studies that the striking wave of increase in cellularity and lymphocyte percentage of efferent lymph of the popliteal lymph node following a footpad injection of SRBC was not accompanied by any perceptible changes of this kind in the afferent lymph of this node, 4 days after injection of the antigen, and that on injection into only one footpad, no antibody could be found in extracts of the contralateral popliteal lymph node or in its efferent lymph (8, 9). Hall and Morris (10) have also obtained evidence, in studies involving perfusion of sheep lymph nodes with thymidine-<sup>3</sup>H, that normally only 4% of efferent lymph cells were produced in the local lymph node; but after antigenic stimulation, the cells of efferent lymph which showed structural evidence of activity arose entirely in the local lymph node.

On the assumption, then, that the PFC we have been studying here arise essentially in the popliteal lymph node, the following considerations are pertinent. First, the study referred to above (1) indicated that, in a lymph node examined under the same experimental conditions, the great majority of the PFC were plasma cells. The present study indicates that the PFC of the lymph emerging from that node are almost entirely lymphocytic, and, in addition, that PFC are emerging from this node at a rate which is substantial in relation to the number of PFC contained in the node (Table I). (Cunningham et al. [6] have also found a considerably higher frequency of PFC among cells of the lymph emerging from an active lymph node than among the cells of the node itself.) If we consider not only the PFC contained in the node at a given time, but rather, the entire population of PFC being produced in that node, then the rate of production of PFC which are lymphocytic would appear to be well above the 13% found within the node in the earlier study (1). The degree to which this percentage exceeds the level of 13% would, of course, depend on the relative rates of production of PFC destined to leave the node and those which will remain within the organized structure of the lymph node. This parameter is not available at present, but these considerations, as well as others involved in the dynamics of antibody-producing cells, would make it very desirable to obtain such a measurement.

Second, by thymidine-<sup>3</sup>H labeling of PFC of the mouse we have recently obtained evidence for a line of differentiation from an antibody-producing lymphocyte to a plasma cell, in the antibody-producing lymph node. In con-

junction with that evidence, the present data would suggest that in such a lymph node there are two paths of differentiation for the nascent lymphocytic PFC. One of these paths would be to differentiate to the plasmacyte and remain in the tissues of the node; the other course would be to enter the lymph as a lymphocytic PFC, perhaps with some increase in the development of organelles. The 7% of lymph PFC which showed so many features of the classical active lymphocyte except for the well-structured ER might represent this advanced stage of development of the antibody-producing lymphocyte. It may be relevant in this connection that cells with this combination of features were found, in a previous study, in the peripheral blood, farthest removed from the lymph node of origin.

Third, the senescent lymphocytic PFC which we found in the lymph had not been seen in these studies before. In PFC isolated from the tissue of lymph node and spleen we had seen cells with such signs of ageing only among the plasmacytic forms. These two sets of observations are consistent with the hypothesis set forth in the previous paragraph, because under those circumstances only the cells destined to differentiate into plasma cells would remain in the node, and therefore only plasma cells would be found among the senescent PFC encountered within it. In contrast, the lymph, where lymphocytic PFC are continuing in their original morphologic character, would contain lymphocytic PFC in all stages, including senescence.

The observations made in this study are of interest to us in terms of widely held positions on the classification and function of cells involved in the immune response. Such functions as the memory cell (11) or the precursor cell in the immune response have generally been ascribed to the lymphocyte. However, in considering the fully responding cell of the immune response, it has been suggested in several schemes that the lymphocyte is the effector cell in delayed hypersensitivity, graft rejection, and the graft-*versus*-host phenomenon, but that the plasma cell is the producer of immunoglobulin and antibody (12-14). The electron microscope has now demonstrated cells clearly of the lymphocytic category as the sole cells in the centers of hemolytic plaques, and therefore the cells which have obviously synthesized and secreted humoral antibody, in several situations: in a few cells of the lymph node (2) and lymph (3), in 13% of several hundred PFC of the lymph nodes and spleens (1), and now as almost the entire population in the efferent lymph of a lymph node engaged in a vigorous immune response. These observations indicate that the lymphocyte is, in fact, an active producer of antibody, confirming evidence we obtained some years ago by extraction of cells of the efferent lymph (15, 16)

#### SUMMARY

Efferent lymph of the popliteal lymph nodes of rabbits was collected 4 days after a single footpad injection of SRBC. Thin-layer agar plating was done to isolate plaque-forming cells of the lymph for electron microscope

examination, and the numbers of plaque-forming cells (PFC) in cells from the lymph and lymph nodes were determined.

Of 71 PFC of lymph isolated and examined, 93% were lymphocytes, most of them with signs of substantial levels of physiologic activity. The cytoplasm showed an abundance of free ribosomes and many finger-like projections. The endoplasmic reticulum (ER) was barely detectable in most of the active lymphocytic PFC, and in some, a few short narrow channels of ER could be seen. Approximately one-fifth of the lymphocytic PFC presented an appearance of senescence, with signs of degeneration: rounded cells, with amorphous nuclear chromatin, and very few microvilli. The remaining 7% of the PFC of the lymph showed an unusual combination of features: small round cells with a narrow ring of cytoplasm which, however, contained well-organized channels of ER. Such cells had been found only among PFC of peripheral blood of the rabbit.

The number of PFC per million cells was higher in the lymph than in the suspensions of lymph node cells. In both the contralateral lymph node and its efferent lymph, the number of PFC was less than 1% that of the injected side.

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