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Quantitative Studies on the Proliferation and Differentiation of Antibody-Forming Cells in Lymph

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The transforming cells that appear in the efferent lymph from a lymph node responding to an antigenic challenge are part of a heterogeneous population which changes as the response progresses. Some cells containing small amounts of antibody appear early in the response and these cells have the cytologic characteristics of small and medium lymphocytes. They are, however, actively synthesizing DNA. As the immune response progresses, the antibody content of the cells in lymph increases. When incubated *in vitro*, cells in lymph appearing late in the response released 20 times more antibody per cell than those appearing early in the response. Large blast cells are the predominant antibody-forming cell in lymph. At the peak of a secondary challenge with horseradish peroxidase, up to 40% of the cells in lymph may be blast cells and, of these, two-thirds may contain specific antibody. It seems probable that most if not all of the blast cells responding to the antigen are involved directly in antibody and DNA synthesis. Cells in all stages of ultrastructural differentiation, and even mature plasma cells, were found to incorporate ^{3}H-thymidine into their nuclear DNA. (Am J Pathol 66:1–24, 1972)

IT IS GENERALLY ACCEPTED that an immune response results from the stimulation of immunologically competent cells by antigen and that once stimulated, these cells transform, proliferate and subsequently produce specific antibody. Experimentally, this sequence of events has never been studied in a single living cell. However, by studying the immune response *in vivo* in rats[1] and *in vitro* with fragments or cell suspen-

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sions of lymphoid organs,[2-5] the events of the immune response have been outlined and it has been shown that the cells which eventually produce antibody arise from the division of precursor cells.[2-4]

Cells from the spleen and from lymph nodes that have been shown to contain or to produce antibody represent a wide range of morphologic types and have been categorized variously as small lymphocytes, large lymphocytes, plasmablasts and mature plasma cells.[6-9] Studies on lymph from the popliteal nodes of sheep have shown that after an antigenic challenge, many large basophilic blast cells appear in the lymph.[10,11] Some of these cells contain antibody [10] and are capable of secreting it into their environment.[12] The cells in the efferent lymph are also responsible for the dissemination of the immune response and for the development of systemic immunity and immunologic memory.[11,13]

In the present study, the cell population in efferent popliteal lymph as a whole and individual cells themselves were examined at different times after the lymph node was challenged with horseradish peroxidase or *Salmonella* antigens to determine the relationship between cell differentiation, transformation and antibody formation, and also the relationship between cell division and antibody production.

Materials and Methods

Animals

Merino, Pre-Alp and Rambouillet-Merino cross ewes, 3–5 years of age, were used for the experiments. The sheep were housed indoors in metabolism cages, and fed lucerne chaff, hay and grain oats. They were allowed free access to water at all times.

Surgical Procedures

For each experiment the efferent lymphatic of the popliteal lymph node was cannulated and the lymph coming from the node collected quantitatively over periods of several weeks. The preparation of the animals, the surgical technics and the methods for collecting the lymph have been described previously.[10,14]

Cell Counts

A Coulter, model-B electronic cell counter with a calibrated 100-μ aperture tube was used for cell counts.

Antigens

Two antigens were used, horseradish peroxidase type VI (Sigma Chemical Company, St Louis) and boiled, *Salmonella muenchen* organisms.

Immunization Procedures

Primary and secondary responses to *Salmonella* antigen and secondary responses to horseradish peroxidase were studied. The priming challenge with horseradish

peroxidase was a 10-mg dose emulsified with 0.5 ml of incomplete Freund's adjuvant (Difco Labs, Detroit) injected subcutaneously into the lower part of the hind leg. The secondary challenge, which was given 3–10 weeks later near the same site, was either 10 mg of horseradish peroxidase dissolved in 1.0 ml of 0.9% NaCl solution or 10 mg of horseradish peroxidase in 0.5 ml of incomplete Freund's adjuvant. The efferent duct of the popliteal node draining the injection site was cannulated 2–3 days before the secondary challenge was given.

The priming dose of *Salmonella* antigen was 10^9 boiled organisms injected into the lower hind leg. The secondary challenge was a dose of 10^9 boiled organisms in 0.5 ml of 0.9% NaCl solution given 3 weeks later in the same leg. Again, the efferent duct of the popliteal node was cannulated 2–3 days before the secondary challenge was given.

Detection of Antibody-Forming Cells

Horseradish Peroxidase. Before and after the secondary challenges, the lymph coming from the popliteal node was collected over 8-hour periods and the cells examined for the presence of anti-horseradish peroxidase antibody. When the response in the lymph had died away and only a few antibody-forming cells remained, the lymph nodes were extirpated and examined. This was done usually 120–150 hours after the antigenic challenge.

Antibody to horseradish peroxidase was detected by the method described by Leduc, Scott and Avrameas.[15] Samples of lymph were centrifuged for 5 minutes at 1000 rpm and the cell pellet washed twice with Hanks' solution. Smears were prepared, fixed in methanol-ether for 30 minutes and dried in air. A proportion of the cells was fixed for 30 minutes in 1.25% glutaraldehyde in 0.1 M phosphate buffer at 4 C. The cell suspension and the cell smears were exposed to a solution of 50 μg/ml of horseradish peroxidase for 30 minutes at room temperature with continual agitation and then washed twice with 0.1 M phosphate buffer at room temperature. The smears were treated with a solution of 2.5 mg of 3,3′ diaminobenzidine in 10 ml of 0.05 M Tris-HCl buffer containing 0.01% hydrogen peroxide for 30 minutes at room temperature to reveal the presence of the antigen-antibody conjugates. The suspension of lymph cells was refixed for 15 minutes with 1.25% glutaraldehyde, washed twice in 0.1 M phosphate buffer and then treated with the diaminobenzidine–hydrogen peroxide reagent. The cell suspension was again washed twice with 0.1 M phosphate buffer and fixed further in 1% osmium tetroxide for 30 minutes and then dehydrated and embedded in Epon.

Lymph nodes were divided into cortex and medulla immediately after they were removed from the animal. The pieces of tissue were placed in Hanks' solution at room temperature, cut into fragments and teased apart with dissecting needles. The dispersed cells and tissue fragments were separated by passing them through a stainless-steel 60-mesh sieve. The cells were washed twice in Hanks' solution and fixed in 1.25% glutaraldehyde as was the case for the lymph cells. Subsequently, cells containing antibody were revealed by the procedures described above.

As controls, the lymph cells collected before antigenic stimulation were used, and no positive cells were found in these samples either in light microscopic smears or in electron microscopic sections. For another group of controls, cells were incubated in the presence of hydrogen peroxide and diaminobenzidine without previously exposing them to horseradish peroxidase solution. These preparations were invariably negative when carried out on cells collected before antigenic challenge and on cells collected from 24 hours onwards after antigenic challenge. The presence of antigen in samples of lymph collected immediately after horseradish peroxidase was injected under the skin gave rise to positive reactions on the surface of some cells unless they were washed. This reaction was diffuse and confined to the

surface of the cells and was easily distinguished from the reaction product in antibody-forming cells.

Lymph cells collected during immune responses to *Salmonella* antigens were also reacted with horseradish peroxidase, hydrogen peroxide and diaminobenzidine as a further control. No cells positive for antibody were found.

Salmonella Antigen. Cells making antibody against this antigen were detected by a free-suspension hemolytic plaque assay.[12] Washed red cells from sheep were incubated for 1 hour at 37 C with *Salmonella* lipopolysaccharide prepared by the method of Halliday and Webb[16]; these sensitized erythrocytes were used for detecting cells making antibody against the *Salmonella* antigen.

Samples of the lymph cells were washed in Eagle's medium and resuspended to give a concentration of 10^5–10^7 cells/ml. To 0.5 ml of lymphoid cells, 0.1 ml of sensitized erythrocytes and 0.05 ml of guinea pig complement were added, the mixture placed in ruled chambers and the monolayer of cells incubated for 1 hour at 37 C. The number of plaques was scored and their proportion calculated in the original cell population. This assay gave information concerning the times of appearance of antibody-forming cells in the lymph during immune responses to *Salmonella* antigen and an estimate of the number of cells secreting antibody at any time. It did not provide any evidence as to the cytologic characteristics of the antibody-forming cells.

Labeling Cells with ^{3}H-Thymidine and ^{3}H-Uridine

Samples of cells collected from the efferent popliteal lymph during immune responses were incubated *in vitro* separately with ^{3}H-thymidine and ^{3}H-uridine. Before and throughout immune responses to *Salmonella* antigen and horseradish peroxidase, freshly collected samples of lymph containing between 10^8 and 10^9 cells were centrifuged for 5 minutes at 1000 rpm and the cells resuspended in 1–2 ml of cell-free lymph. ^{3}H-thymidine or ^{3}H-uridine (The Radiochemical Center, Amersham, Australia) was added at a concentration of 10 μCi/ml and the cells incubated for 1 hour at 37 C with intermittent, gentle agitation. After incubation, the cells were centrifuged and separated from the incubation medium, washed three times in lymph and resuspended in a small volume of sheep plasma or in Hanks' solution. Cell smears were prepared and the remainder of the sample fixed with 1.25% glutaraldehyde and then with 1% osmium tetroxide. The cells were stained with 1% aqueous uranyl acetate solution and then dehydrated and embedded in Epon or Araldite.

For determining quantitatively the extent to which ^{3}H-thymidine and ^{3}H-uridine were incorporated into the nucleic acids of lymphoid cells, samples containing 5×10^6 cells were incubated in 1 ml of Eagle's medium for 1 hour at 37 C. The medium contained 10% fetal calf serum and the labeled nucleic acid precursor at a concentration of 2 μCi/ml. After the incubation period, the cells were centrifuged out of the medium and washed twice in Hanks' solution. Carrier protein (0.1 ml of 2% bovine serum albumin solution) was first added to the cell pellet and then 5 ml of cold 5% trichloracetic acid. The cells were centrifuged and washed once with cold 5% trichloracetic acid. The precipitate was dissolved in 1 ml of formic acid and transferred to a scintillation vial which was then filled with 15 ml of Triton-toluene scintillator. Counting was done to an error of 2%, in a Beckman LS-100 liquid scintillation counter.

An alternative procedure was based on a method used for cell cultures.[17] In this method, the washing and precipitation of the nucleoproteins were done in the same way but the final precipitate was dissolved in 0.5 ml of 2 M NH_4OH solution. Volumes of 0.1 ml were added to Whatman glass fiber discs (W & R Balston, Ltd),

and dried at 110 C for 20 minutes. The dried discs were then placed in scintillation vials and 10 ml of toluene scintillator was added.

Autoradiography

Light microscopic autoradiography was done on cell smears and on thin resin sections of about 1 μ thickness. The cell smears were fixed in methanol, coated with a thin layer of carbon and then dipped in a 1:8 dilution of Ilford G5 autoradiographic emulsion or covered with Kodak AR 10 stripping film. The slides were dried thoroughly and left for 2–4 days before being developed for 5 minutes in D19B (Kodak) developer at 22 C. After acid fixation and drying, slides were stained with 0.1% azure A for 15 minutes. In experiments with horseradish peroxidase, some cell smears were reacted with antigen, diaminobenzidine and hydrogen peroxide after they had been incubated with ^{3}H-thymidine. Subsequently, these smears were processed for autoradiography to give a double label, which detected those cells incorporating ^{3}H-thymidine and synthesizing antibody.

Electron microscopic autoradiography was done using the method described by Salpeter and Bachmann.[18] Ribbons of ultrathin sections were placed on glass slides coated previously with a 0.5% or 1.5% solution of collodion in amyl acetate. A layer of carbon approximately 100 Å thick was then deposited over the sections and the slides dipped in a solution of Ilford L4 emulsion diluted about 1:5 with double-distilled water so as to give a layer showing purple interference color. The slides were thoroughly dried, stored for 4 weeks at 4 C and then developed for 2½ or 3 minutes with Microdol X (Kodak) developer at 23 C. After development, the collodion film was floated off the slide onto a water bath and the sections picked up on 300-mesh grids. The grids were treated with amyl acetate for 5–15 minutes to dissolve some of the collodion; this increased the contrast of the sections in the electron beam.

Antibody Assay

Antibody against *Salmonella* antigen was measured by hemagglutination or hemolysin assay. Calcium-magnesium saline was added to Perspex hemagglutination trays and twofold dilutions of samples were made with a Takatsky loop. Red cells from chickens were used as test cells and end points were read after 35 minutes. Lytic antibody was detected by adding 0.025 ml of a 1:4 dilution of reconstituted, lyophilized guinea pig serum, and the trays were read after standing at 4 C overnight.

Electron Microscopy

Sections were examined unstained or stained with uranyl acetate or lead citrate after mounting them unsupported or supported on formvar films on 300-mesh copper grids. A Philips EMU 200 or a Siemens Elmiskop I electron microscope was used at 60 or 80 kV.

Results

Measurement of the Antibody Content of Plaque-Forming Cells in Lymph

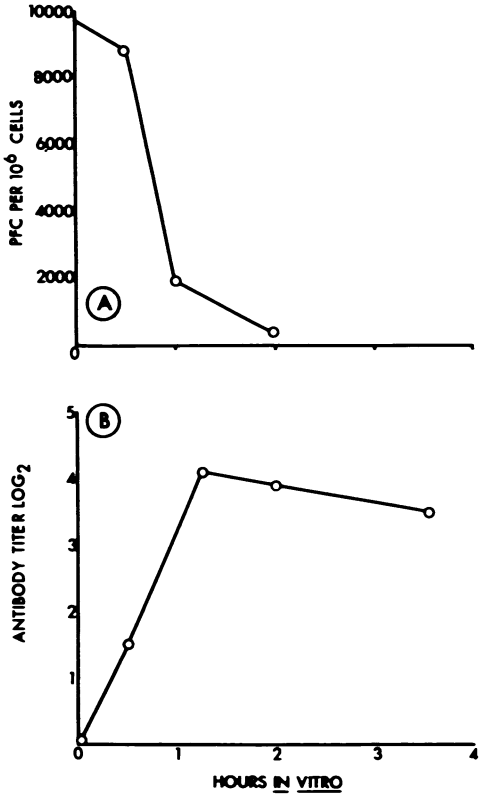
It has been shown that a temporal relationship exists between the appearance of basophilic blast cells, plaque-forming cells and antibody in lymph from the stimulated popliteal node.[10–12] The formal demonstration that the antibody titrated in the lymph actually comes from plaque-forming cells has not been done. Experiments were therefore designed to

show the relationship between plaque-forming cells and titratable antibody released *in vitro*.

Sheep with popliteal lymphatic fistulae were given both primary and secondary challenges with *Salmonella* organisms and the cells collected from the lymph throughout the responses. Duplicate samples of lymph containing 5×10^8–2×10^9 cells were centrifuged and the cells washed twice and then resuspended in a volume of 5 ml of Eagle's medium. The cells were incubated for 4 hours at 37 C; throughout this time the samples were assayed for the amount of antibody released and for the number of plaque-forming cells.

Antibody was released from the cells very rapidly and was detected in the medium within 30 minutes; the highest titer obtained was 1:256. The maximum titer was reached after about 2 hours' incubation; by this time, the number of plaque-forming cells in the cultures had fallen to a low level (Text-fig 1).

The coincident fall in the number of plaque-forming cells and the increase in antibody titer in the culture medium suggested that the cells in

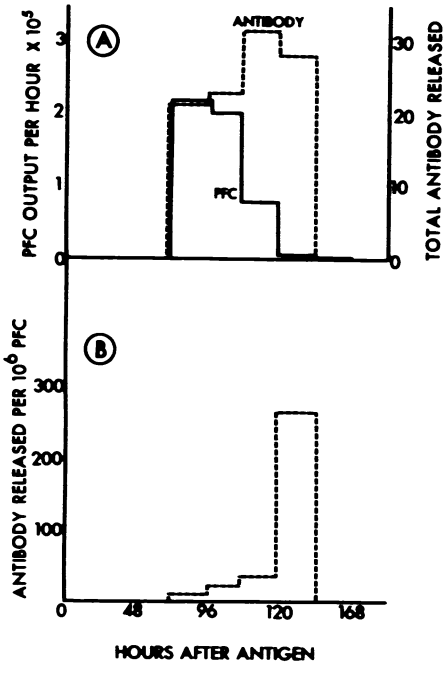


TEXT-FIG 1A—Number of plaque-forming cells (*PFC*) detected in a population of cells obtained from the efferent lymph of the popliteal node 96–120 hours after a primary challenge with *Salmonella*. The cells were incubated in Eagle's medium at 37 C. B—The titer of antibody in the culture medium during incubation of the population of plaque-forming efferent lymph cells shown in Fig 1A. Mean results are given for duplicate cultures.

lymph were releasing mostly preformed antibody *in vitro*. To test the extent of *de novo* synthesis of antibody by the lymph cells, cultures were set up with actinomycin D added at concentrations of 0.1, 0.5, 5.0 and 10.0 μg/ml and puromycin at 1, 10 and 100 μg/ml. No significant suppression was found in either the antibody titers in the medium or in the number of plaque-forming cells detected when compared with cultures done in Eagle's medium alone. Although these assay methods would not detect small levels of protein synthesis, it was concluded that most of the antibody measured *in vitro* had been formed before the cells were incubated.

Antibody Content of Plaque-Forming Cells at Different Stages of the Immune Response

By calculating the total number of plaque-forming cells before incubation, it was possible to measure the amount of antibody released by a given number of plaque-forming cells *in vitro*. Samples of cells collected sequentially throughout immune responses to *Salmonella* organisms were incubated in Eagle's medium and the antibody released *in vitro* was titrated. Text-fig 2 shows the output of plaque-forming cells from the popliteal node during a primary response to *Salmonella in vivo* compared with the amount of antibody released by these cells *in vitro* and the amount of antibody released by 10^6 plaque-forming cells collected at different times during the response.



TEXT-FIG 2A—Output of plaque-forming cells from the popliteal node during a primary immune response to *Salmonella* in relation to the total amount of antibody released *in vitro*. B—Amount of antibody released *in vitro* by 10^6 plaque-forming cells at different stages of a primary immune response to *Salmonella*.

The amount of antibody released by the lymph cells ($\log_2$ titer $\times$ 5 ml culture medium) increased as the response progressed. The maximum amount of antibody released (Text-fig 2A) came from the cell collections made over the 24-hour period after the peak output of the plaque-forming cells and after the proportion of plaque-forming cells to total cells reached a maximum in the lymph.

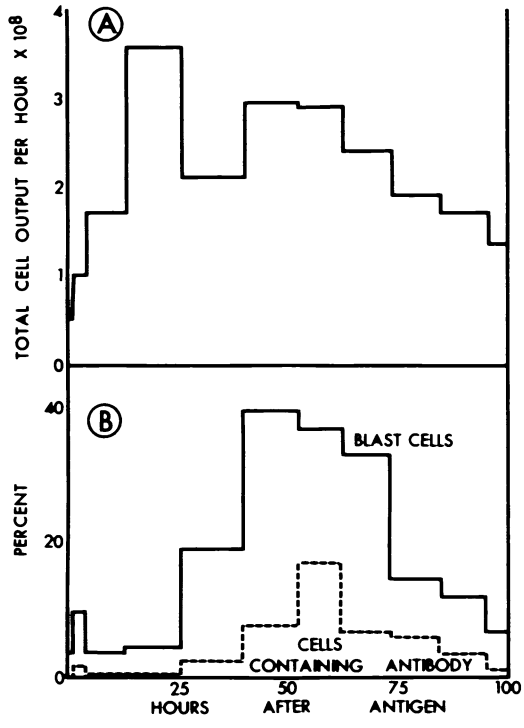
The amount of antibody released *in vitro* per 10^6 plaque-forming cells increased throughout the immune response, and the plaque-forming cells that appeared late in the response were found to contain some 20 times more antibody per cell than those that appeared first (Text-fig 2B). This was found to occur in both primary and secondary responses to *Salmonella*.

Number of Antibody-Forming Cells Appearing in Lymph During an Immune Response

Secondary responses to horseradish peroxidase were studied in the efferent lymph from the popliteal node of sheep in order to measure the number of antibody-forming cells released from the node. This antigen was chosen because the histochemical reaction that detects the presence of antibody against horseradish peroxidase is highly sensitive and it was thought that cells producing minimal amounts of antibody would be detected by this method more readily than by the hemolytic plaque assay.

A secondary response to horseradish peroxidase is shown in Text-figure 3. This challenge was given 6 weeks after a primary stimulation with antigen and incomplete Freund's adjuvant. The background level of blast cells and cells containing antibody in the lymph early in the response was due to a continuing reaction from the chronic granuloma produced by the primary injection. The secondary response to this antigen always reached its maximum before 60 hours. In this particular experiment, the antibody-containing cells constituted about 17% of the total cell population at the height of the response. Approximately 37% of the cells in the lymph were classified as blasts at this time and 1 out of 2 of these cells contained antibody. The peak of the blast cell response occurred before the peak number of antibody-containing cells appeared; it was thought that this was most likely due to the fact that the population of cells appearing later in the response contained more antibody and were thus more likely to be scored as positive in the light microscope. In vigorous secondary responses to horseradish peroxidase in some sheep, as many as two out of three of the blast cells in the lymph at the peak of the immune response were positive for antiperoxidase antibody (Fig 1A and B).

When samples of cells obtained from efferent popliteal lymph during

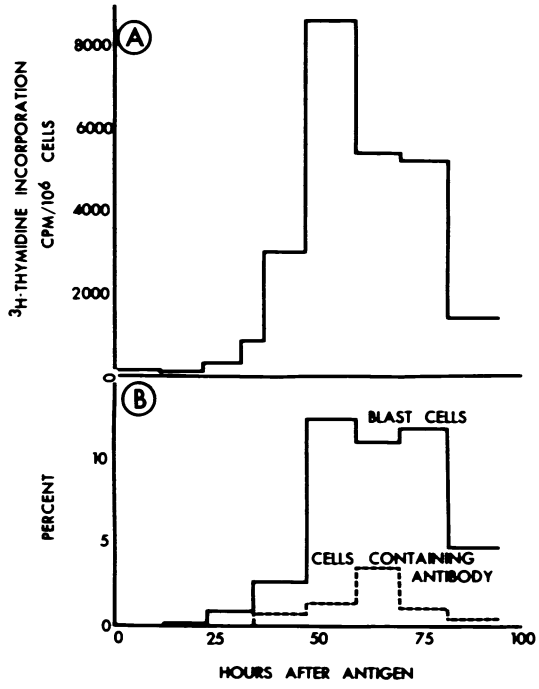


TEXT-FIG 3—A secondary immune response to horseradish peroxidase in the efferent popliteal lymph of the sheep. The total cell output in the lymph is shown in A and the percentages of blast cells and cells containing antibody are shown in B.

secondary responses to horseradish peroxidase were examined in the electron microscope, it was found that the number of antibody-containing cells was even higher than was apparent from the light microscopic smears. Additional cells with only minimal amounts of antibody in their cytoplasm could be detected at the higher magnifications of the electron microscope (Fig 1C and D).

Text-fig 4 shows a secondary response to horseradish peroxidase in which the priming challenge had been given 10 weeks before. This response was less vigorous than secondary responses occurring 3–6 weeks after the primary challenge and there was no background of blast cells or antibody-containing cells in the lymph at the time of the secondary challenge. As in other responses, the antibody-containing cells reached a peak later than the peak of the blast cell response.

In all secondary challenges with peroxidase, the rates of lymph flow always increased considerably during the response and often were maintained at up to five times the normal rate of flow for periods of a week. This hypersensitivity response did not usually occur until the second day after challenge.



TEXT-FIG 4—A secondary immune response to horseradish peroxidase in the efferent popliteal lymph showing the temporal relationship between the changes in ^{3}H-thymidine incorporation by the cell population and the appearance of blast cells and cells containing antibody.

Incorporation of ^{3}H-Thymidine and ^{3}H-Uridine into Cells in Lymph

During immune responses to *Salmonella* and horseradish peroxidase, efferent lymph cells were tested for their ability to incorporate ^{3}H-thymidine and ^{3}H-uridine and to produce antibody. The cells in lymph showed an increased incorporation of ^{3}H-thymidine before plaque-forming cells and blast cells appeared (Table 1). A similar result was obtained in responses to horseradish peroxidase (Text-fig 4). These results were interpreted to mean that some cells appearing early in the response were synthesizing DNA before they contained or were capable of releasing detectable antibody and before they had transformed into basophilic blast cells.

The incorporation of ^{3}H-uridine into TCA-precipitable material from the lymph cells increased throughout the response, although to a smaller and more variable extent than that for ^{3}H-thymidine. The peak period of ^{3}H-uridine incorporation occurred at the same time in the response as that for ^{3}H-thymidine; the increased incorporation of uridine over prestimulation levels was about sixfold, however, compared with a 60-fold increase in thymidine incorporation (Table 1). Incorporation of ^{3}H-thymidine had increased significantly by the second day after antigenic stimulation, whereas, possibly because of the more variable results, a significant increase in ^{3}H-uridine could not be detected until the third day.

Table 1—Incorporation of ^{3}H-Thymidine and ^{3}H-Uridine into Lymphocytes from the Efferent Popliteal Lymph During Primary Response to **Salmonella***

Time	Blasts (%)	PFC†/10^6	^{3}H-thymidine (cpm/10^6)	^{3}H-uridine (cpm/10^6)
Before antigen	<1	0	109 ± 6.6	134 ± 17.5
Day 1	<1	0	91 ± 5.5	228 ± 55.8
Day 2	1.0	0	204 ± 14.8	168 ± 14.8
Day 3	6.8	430	3,920 ± 240.8	408 ± 18.9
Day 4	31.3	6,500	6,327 ± 605.3	705 ± 36.8
Day 5	20.1	939	1,404 ± 125.9	76 ± 13.0
Day 6	2.6	262	395 ± 139.3	227 ± 21.2

* The activity of TCA-precipitable material was determined after incubating for 1 hour with the radioactive precursor. Mean values of triplicate samples are expressed ± SD.
† Plaque-forming cells.

The Proliferation of Antibody-Forming Cells

Throughout the immune responses, some cells in the lymph were undergoing mitosis. Many of these cells, in responses to horseradish peroxidase, could be identified, in both light microscopic smears and in the electron microscope, with antibody in their cytoplasm. Although some of the cells found in mitosis were positive for antibody, mitotic figures negative for antibody were also present; this demonstrated either that there were cells proliferating in the response before becoming involved in antibody synthesis or that some cells were responding to antigens other than the one being tested.

Light Microscopic Autoradiography

In order to have a more accurate idea of the proliferative potential of the cells in lymph and to establish the number of cells entering the S phase of the mitotic cycle at the same time as they were synthesizing antibody, samples of lymph cells were incubated with ^{3}H-thymidine and those containing antibody were revealed with diaminobenzidine and hydrogen peroxide.

After 1 hour of incubation with ^{3}H-thymidine, between 70 and 80% of the large blast cells were found to have incorporated the radioactive label into their nuclear DNA. At the peak of the response, when the maximum number of antibody-forming cells was present, it was found that about half of the blast cells were both incorporating thymidine and synthesizing antiperoxidase antibody. In some of the blast cells, the distribution of antibody was sparse, suggesting that antibody synthesis had been proceeding for only a short time. On the other hand, a good number of cells stained intensely for antiperoxidase antibody and also were synthesizing DNA.

A small proportion of the cells taking up ^{3}H-thymidine were cells resembling small and medium lymphocytes. Some of these contained antibody. As a group, the smaller antibody-positive cells did not incorporate thymidine to the same extent as the larger blast cells.

Electron Microscopic Autoradiography

It was apparent from the ^{3}H-thymidine studies that, for many of the blast cells appearing in lymph during the secondary response to horseradish peroxidase, antibody synthesis and cell division went hand in hand. These cells could be considered therefore as differentiated cells in the biochemical sense but undifferentiated cells in the ultrastructural sense.

To test whether the development of a highly organized ergastoplasm storing specific antibody, such as that in plasma cells, precluded a cell from synthesizing DNA, lymph cells were collected coming from popliteal nodes of sheep hyperimmunized with *Salmonella*. With this antigen, as with influenza virus, [20] it is possible to find, at the peak of the response, a small proportion of antibody-forming cells in the lymph with highly developed, dilated ergastoplasm similar in may respects to that of mature plasma cells.

Samples of lymph were obtained during vigorous secondary responses to *Salmonella*; the cells were incubated with ^{3}H-thymidine and then exposed for electron microscopic autoradiography. Examples of cells in all stages of ultrastructural differentiation could be found synthesizing DNA (Fig 2 and 3). However, in addition to the abundant labeled blast cells, cells that could only be classified as plasma cells on cytologic and immunologic grounds were also found to incorporate ^{3}H-thymidine as actively as many of the immature blast cells. Many of these labeled cells contained Russell bodies as well as a dilated concentric ergastoplasm filled with secretion products.

Characteristics of Individual Antibody-Containing Cells in Lymph

During the immune responses to horseradish peroxidase, some cells containing antibody were present which had many of the ultrastructural characteristics of small and medium lymphocytes. These cells often contained minimal amounts of antibody, which was mostly confined to the perinuclear space. The nuclear chromatin of these cells was condensed, particularly around the periphery of the cell, and the nuclear-cytoplasmic ratio was low. The ribosomes in the cytoplasm of these cells showed some degree of aggregation into polyribosomal clusters, distinguishing them from normal lymphocytes although there were still many single

ribosomes present. The endoplasmic reticulum was undeveloped and usually amounted to only a few short profiles. Cells of this type appeared early in the response and were present in the first lymph samples in which antibody-containing cells were identified. Examples of this type of cell are shown in Fig 4.

Large basophilic blast cells were the predominant antibody-containing cell throughout the whole of the response and these cells contained amounts of antibody varying from groups of positive ribosomes to cells replete with reaction product (Fig 5). No cells with the ultrastructure of plasma cells appeared in the lymph, even though this type of cell, filled with antibody, was abundant in the lymph node.

As with the blast cells, the antibody present in cells actually in mitosis varied from minimal amounts detectable only in the electron microscope to extensive deposits easily visible in the light microscope. The antibody extended throughout the cytoplasm of these cells, intermingling with the chromosomes. In prophase cells, the antibody tended to be concentrated around the periphery.

Discussion

The term *undifferentiated cell* implies that a cell, given the appropriate stimulus, has the potential to develop along one of several different pathways. Is the lymphocyte then a differentiated cell? If it is assumed that a lymphocyte is a precommitted, unipotential cell [21] which can only respond to a single antigen, then the lymphocyte can be thought of as being differentiated. However, if it is assumed that a lymphocyte is capable of being stimulated to transform by a range of antigens and that it is multipotential, then it is an undifferentiated cell. At this time, no definitive answer can be given to this question of unipotentiality versus multipotentiality and so for the purpose of this discussion, the differentiation of lymphocytes is considered to be a process initiated by antigen and concerned with the expression of the cell's ability to synthesize specific antibody. In this sense, the lymphocyte is an undifferentiated cell.

In general terms, the cellular response in the popliteal efferent lymph to a secondary challenge with horseradish peroxidase resembled secondary responses to *Salmonella* and other antigens. The number of specific antibody-forming cells involved in the response was, however, an order of magnitude higher than the numbers detected in responses to *Salmonella* antigens in the same experimental system,[12] and several orders of magnitude greater than the numbers of antibody-forming cells reported by other workers using different systems. Figures for antibody-form-

ing cells in the spleen and lymph nodes are usually of the order of 1:1000 to 1:10,000.[22-24]

Using the plaque assay, up to 6% of the cells in efferent lymph were shown to be releasing antibody to *Salmonella* and the proportion of cells stained with fluorescent anti-sheep globulin was of a similar order of magnitude.[25]

The most likely reasons for the high proportion of antibody-forming cells detected in the present experiments with horseradish peroxidase were the sensitivity of the immunochemical reaction coupled with the use of electron microscopy and the specific release of the antibody-forming cells into the efferent lymph. It seems unlikely that the essential characteristics of immune responses to horseradish peroxidase differ from responses to other antigens and the proportion of antibody-forming cells to the total number of differentiating cells produced in secondary immune responses to other antigens might well be as high or higher than those found in the present experiments, provided sufficiently sensitive systems could be devised to detect them.

The present results showed that at the height of an immune response to horseradish peroxidase, some 40–50 million cells containing antibody may leave the popliteal lymph node each hour via the lymph and these cells may comprise up to two-thirds of the dividing blast cell population.

The exact relationship between antibody-forming cells to other types of antigen-sensitive cells and antibody-forming cell precursors is uncertain, but current immunologic thought appears to favor the idea that an interaction occurs between antigen and distinct populations of lymphoid cells from the thymus and bone marrow which, in some way, leads to the development of differentiated plasma cells that make antibody.[26-32] There is good evidence that the antibody-forming cells are the outcome of cellular proliferation [2,3,5,6,33] although it is held that the most extensive proliferation occurs among "antigen-sensitive" cells and "antibody-forming cell precursors." [34-36] Actual antibody-forming cells have been thought to have only limited proliferative potential once they begin to synthesize specific antibody.[1]

Both antigen-sensitive cells and antibody-forming cell precursors have been shown to be present in lymph as part of the recirculating cell population.[31] The present results show two things clearly. The first is that many of the cells in lymph that show evidence of transformation in response to antigen are proliferating cells and the second is that many of these cells are also actually synthesizing antibody. In view of the joint commitment of the transforming cells in lymph to division and antibody synthesis, it is difficult to find a place for antigen-sensitive cells

and antibody-forming cell precursors among the proliferating cell population in lymph.

The characteristics of the antibody-forming cells in lymph change as the immune response proceeds. Since significant numbers of antibody-containing cells with the cytologic characteristics of lymphocytes were present early in the response, it seems most probable that the lymphocyte is the precursor of at least a proportion of the antibody-forming cells throughout the entire response. This appears also to be so in the rat responding to red cell antigens from sheep.[37] From a cytologic viewpoint, the majority of the antibody-forming cells (large blasts) would be considered as undifferentiated cells because of their nuclear structure, dispersed chromatin and well-developed nucleoli and also because of the abundance of polyribosomes in their cytoplasm. However, from a biochemical viewpoint, since these cells have acquired the capacity to synthesize specific antibody, they must be considered to be differentiated.

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[*Illustrations follow*]

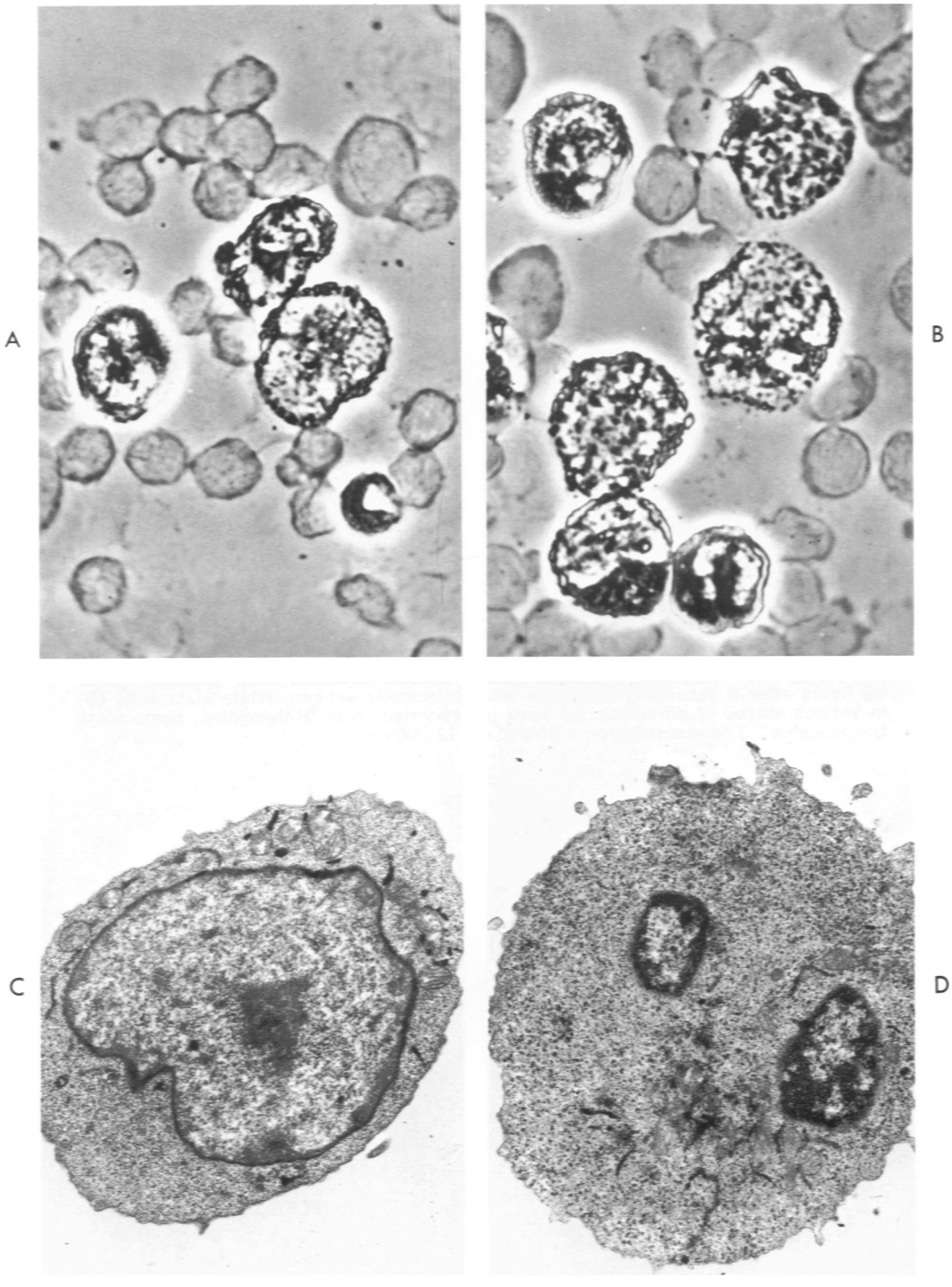
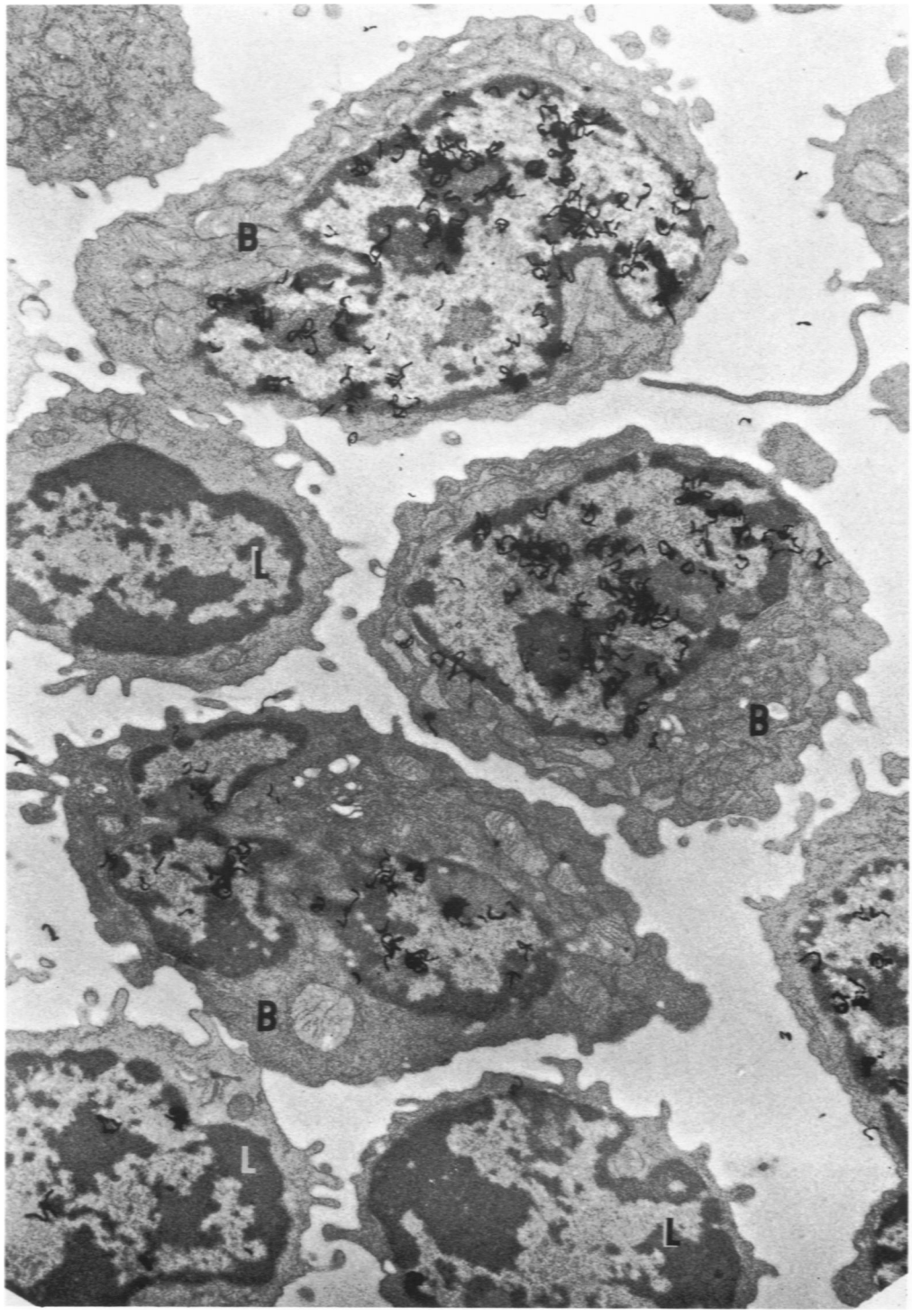


Fig 1A and B—Phase microscope pictures of cells collected from the popliteal lymph during secondary immune responses to horseradish peroxidase. Most of the blast cells contain antiperoxidase antibody. (× 1600). **C and D**—Examples of lymph cells containing minimal amounts of antiperoxidase antibody detected by electron microscopy (× 11,000).

Fig 2—Electron microscopic autoradiograph of cells collected from popliteal lymph 96 hours after a secondary challenge with *Salmonella* antigen. While blast cells (*B*) in various stages of differentiation have incorporated most ^{3}H-thymidine, some small lymphocytes (*L*) have also become labeled (× 12,000).


B
L
B
B
L
L

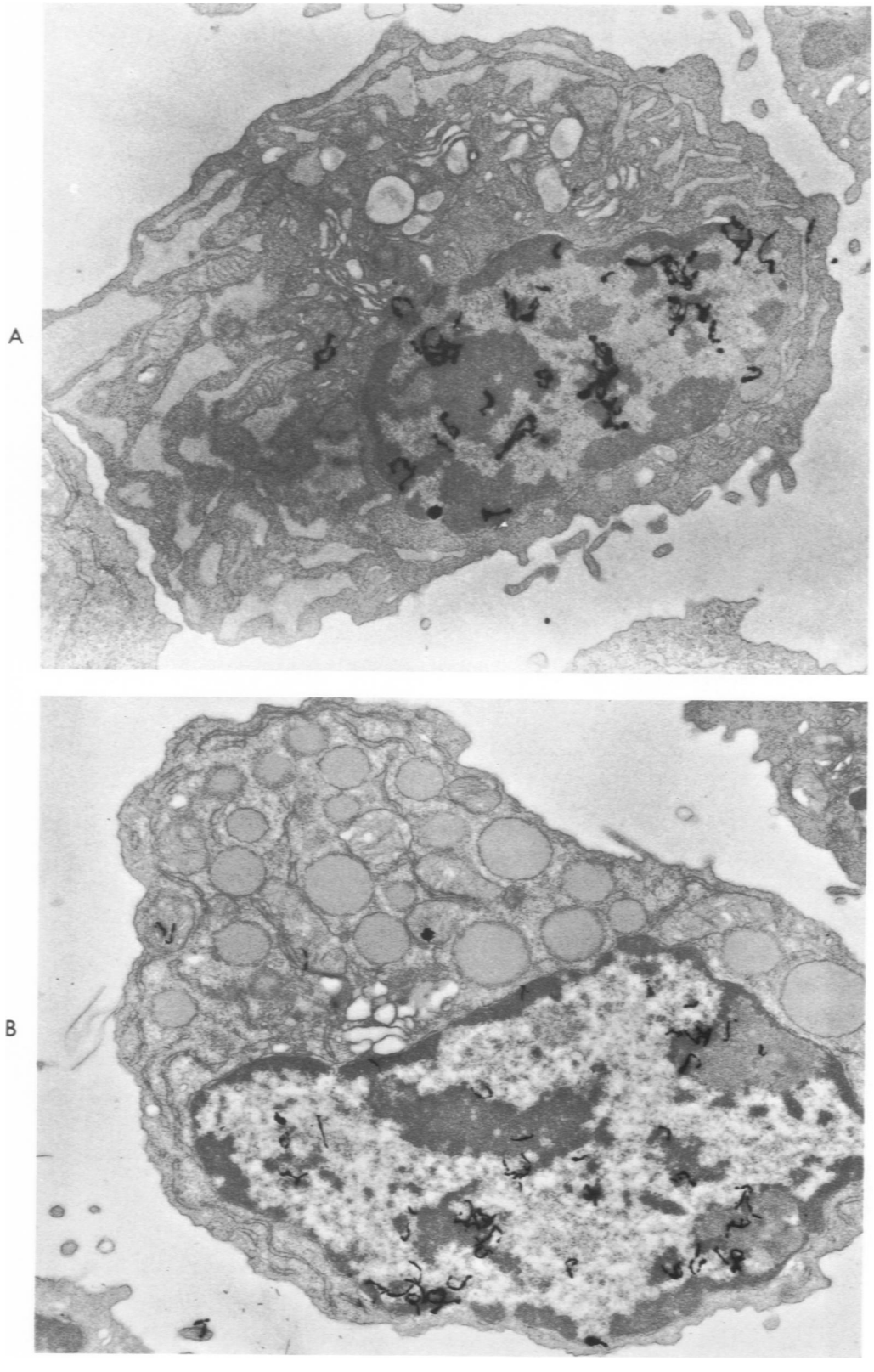


Fig 3A and B—Electron microscopic autoradiographs of plasma cells incorporating ^{3}H-thymidine. The ergastoplasm of these cells is extensively developed and filled with secretory products (A × 16,000; B × 18,000).

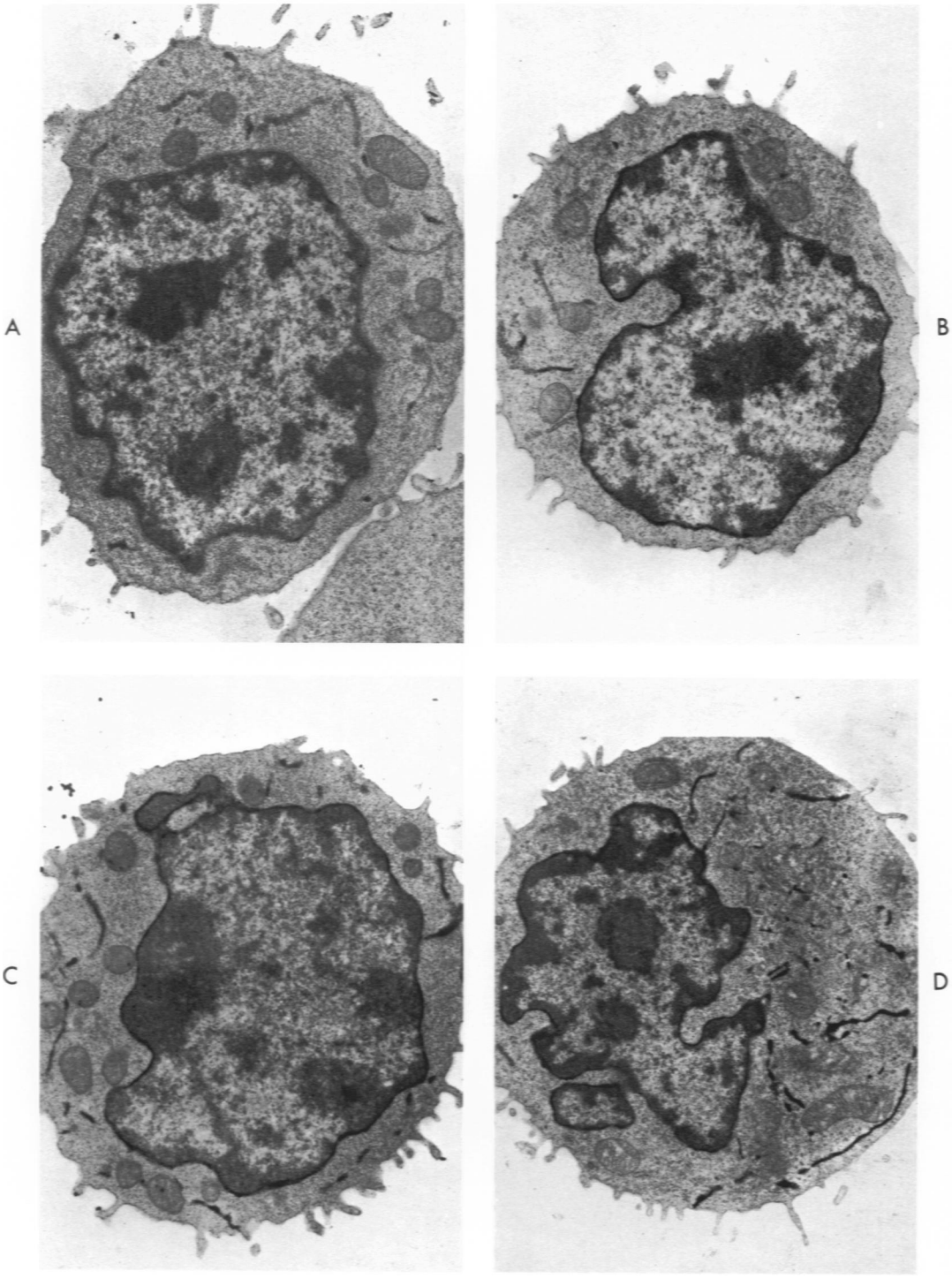


Fig 4—Examples of small and medium lymphocytes containing specific antibody against horseradish peroxidase. The cells were collected from popliteal lymph 50 hours after a secondary challenge (× 9000).

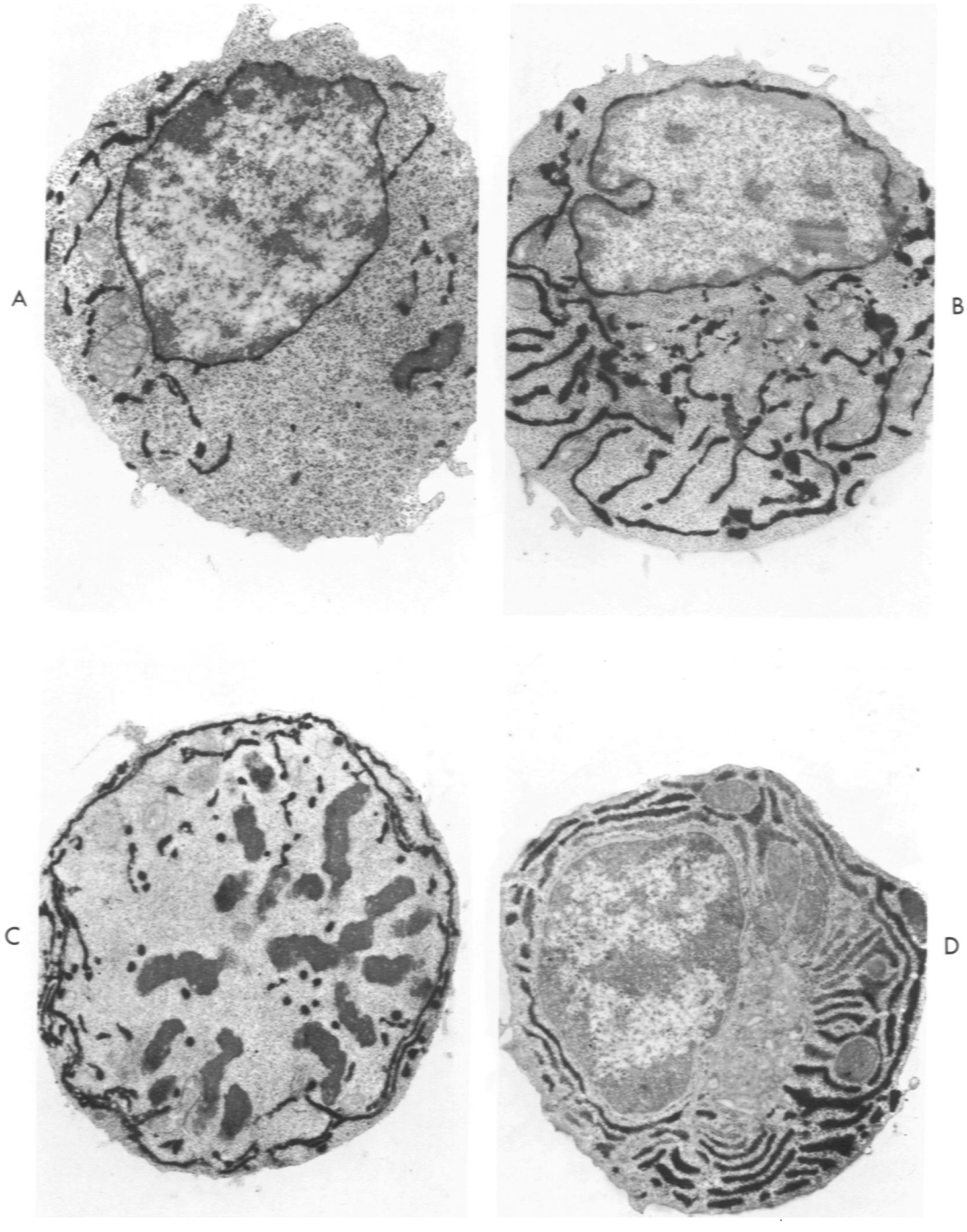


Fig 5A and B—Blast cells containing varying amounts of specific antibody against horseradish peroxidase collected from efferent popliteal lymph during secondary responses. (× 11,000). **C**—A cell in mitosis containing antibody (× 10,000). **D**—A plasma cell filled with antibody from a lymph node 120 hours after antigenic challenge (× 10,000).