THE ORIGIN OF THE CELLS IN THE EFFERENT LYMPH FROM A SINGLE LYMPH NODE

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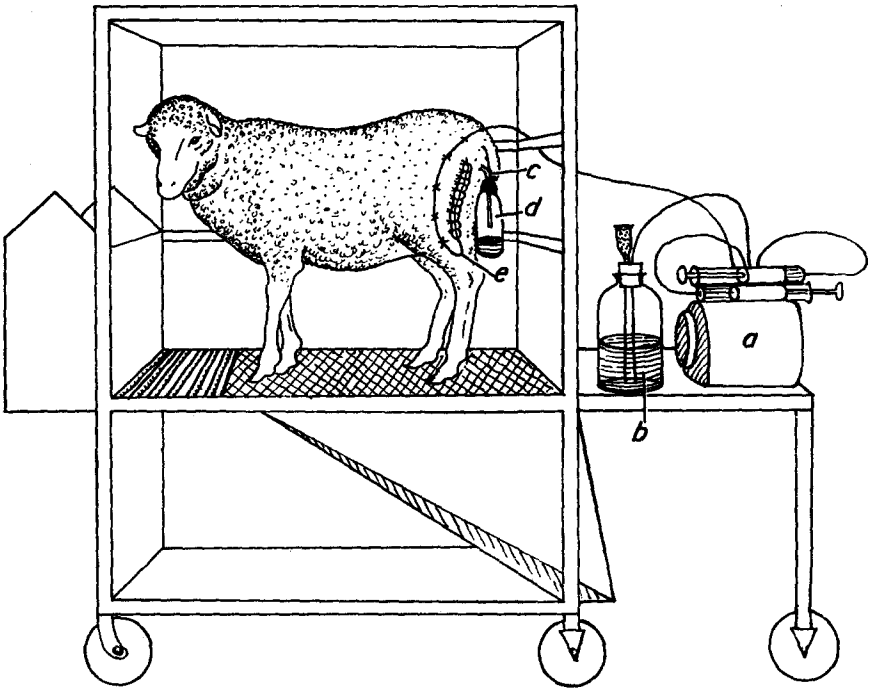
The work of Gowans *et al.* (1–6) has established that in the rat lymphocytes constantly recirculate from blood to lymph through the substance of the lymph nodes. The lymphocytes in the blood reenter the lymphatic system by traversing the endothelium of specialized postcapillary venules in the deeper parts of the cortices of the lymph nodes. These findings were the results of experiments based on an investigation of the conglomerate lymphocyte population of thoracic duct lymph and the study of fixed lymphoid tissue. It is not known however to what extent this recirculation of lymphocytes occurs in individual lymph nodes, nor is it known how many lymphocytes are being continually formed within a lymph node.

The development of techniques for monitoring the lymphocyte output of a single popliteal lymph node in sheep (7, 8) enabled an experiment to be designed to measure directly the contributions of lymphocyte production in the node and lymphocyte recirculation from the blood to the total lymphocyte population of the efferent lymph. The experiment involved the continuous perfusion of the popliteal node of a sheep with a solution of ^{3}H-thymidine, *via* an afferent lymphatic, so as to establish a high concentration of ^{3}H-thymidine locally in the tissue fluid of the node for several days. In this way the ^{3}H-thymidine was incorporated into the nuclear DNA of the cells produced in the node during the period of the perfusion. When these cells were released into the efferent lymph they were identified by autoradiography and their contribution to the total cell output was determined.

Materials and Methods

General Experimental Plan.—The efferent ducts from the popliteal nodes of the two hind legs of a sheep were cannulated. At the same time a further cannula was inserted in the direction of lymph flow into an afferent lymphatic of one popliteal node so that the node could be perfused. As soon as the sheep had recovered from the anaesthetic and was standing normally in its cage, a 0.9 per cent NaCl solution containing ^{3}H-thymidine was infused continuously into the perfusion cannula for as long as the preparation functioned, usually about 100 to 150 hours. Throughout the period of the experiment the efferent lymph from both the perfused node and

the unperfused, control node of the opposite leg was collected quantitatively over 12-hour collection periods. On each volume of lymph collected total and differential white cell counts were made and autoradiographs of cell films were prepared. The amount of radioactivity in the lymph plasma and the specific activity of the DNA of the lymph cells were also measured. The perfusion was stopped when radioactivity disappeared from the efferent lymph of the perfused node, showing that the perfusion circuit had been broken. After the perfusion had been stopped the experiment was continued until no radioactivity was detectable in the DNA of the cells



TEXT-FIG. 1. Diagram of the animal preparation used in the experiments; (*a*) pump, (*b*) reservoir of perfusion fluid, (*c*) cannula leading from the efferent duct of the popliteal lymph node, (*d*) collecting bottle, (*e*) perfusion cannula leading into an afferent lymphatic.

coming from the perfused node. At this point the perfused and control nodes were removed, sectioned, and prepared for examination by autoradiography. In some experiments the nodes were removed immediately the perfusion was stopped.

The surgical techniques used and the methods of counting and classifying the lymph cells have been described previously (7, 8).

A diagram of the experimental preparation is shown in Text-fig. 1.

Animals.—Full-mouth merino ewes were obtained from local flocks and used for all the experiments.

Perfusion Fluid.—^{3}H-thymidine (1.7 c per mmole) was added to a 0.9 per cent NaCl solution to give a final concentration of radioactivity of 2 μc per ml. The perfusion fluid was made up in sterile, 1000 ml transfusion bottles which served as a reservoir for the perfusion pump.

Perfusion Pump.—A "unita 1" continuous infusion apparatus (Braun Apparatbau, Melsun-

gen, West Germany) which consisted of a pair of reciprocating 50 ml syringes was used. The pump was connected to the reservoir containing the ^{3}H-thymidine solution and not disconnected until the experiment was stopped. The pumping rate was 3.75 ml per hour.

Isotope.—Thymidine-6-^{3}H was obtained from Schwartz Bioresearch Inc., New York.

Collection of Lymph.—Lymph was collected into sterile polythene bottles to which penicillin and heparin had been added. Enough unlabelled thymidine was added to the collecting bottles into which the lymph from the perfused node was collected to ensure a 500-fold reduction in the specific activity of the infused ^{3}H-thymidine even if this was recovered quantitatively in the efferent lymph. This was done to prevent the occurrence of *in virto* labelling of the collected cells.

Autoradiographs.—These were prepared by the method of Pelc (9) using kodak AR 10 fine-grain stripping film. Cells from the perfused and control nodes were spread on the same slide in two parallel films. After the stripping film was applied the slides were kept at 0°C in light-tight boxes for 3 weeks before being developed and stained through the emulsion with a 0.1 per cent solution of azure A.

Assay of Radioactivity in Lymph Plasma.—The radioactivity in the lymph was measured after processing the lymph plasma by an unvarying procedure which was aimed at the preparation of planchets containing material of identical composition and distribution so that their counting rates could be compared directly.

To 4.5 ml of lymph plasma 0.5 ml of trichloroacetic acid (TCA) solution (100 gm per 100 ml solution) was added and the precipitated protein removed by filtration. The filtrate was washed twice with 8 ml ether to remove the TCA. Portions of the washed filtrate (1 ml) were pipetted into metal planchets and evaporated to dryness at 98°C. The radioactivity was then counted in a "tracerlab" model SC50-B continuous gas-flow, proportional counter.

The amount of radioactivity infused was measured by diluting the perfusion fluid in non-radioactive lymph plasma and then processing it as described above. By trial and error a dilution was found which gave a counting rate equal to that of the specimen under test and from this measurement the percentage of the infused radioactivity recovered in the efferent lymph was calculated.

Determination of the Specific Activity of ^{3}H-labelled DNA.—Measurements of the specific activity of the DNA of the cells in the efferent lymph were made at intervals throughout the experiments to give an immediate estimate of the extent of DNA synthesis by the lymph cells.

Lymph cells were deposited by centrifugation and washed twice in 0.9 per cent NaCl solution. The nucleic acids were extracted from the cells and the DNA measured by the diphenylamine reaction after the method of Schneider (10). The only modification of the published method was that the 5 per cent TCA extract containing the nucleic acids was washed twice with an equal volume of ether and once with an equal volume of 2:5 butanol-chloroform mixture to remove the TCA and residual protein. 1.0 ml of the washed extract was pipetted into a planchet evaporated to dryness and the radioactivity counted and 1.0 ml was taken for the diphenylamine reaction. The standard deviation of replicate estimations of the specific activity of the DNA was 8.5 per cent of the mean.

Sterile Precautions.—All operations were performed in a properly equipped operating theatre under strict aseptic conditions. Immediately after the perfusion cannula was inserted into the afferent lymphatic its free end was sealed in a flame. All the external cannulae of the pumping circuit were boiled before use and the assembled pumping circuit was flushed through with surgical spirit and then with boiling water immediately before use.

When the sheep had recovered from the anaesthetic the free end of the perfusion cannula was unsealed and immediately connected to the pumping circuit and the perfusion was begun. From then on the pumping circuit was not interfered with until the experiment was over; in this way the entry of extraneous antigenic material into the system was minimised. Air entered the reservoir of perfusion fluid through a filter of sterile gauze.

RESULTS

The experiment as outlined was carried out successfully in 7 sheep.

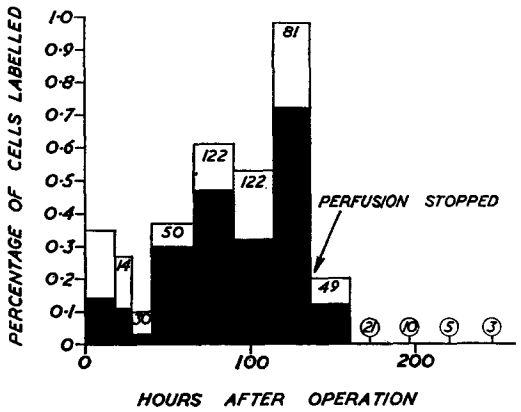
The amount of radioactivity recovered in the efferent lymph from the perfused nodes was always small, usually about 1 to 2 per cent of the amount infused. Most of the ^{3}H-thymidine left the lymph as it passed through the node presumably entering the blood stream. In spite of this no radioactivity was detected in the lymph collected from the unperfused, control nodes and no unequivocally labelled cells were seen in autoradiographs of cell smears prepared from the lymph from these nodes. Similarly no labelled cells were seen in autoradiographs prepared from sections cut from the unperfused control nodes. Some radioactivity was detected in the DNA extracted from the cells in the efferent lymph from the control nodes but the specific activity of this DNA was never more than 5 per cent of that of the DNA obtained from cells in the efferent lymph from the perfused nodes and usually it was much less. In view of these findings it was relatively certain that the labelled cells which came from a perfused node were produced in that node and nowhere else.

As the cannulation of afferent lymphatic vessels was carried out below the level of lymphatic drainage to the popliteal node a direct route was established by which antigenic contaminants could reach the node. Thus fortuitous antigenic stimulation of the node occurred frequently and in consequence, plasma cells and their precursors appeared in the lymph in considerable numbers (8). However in 2 of the 7 experiments the perfused node remained unstimulated and the cell population of the lymph remained entirely lymphocytic. The detailed results of these 2 experiments were very similar; the results of 1 of these is shown graphically in Text-fig. 2. In the experiment illustrated the popliteal node was perfused for 136 hours. In the autoradiographs of the cells in the efferent lymph the number of labelled cells never accounted for more than 1 per cent of the total. About half of the labelled cells were large lymphocytes the remainder being normal, "mature" lymphocytes. All the cells which incorporated the ^{3}H-thymidine were labelled so intensely that the silver grains often formed a confluent mass over the nucleus of the cells. A photomicrograph of labelled lymphocytes in the efferent lymph from a perfused node is shown in Fig. 1.

The maximum percentage of labelled cells occurred after the node had been perfused for about 110 hours; at this time the specific activity of the DNA had reached a plateau level. Of the large lymphocytes present in the lymph at this time about 30 per cent were labelled. If it is assumed that all the large lymphocytes in the efferent lymph are formed in the node, this result could be interpreted as indicating that only 30 per cent of the node was being perfused effectively. Once the perfusion was stopped very few labelled cells appeared in the lymph and within 40 hours the labelled cells had disappeared completely

from the lymph. Similarly the specific activity of the DNA fell rapidly after the perfusion was stopped although small amounts of radioactivity were detectable for the following 160 hours.

In the 2nd experiment, the node was perfused for 180 hours without any antigenic stimulation occurring. In this case the labelled cells never accounted for more than 0.6 per cent of the total and this value was reached after the node had been perfused for about 100 hours at which time the specific activity of the lymphocyte DNA had reached a plateau. Although the perfusion was continued for a further 80 hours there was no further increase in the percentage



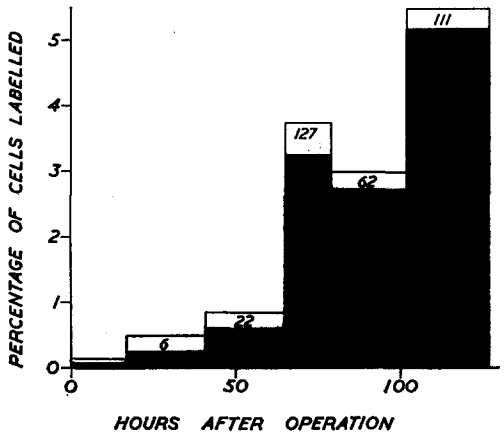
TEXT-FIG. 2. The appearance of labelled lymphocytes in the efferent lymph from an unstimulated popliteal node during its continuous perfusion *via* an afferent lymphatic, with a solution containing ^{3}H-thymidine. The proportion of the labelled cells which was made up of large, immature lymphocytes is shown by the blocked area. The numerals in the histograms denote the specific radioactivity (CPM per μg DNA-phosphorus) of the DNA extracted from the lymphocytes in the efferent lymph.

of labelled cells or in the specific activity of the DNA. Again about half the labelled cells were large lymphocytes and about 40 per cent of all the large lymphocytes in the lymph were labelled. When the perfusion was stopped the percentage of labelled cells and the specific activity of the DNA again fell rapidly.

In the other experiments the perfused nodes became stimulated. The results of one of these experiments is shown in Text-fig. 3. Until plasma cells began to appear in the lymph the pattern of labelling of the cells was much the same as in the previous experiments. When the immune response was manifest in the lymph several per cent of the total cells were found to be labelled. However, in these circumstances nearly all the labelled cells were transitional cells, large lymphocytes, or cells of the plasma series (8). The proportion of the total cells accounted for by labelled, mature lymphocytes never exceeded 1 per

cent. In the experiment illustrated 5.5 per cent of all the cells in the lymph were labelled after the node had been perfused for 130 hours but of these labelled cells only 7 per cent were normal mature lymphocytes. At this time about 40 per cent of all the immature cells and plasma cells were labelled. A photomicrograph of a labelled, immature plasma cell is shown in Fig. 2.

In the final experiment of this series the ^{3}H-thymidine solution was perfused simultaneously through 3 separate afferent lymphatics of the popliteal node in order to ensure that it was distributed as widely as possible through the node. In this experiment a vigorous immune response occurred and after 86



TEXT-FIG. 3. The appearance of labelled cells in the efferent lymph from an antigenically stimulated popliteal node during its continuous perfusion *via* an afferent lymphatic, with a solution containing ^{3}H-thymidine. The proportion of the labelled cells accounted for by transitional cells, cells of the plasma series and large lymphocytes is shown by the blocked area. The numerals in the histograms denote the specific radioactivity (CPM per μg DNA-phosphorus) of the DNA extracted from the cells in the efferent lymph.

hours, 48 per cent of all the cells in the efferent lymph were cells of the plasma series; 42 per cent of all the cells in the lymph were labelled and of these only 2 per cent were mature lymphocytes.

The Autoradiographic Appearance of Sections Cut from Perfused Lymph Nodes.—Sections were cut from 3 lymph nodes which had been perfused with ^{3}H-thymidine for 1 hour and removed immediately. These showed that some of the cells in the lymph node had already become labelled. The labelled cells were confined to the lymphoid parenchyma of the cortex; some of these cells were in germinal centres but many were scattered throughout the cortex in an apparently random fashion.

Sections cut from lymph nodes which had been perfused for several days and then removed showed more extensive labelling. The labelled cells were

nearly all lymphoid cells in the parenchyma but a few of the reticuloendothelial cells which lined the pericortical sinus and the lymph sinusoids were also labelled. Most of the labelled lymphoid cells were randomly distributed through the node but some were grouped together in the lymphoid nodules. In the nodules the labelled cells were usually larger than the unlabelled lymphocytes which surrounded them and some of the labelled cells in the germinal centres were very large cells indeed. As these nodes had been stimulated the cellularity of their medullae had increased. Labelled cells were scattered throughout the medullary region but were more sparse than in the cortex. The labelled cells in the medullae did not occur in groups and again for the most part they appeared to be immature being larger than the cells which surrounded them.

Sections cut from perfused nodes which had not been removed until 140 hours or so after the perfusion had been stopped showed that few labelled cells remained in the lymphoid parenchyma. Some of the reticuloendothelial cells lining the sinusoids and some connective tissue cells in the capsule and trabeculae however were still heavily labelled.

The Distribution of ^{3}H-Thymidine within the Nodes.—Apart from the 1 experiment in which the node was perfused through several cannulae the labelling pattern of the cells in the lymph suggested that the ^{3}H-thymidine may not have been uniformly distributed to all parts of the node. This appeared to be borne out by the autoradiographs of sections cut from the perfused nodes as the labelled cells were not uniformly distributed and some areas of the node contained no labelled cells at all. It was estimated that about one third of the lymphoid parenchyma was perfused in the experiment but to interpret the results in a sufficiently critical way the assumption has been made that one quarter of the node tissue was in fact effectively perfused.

DISCUSSION

In the 2 experiments in which the perfused nodes remained unstimulated the maximum numbers of lymphocytes which became labelled were 1.0 and 0.6 of the total cell population of the efferent lymph. Even if it is assumed that only one quarter of the lymph node was adequately perfused this result indicates that the unstimulated lymph nodes produced no more than 2 to 4 per cent of all the lymphocytes that were released into the efferent lymph. The cells in the lymph that became labelled were mostly large lymphocytes. These large lymphocytes normally make up about 2 per cent of the cells in lymph and being immature cells they might reasonably be suspected of being of recent origin and of having the potentiality for dividing to supply a number of small lymphocytes (7, 8). It cannot be argued that the reason why only a small number of labelled lymphocytes appeared in the efferent lymph was due to the failure of newly formed cells to leave the lymph node for some time after

they were formed. The rapid fall in both the number of labelled cells and in the specific activity of the cellular DNA after the perfusion was stopped showed that there was no large pool of freshly made but temporarily immobile lymphocytes within the node. This conclusion was supported by the fact that only a small number of labelled lymphoid cells were found in autoradiographs cut from sections of nodes which were removed 140 hours or so after the perfusion had been stopped. In any case it had been shown previously that if all the lymphocytes in the efferent lymph of the popliteal node of sheep were produced in the node itself every lymphoid cell within the node would have to replicate itself at least once every 70 hours (11). Since even after the nodes in these experiments had been perfused with ^{3}H-thymidine for 120 to 180 hours only 1 per cent or less of the cells in the efferent lymph were labelled it is apparent that the *de novo* production of cells in a lymph node does not contribute many lymphocytes to the cell population of efferent lymph. Since the number of lymphocytes conveyed to the node by the afferent lymph is very small (7, 8) the only other source of lymphocytes is the blood. It is from there that most of the lymphocytes in the efferent lymph must have come.

There are no experiments in the literature with which these results can be compared directly. Gowans (2) administered a continuous intravenous infusion of ^{3}H-thymidine to a rat for the first 12 hours after he had cannulated its thoracic duct. Autoradiographs of the cells in the thoracic duct lymph which had been collected throughout the 48 hours after the infusion was stopped showed that 5 per cent of the lymphocytes were labelled. Of these labelled cells 80 per cent were large immature lymphocytes and 90 per cent of all the large lymphocytes in the lymph were labelled. Everett, Reinhardt and Yoffey (12) gave a series of guinea pigs a single injection of ^{3}H-thymidine and sampled the thoracic duct lymph at various times after the injection. The percentage of labelled cells achieved a maximum value of 7 per cent 7 to 12 hours after the injection and about half the labelled cells were immature. These results are difficult to interpret quantitatively because the isotope was not available to the cells long enough for a steady state to be achieved, but both experiments indicate that the bulk of the cells which became labelled were immature forms and that the majority of the smaller lymphocytes which are found in lymph are not of very recent origin.

In the experiments where the perfused nodes received fortuitous antigenic stimuli many more labelled cells appeared in the lymph but these cells were nearly all large immature lymphocytes or cells of the plasma cell series. The labelling pattern of the plasma cells could not be correlated with antibody production since the nature of the antigens and the time at which they impinged on the nodes were unknown. In the absence of a proper experimental design it is impossible to discuss the labelling of the plasma cell series in detail, however, there seems to be little doubt that these cell types were all produced in the node.

The changes in the cell output in the efferent lymph of the popliteal node of the sheep following antigenic stimulation have been described (8). Some of these changes can now be interpreted in terms of the extent of recirculation of lymphocytes through the node in response to the presence of an antigen in the node. The average output of lymphocytes from an unstimulated popliteal node weighing 1 gm is 30×10^6 cells per hour (11). As almost all of these cells are derived from the blood something of the order of 10 per cent of the lymphocytes in the blood passing through the node must migrate out of the blood stream. The extent of this migration probably increases to the order of 30 per cent following antigenic stimulation. As lymphocytes do not appear to show chemotactic responses this large scale migration might well be controlled by the activities of capillary endothelium within the node. Although the migration of lymphocytes apparently occurs most spectacularly through the postcapillary venules within lymph nodes, the endothelium of the blood vascular system in general allows the migration of very large numbers of lymphocytes into tissues throughout the body. In this regard the effective establishment of localized immune responses to antigens such as homografts probably depends on the migration of lymphocytes from blood capillaries around the homografts. It would appear that of the large numbers of cells which recirculate only a small proportion become involved in initiating the immune response within the node. The profligate nature of this recirculation suggests that there is no specific attraction between antigen and lymphocyte insofar as migration from the blood stream is concerned. It seems that from a heterogeneous population of lymphocytes with diverse immunological potentials an appropriate few are selected and retained in the node where they give rise to lymphoid blast cells and cells of the plasma series which subsequently produce specific antibody (13).

SUMMARY

The popliteal nodes of sheep were perfused continuously for over 100 hours with a solution containing ^{3}H-thymidine. The labelling pattern of the lymphocytes in the efferent lymph from the perfused nodes showed that under normal conditions not more than 4 per cent of these lymphocytes were actually produced in the node. The transitional cells, large lymphocytes and cells of the plasma cell series which appeared in the lymph following antigenic stimulation of the node were all produced in the node.

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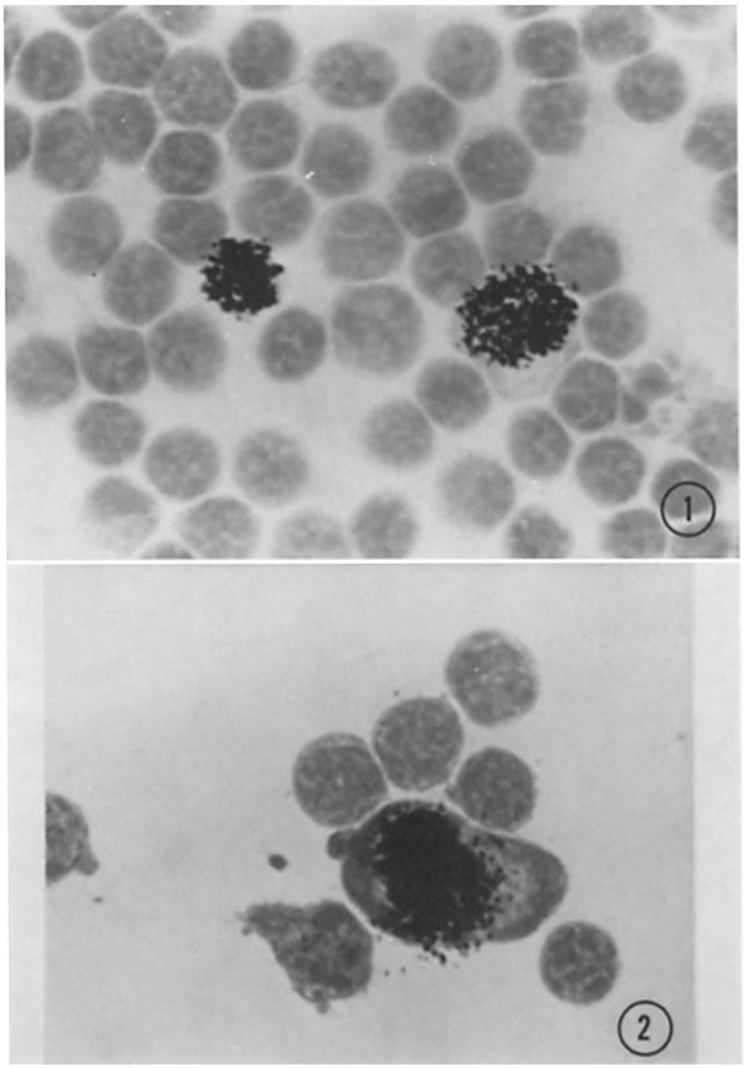
EXPLANATION OF PLATE 73

FIGS. 1 and 2. High power photomicrographs of autoradiographs of cell films prepared from the efferent lymph of popliteal lymph nodes which were being perfused with ^{3}H-thymidine. Approximately × 2200.

FIG. 1. Lymph from an unstimulated node. A large lymphocyte and a normal, mature lymphocyte are heavily labelled but the other lymphocytes contain no detectable labelling. The node had been perfused for 136 hours when this specimen was obtained.

FIG. 2. A heavily labelled, immature plasma cell in lymph from an antigenically stimulated node. The node had been perfused for 95 hours when this specimen was obtained.



(Hall and Morris: The origin of lymph cells)