STUDIES OF THE CELLS IN THE AFFERENT AND EFFERENT LYMPH OF LYMPH NODES DRAINING THE SITE OF SKIN HOMOGRAFTS

By J. G. HALL, M.B.

(From the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge, England)

Plates 90–92

(Received for publication 19 December 1966)

In 1942 Ehrich and Harris (1) attempted to investigate the number and types of cells in the lymph from an antigenically stimulated node by collecting lymph from the efferent duct of the popliteal nodes of rabbits. However the volumes of lymph that could be collected by this method were too small to allow a study of the morphology of the lymph cells.

In 1962 Hall and Morris (2) developed a method for collecting lymph from the popliteal nodes of sheep under physiological conditions for long periods of time. Further experiments (3) showed that the stimulation of the popliteal node with conventional antigens was followed by characteristic changes in the number and types of cells in the efferent lymph together with the appearance of both circulating and intracellular antibodies. The main feature of this cellular response was the appearance of large cells which had intensely basophilic cytoplasm, prominent nucleoli, and frequent mitotic figures. Similar cells were found in afferent (peripheral) popliteal lymph draining from the sites of antigen injection, though in this situation the number of cells was very small. A later electron microscope study (4) showed that these cells lacked the typical concentric endoplasmic reticulum of classical plasma cells. Instead they contained large numbers of free polyribosomes and only a small amount of endoplasmic reticulum.

Preliminary experiments (5) in which the popliteal nodes of sheep were stimulated by the application of skin homografts to the lower leg showed that the changes in the cell population of the efferent lymph during the rejection of the homografts were qualitatively similar to those which followed stimulation with conventional antigens. Similar findings have been reported by Rymaszewska et al. (6) who collected the lymph of rabbits.

In the present work a more thorough study has been made of the cells in lymph draining from the sites of skin homografts. Also, in some experiments the basophilic cells released into the efferent lymph were labeled in vitro with thymidine-^{3}H and returned to the animal by intravenous injection. The arrival

of the labeled cells in the skin homografts and other tissues was then monitored by liquid scintillation counting and radioautography of biopsy and postmortem specimens.

Materials and Methods

Animals.—Yearling Clun Forest wethers weighing 36–46 kg were used for the experiments. The sheep were kept in separate pens in an animal house and fed a standard diet of chaff and oats with water and mineral lick *ad libitum*.

The sheep were taken from what is essentially a commercial flock, somewhat inbred. The number of rams used for breeding is small and some of the ewes may have been backcrossed with their sires. The blood group factors of sheep from this flock have been found to be relatively homogenous but evidence for an increased histocompatibility between individual sheep has not been detected in terms of the rejection times of homografts of skin.[1]

Surgical Procedures.—All lymphatic cannulations and skin grafting operations were done under aseptic conditions and general anesthesia.

Anesthesia was induced by injecting Nembutal (Abbott Laboratories Ltd., Queenborough, Kent, England) into the jugular vein and when necessary was maintained with an oxygen-halothane mixture administered through an endotracheal tube which was connected to a Boyle apparatus.

The technique of cannulating efferent and afferent lymphatic vessels of individual lymph nodes and the methods of lymph collection and cell counting have been described in detail previously (2–5).

In sheep, skin grafting presents some problems. In the preliminary experiments (5) the grafting of either full or split thickness skin from wool-bearing areas proved unsatisfactory. Although, on occasion, successful autografts were established the appearances of the grafts were difficult to interpret because of the variable behavior of the wool follicles and a peculiar and patchy type of hyperkeratinization. Therefore in the present study skin grafts were always taken from the bare areas of skin in the groins and axillae. The skin of these areas is thin and mobile; it bears no wool, only a few sparse hairs. Full thickness grafts were cut from such donor sites and pinned with the epidermal surface downward on a cork board so that the subdermal connective tissue could be trimmed away. The trimmed grafts were then applied to full thickness beds in the lower leg or flank of the recipient sheep and sutured in place with 5/0 black silk. The skin grafts were rectangular, those applied to the leg measured 1½ × 2½ cm while those applied to the flank measured 7 × 2 cm. All grafts were covered for 12 days with dressings of several layers of tulle gras covered with gauze swabs. In the case of grafts on the legs the dressings were held in place by a "Lastonet" bandage (Lastonet Products, Redruth, Cornwall, England). Dressings on grafts on the flank were held in place by rubber bands which were hooked to appropriately placed skin sutures. Both these methods kept the dressings firmly applied to the grafts yet permitted the dressings to be changed and the graft inspected quickly and with minimal trauma.

Criteria of the Rejection of Skin Homografts.—In this study only the rejection of primary homografts was investigated. Grafts were first inspected after they had been in place for 5 days. At this time both homografts and autografts appeared pink, well vascularized, and healthy. Thereafter autografts became slighly less pink, healed into place, and ultimately a normal growth of hair was established. The first signs of rejection of homografts appeared 5–10 days after grafting. The original pink color became rather deeper and soon took on a

[1] Tucker, E. M. 1966. Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge. Personal communication.

bluish tinge. This color change was usually associated with a patchy, oedematous thickening of the graft. The development of the blue coloration was taken as the earliest signs of homograft rejection. Later the blue color of the homograft deepened and the graft ultimately took on a dirty grey "wash-leather" appearance. When the dressings were removed after 12 days the homografts soon dried out and became dark colored eschars which, with a little encouragement, fell away on the 14th–18th day.

The Labeling of Cells with Thymidine-^{3}H In Vitro.—Tritiated thymidine (16 c per mM, Radio Chemical Centre, Amersham, England) was added to whole, heparinized lymph to a concentration of 1 μc per ml. The lymph was incubated for 1 hr at 39°C. The cells were then deposited by centrifugation, resuspended in 20 ml of sterile Hank's solution, and injected immediately into the jugular vein of the sheep. A measured portion of the suspension of labeled cells was retained for radioautography and the radio assay of an extract of the nucleic acids.

Radioautography.—Radioautography of histological sections and methanol-fixed films of cells was carried out according to Speirs et al. (7) using Kodak NTB 2 emulsion. After developing and fixation, cell films were stained through the emulsion with May-Grünwald Giemsa and the sections with hematoxylin and eosin.

Adequate results could be obtained from radioautographs of films of the cells which had been labeled in vitro after a 24 hr exposure, although it was usually convenient to allow an exposure period of 5 days. Radioautographs of sections of biopsy material were first examined after 7 days' exposure. At this time the intensity of labeling was quite sufficient for experimental purposes but for the preparation of photomicrographs the radioautographs were exposed for up to 3 wk in order to increase the contrast between labeled and unlabeled cells.

The specific activity of the thymidine-^{3}H used in these experiments was much higher than necessary for radioautography because relatively high specific activities were needed to ensure that the radioactivity of the small biopsy fragments was sufficient for accurate counting in the liquid scintillation system.

Preparation of Material for the Assay of Radioactivity.—The nucleic acids were extracted from the material under test by the method of Schneider (8) so that the actual material assayed was in every case a solution of nucleic acids in 5% trichloracetic acid (TCA). However, each type of material required a different treatment to render it susceptible to the extraction procedure; these treatments were as follows:

Skin graft biopsies: Under general anesthesia biopsies were cut transversely across the flank grafts to include the normal skin on either side and the underlying cutaneous muscle (panniculus carnosus). A part of this material was immediately fixed in 10% formol-saline and used for the preparation of histological sections. The margins of normal skin and all but the uppermost fibers of the cutaneous muscle were cut from the remaining portion of each biopsy specimen so that a rectangular block consisting only of the grafted tissue and the graft bed remained. The surface area of this block was measured so that the proportion of the total graft in the specimen could be determined. The specimen was then minced into small fragments and further disintegrated by placing it in 1 N NaOH overnight, at room temperature. Following this the NaOH was neutralized with 1 N HCl and the nucleic acids were extracted by the standard procedure. This method did not, of course, result in an absolutely quantal extraction of the DNA. Following the extraction procedure the residues of highly radioactive specimens were treated with 5 N NaOH at 100°C for 2 hr and the resulting solution assayed for residual radioactivity. It was found that 90% of the detectable radioactivity had been recovered by the extraction of the nucleic acids. The residues from low activity specimens contained traces of radioactivity that were too small for accurate counting.

Postmortem specimens: At the end of experiments in which labeled cells had been used the sheep were slaughtered so that specimens of the liver, spleen, and lungs could be obtained. Each of these organs was weighed and then several small pieces were removed at random so that in

each case about 500 mg (wet weight) of representative tissue was obtained. This material was weighed and then minced and treated with 1 N NaOH, as above.

Suspensions of lymph cells: Prior to extraction of the nucleic acids the washed cells were deposited by centrifugation and completely disrupted by shaking with distilled water.

Blood leukocytes: 40 ml of blood were collected by jugular venipuncture. The red cells were lysed by treatment with a hypotonic saline solution. This method (9) preserves many of the leukocytes, though some of the granulocytes are destroyed so that 50–70% of the surviving leukocytes are mononuclear cells. The surviving leukocytes were deposited by centrifugation and then disrupted with distilled water.

In each case 1 ml of the nucleic extract was used for the diphenylamine reaction (8). The optical density at 600 mμ of the resulting blue color gave a measure of the deoxyribonucleic acid (DNA) content. The concentration of the extracts was adjusted so that this optical density did not exceed 0.900 or fall below 0.100.

Assay of Radioactivity and Calculation of Final Results.—The radioactivity of the nucleic acid extracts was measured in a "Tricarb" liquid scintillation counting system, Model 314E (Packard Instrument Co. Inc., Downers Grove, Ill.). 1 ml of the extract was added to 10 ml of "D.E.M." phosphor scintillating fluid (Panax Equipment Ltd. Nucleonic Instrument Engineers, Holmethorpe Industrial Estate, Redhill, Surrey, England). This was done immediately before counting because after 12–24 hr some of the extracted material was thrown out of solution so that a rapid decline in the counting rate occurred.

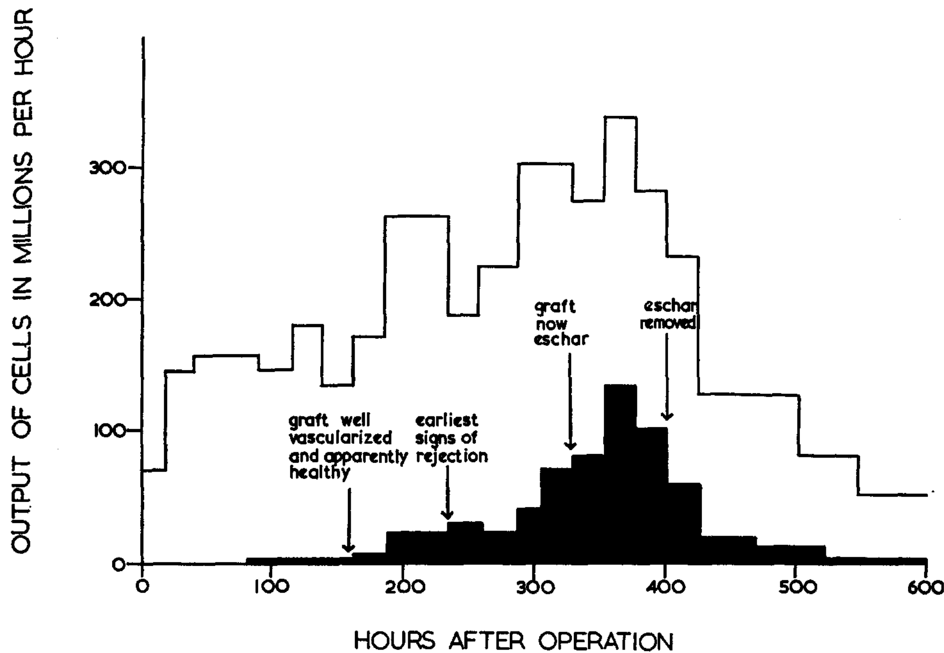
The counting rate was corrected for background (40 cpm) and the cpm per ml of extract was calculated. This value, when divided by the optical density of the diphenylamine color gave a measure, in arbitrary units, of the specific radioactivity of the sample. For example in the experiment described in Results the corrected counting rate of the extract of the most radioactive biopsy of the homograft was 2025 cpm per ml. The optical density of the color produced when 1 ml of the extract was allowed to react with 2 ml of diphenylamine reagent was 0.475. The specific radioactivity of the extract was thus $2025/0.475 = 4263$. The total amount of extractable radioactivity in the graft at the time the biopsy was taken was calculated by multiplying the observed counting rate per ml by the total volume of the extract and by the factor obtained by dividing the total surface area of the graft by the surface area of the biopsy specimen. The resulting figure could then be expressed as a percentage of the total radioactivity injected in the form of labeled cells.

RESULTS

Efferent Lymph.—The same experiment was performed on each of four sheep. At operation bilateral cannulations of the efferent ducts of the prefemoral lymph nodes were performed. A skin homograft was applied to the prefemoral area of the right flank and a skin autograft was applied to the same area on the left flank. The lymph from each side was collected quantitatively for about 600 hr (25 days) after the operation so that the number and types of cells in the lymph could be studied.

The results were generally the same in all experiments. On average the lymph flowed from the cannulae at about 5 ml per hr. There were no significant changes in the cell population of the lymph draining from the side on which the autograft was situated; the cells were nearly all normal lymphocytes, indistinguishable from those in the peripheral blood, with 1–2% of large lymphocytes and occasional granulocytes.

The changes in the numbers and types of cells in the lymph draining from the side on which the homograft was situated were similar in all four sheep. The results of a typical experiment are shown graphically in Text-fig. 1. The main change was the appearance of the large basophilic cells which were indistinguishable from those that appear during immune responses to conventional antigens. The appearances of these cells in the light and electron microscope have already been exhaustively described (3, 4) and will not be repeated here; it would suffice



TEXT-FIG. 1. The responses in the efferent lymph of the prefemoral lymph node to a homograft of skin placed on the flank of a sheep. The unhatched area represents the total output of lymphoid cells, the blocked area the output of basophilic lymphoid cells.

to say that the cells seen in the lymph during the experiments described here were morphologically identical with those seen during responses to conventional antigens. A photomicrograph of the cells is shown in Fig. 1.

Compared with responses to conventional antigens the responses to the skin homografts lasted a long time. A single injection of conventional antigen is followed by a response that is maximal within 100–120 hr and over by 200 hr. The response to a homograft lasted for up to 600 hr. Furthermore during a primary response to conventional antigens the number of basophilic cells in the lymph rarely exceeded 15% of the total count, whereas in the homograft situation these cells sometimes accounted for 40% of all the cells present in the

lymph. In one of the experiments the node was discharging 200 million of these basophilic cells every hour at the peak of the response. Thus in terms of the magnitude of the cellular reaction the stimulus provided by a skin homograft is apparently very potent.

An unexpected and important feature of these cellular changes was their timing in relation to the changes in the graft. From Text-fig. 1 it can be seen that the peak of the cellular response occurred well after the process of rejection was established. Although in absolute terms the actual times of the various stages of graft rejection varied in different experiments this relationship between the changes in the graft and the changes in the lymph held good in each experiment. Thus, at the time when unequivocal signs of graft rejection were already present, the cellular changes in the lymph were unimpressive in comparison with those that occurred later. It should be stressed that the changes in the grafts were assessed only on the basis of their macroscopic appearances. In these experiments it would have been inadvisable to take serial biopsies of the grafts because such traumatic procedures would almost certainly have been followed by changes in the cell picture in the lymph which might have confused the issue.

In considering the time taken for the homografts to be rejected it is necessary to make it clear that in these experiments there was no evidence that the continuous drainage of the lymph had prolonged the survival of the graft. The rejection times of the grafts were similar to those recorded from homografting experiments in uncannulated sheep. It is, in any case, apparent that the lymphatic drainage from a graft on the flank is not confined to the prefemoral node and some of the lymph must necessarily drain to other lymph nodes.

Afferent Lymph.—In these experiments afferent popliteal lymph was collected from the lower leg following the application of skin grafts to the lateral aspects of the cannon. At operation the proposed site of the graft bed received an intradermal injection of Evan's blue so that the actual lymph vessel draining this area could be identified and cannulated. When, and if, the cannulation of this lymphatic had been successfully carried out the graft was applied to a full-thickness bed cut in the marked area. In this way peripheral lymph (afferent to the popliteal node) was collected from both hind legs of each of three sheep which had received an autograft on the left leg and a homograft on the right leg. Two of these preparations functioned for about 150 hr but in one animal although the lymph from the cannula draining the autograft ceased flowing after 150 hr the homograft side functioned for nearly 500 hr. In order to collect lymph during the later stages of graft rejection two further experiments were carried out in which the grafts were applied 150 hr before the lymphatics were cannulated. These preparations functioned for 250 hr i.e., until the grafts had been in place about 400 hr (17 days).

From all these preparations the lymph flowed at a rate of 1–3 ml per hr. Since lymph flows from the efferent duct of the popliteal node at an average rate of

7 ml per hr it is clear that the afferent lymph was not being collected quantitatively. Presumably it was for this reason that the rejection time of the homografts was not influenced by the draining away of the lymph. In general the timing of the events of the rejection of the homografts was similar to those shown in Text-fig. 1.

In all experiments the flow of lymph was continually interrupted by blockage of the cannula. These blockages were caused by aggregates of dead white cells and not primarily by fibrin thrombi, and it was usually easy to remove them and reestablish a normal flow of lymph. The white cell count of afferent lymph was always low but ranged between 300–1000 cells per mm^3 together with a roughly equal number of red cells. Because of the low cell counts and the low rate of flow of lymph it was often difficult to obtain enough fresh cells to prepare good cell films, and this difficulty was increased by the tendency of the cells in the afferent lymph to adhere to one another so that they were difficult to redisperse and smear after being collected by centrifugation. For these reasons one can have little confidence in differential cell counts expressed to the nearest 1% and it is thus necessary to describe the cell picture in rather general terms. However before describing the cell population of afferent lymph draining from the sites of skin homografts in any detail it is necessary to describe briefly the cell population of normal afferent lymph and the changes that occur following the subcutaneous injection of conventional antigens. Some details of these subjects have already been published (3).

About 75% of the white cells in normal afferent lymph are small lymphocytes. The remainder is made up of roughly equal numbers of macrophages and neutrophil polymorphonuclear cells with occasional eosinophil polymorphs. The term macrophage is used here to denote a particular type of morphology and does not relate necessarily, as it strictly should, to function. These cells are characterized by bilobed and often horseshoe-shaped nuclei. The cytoplasm is relatively abundant, stains grey-blue with Romanowsky stains, and often contains inclusion bodies. The cytoplasmic borders are often irregular and indistinct and are in fact frequently arranged in numerous, long tentacles. The latter feature is usually absent in fixed preparations but is easily seen in living preparations examined by phase microscopy.

Following the injection of conventional antigens into the lower leg the changes in the cell picture, apart from an initial transient increase in neutrophil polymorphs, mimic those that occur in efferent lymph under the same circumstances, although the number of cells in the afferent lymp is always very small.

In the present experiments the afferent popliteal lymph draining from the autografts showed little change. There was an initial increase in the number of neutrophil polymorphs which was presumably due to the trauma of the grafting procedure. Thereafter the cell picture remained essentially normal. In the homograft situation the same initial polymorph leukocytosis occurred during the 48

hr after grafting. This was then followed by a gradual increase in the percentage of the macrophage type of cell, so that in some experiments these cells accounted for nearly 75% of all the cells in the afferent lymph. This increase in the number of macrophages did not become very evident until the homograft was showing obvious signs of rejection. A photomicrograph of cells in afferent lymph draining from the site of a skin homograft is shown in Figs. 2 and 3.

It should be emphasized that the total number of cells in the lymph did not increase at any time so that from a quantitative standpoint these changes must be regarded as relatively trivial. The surprising feature of the response to the homografts was the lack of definite signs of a lymphoid cell reaction. Large basophilic cells were seen but they were rare, usually less than 1 in 1000 and they never exceeded about ½ a per cent. It is interesting that the skin homograft which seems such a powerful stimulus to the lymphoid cells in the lymph node causes only a feeble lymphoid reaction in the peripheral lymph, less, in fact, than is caused by a single injection of heterologous serum protein.

Another feature of afferent lymph that deserves comment is the relatively large amount of amorphous debris that is contained in it. When the cells were allowed to settle out of afferent lymph the supernatant lymph plasma often remained slightly turbid. This contrasts with efferent lymph which, under similar circumstances, yields a perfectly clear supernatant. Examination of this suspended matter under the microscope showed some of it to be amorphous cell debris with the staining characteristics of nuclear material and some of it had a pseudocrystalline appearance. It seems unlikely that this material is released by the degenerating white cells in the collecting bottle since a drop of fresh lymph, taken direct from the tip of the cannula into a counting chamber contained a similar quantity and type of material. The presence of this debris was another factor that interfered with the preparation of satisfactory cell films. It is not possible to say definitely if the amount of this material was increased in the lymph draining from homografts. Similar material is present to some extent in normal afferent lymph but the impression gained was that there was more of it in lymph from sites where the homograft was showing obvious signs of rejection.

The Fate of the Cells Released into the Efferent Lymph.—In order to investigate the fate of the cells released into the efferent lymph in response to skin homografts these cells were labeled in vitro with thymidine-^{3}H then returned to the animal by injecting them into the jugular vein.

In each of these experiments the efferent duct of the right prefemoral node was cannulated and at the same time a skin homograft and a skin autograft were applied to the right flank. The homograft and the autograft were placed close together with a 1 cm margin of normal skin between them.

When the number of basophilic cells in the lymph had reached about 10% of the total count the cells were labeled with thymidine-^{3}H by the method described. The basophilic cells took up the isotope avidly and within 1 hr 60% of them

became labeled (Fig. 4). In general between 10 and 15% of all the cells injected were labeled. In order to ensure the arrival of countable levels of radioactivity in the homografts it was necessary to label and inject three consecutive 8-hr collections of lymph cells. After the injections of labeled cells had been given, biopsies of the homografts, autografts, and adjacent pieces of normal skin, together with venous blood samples, were collected at appropriate time intervals. When there was no more homograft left to biopsy the sheep was killed and postmortem specimens of the lungs, liver, and spleen were collected. The radioactivity of these specimens was assayed by the methods described and radioautographs of the biopsies of the graft and skin were prepared.

Three such experiments were performed. The results were generally similar in all cases; the regimen and results of a typical experiment are outlined in Table I.

Following the injection of labeled cells no radioactivity could be found in biopsies of the autografts or in pieces of normal skin by liquid scintillation counting. A very few, isolated labeled cells were seen in some radioautographs of sections of autografts but most of such sections contained no labeled cells. No labeled cells were seen in radioautographs of sections of normal skin.

Radioactivity was always easily detectable in the biopsy specimens of the homografts but as a percentage of the total radioactivity injected even the maximum amounts in the homografts were small, ranging from 0.336% in the experiment illustrated to 0.200 and 0.194% in the other two experiments. Labeled cells were easily found in radioautographs of sections of the biopsies of the homografts. The labeled cells appeared to be randomly scattered throughout the extensive mononuclear cell infiltrate in the base of the graft (Fig. 5).

In all the experiments the specific radioactivities of the DNA samples extracted from the blood leukocytes was about half that of the extracts of the homografts. However 30–50% of the DNA extracted from the blood leukocytes would have been derived from unlabeled granulocytes so, in fact, the actual specific activity of the DNA from the blood mononuclear cells must have approached that of the DNA in the homografts. Furthermore the radioactively labeled cells in the grafts would have been cumulatively recruited from labeled cells in the arterial blood for periods of many hours whereas the specific activities of the DNA extracted from the leukocytes in the blood samples merely reflect the number of labeled cells present in jugular venous blood at a single instant in time. The actual relationship between the specific activities of the blood leukocyte DNA and the homograft DNA is thus difficult to assess; allowing for the errors involved, these specific activities must be considered to be of the same order of magnitude.

The examination of the postmortem specimens showed that in every case between 4–6% of the radioactivity of the labeled cells, which had been injected

TABLE I

Data Obtained after the Injection of Thymidine-³H-Labeled Lymph Cells

The procedures and main results of a typical experiment in which the cells in the efferent lymph from the prefemoral node draining the site of a skin homograft were labeled in vitro with thymidine-^{3}H and returned to the sheep by injecting them into the jugular vein.

All measurements of radioactivity were made on TCA extracts of the nucleic acids of the particular specimens.

Time after operation	Procedure	Results
hr		
200	1st injection of labeled cells: 2.4×10^9 cells containing 4×10^6 cpm injected	
210	2nd injection of labeled cells: 2.4×10^9 cells containing 3.4×10^6 cpm injected	
220	3rd injection of labeled cells: 1.8×10^9 cells containing 5.7×10^6 cpm injected	Total cells injected = 8.2×10^9 Total cpm injected = 13.1×10^6
220	1st biopsy of grafts and blood sample taken	Specific activity of homograft DNA = 1926 Specific activity of blood leukocyte DNA = 960
240	2nd biopsy of grafts and blood sample taken	Specific activity of homograft DNA = 4263 Specific activity of blood leukocyte DNA = 2237
265	3rd biopsy of grafts and blood sample taken	Specific activity of homograft DNA = 3355 Specific activity of blood leukocyte DNA = 1870
314	4th biopsy of grafts and blood sample taken	Specific activity of homograft DNA = 2711 Specific activity of blood leukocyte DNA = 1743
315	Sheep slaughtered	Specific activity of lung DNA = 340 " " " liver DNA = 60 " " " spleen DNA = 74

Final Result: The highest amount of radioactivity in the homograft was present at 240 hr. It was calculated that at this time the homograft contained 0.34% of the total radioactivity injected. At the time of death the lungs contained 5.2% of the injected radioactivity; the liver contained 1.1%, and the spleen 0.6%.

at least 100 hr previously, was still present in the lungs. A further 2% was present in the liver and spleen.

In all of these experiments the prefemoral lymph node draining the homograft was removed at the postmortem. No radioactivity was detected in the node by the scintillation counting and no labeled cells were seen in radioautographs that had been exposed for up to 4 wk. The absence of detectable labeling in the rapidly proliferating lymphoid tissue of these activated lymph nodes makes it relatively certain that "reutilization" of the radioactive label was not contributing significantly to the amount of label detected in the actual experimental material.

In general terms these experiments showed that although hardly any labeled cells entered the autografts only 2–3 in 1000 of the labeled cells actually entered the homograft and at least 1 in 20 were trapped in the lungs.

It seemed possible that one of the reasons for the entry of only small numbers of labeled cells into the homografts was the timing of the injection of the labeled cells. Activated lymph cells capable of taking up label in vitro, only appeared in significant numbers after the process of graft rejection was well established. It was thought that by this time the grafted tissue might have already degenerated so far as to have lost its "antigenic attraction" for lymphoid cells. For this reason a further experiment was performed in which a second homograft was applied 4 days after the first. 4 days after this, i.e. 8 days after the first grafting, there were abundant basophilic cells in the lymph and yet the process of rejection was not yet apparent in the second graft. The experiment was then continued as above. However the results were the same, about 2 in 1000 of the labeled cells entered the homografts and the distribution of radioactivity between the first and second homografts was very similar. Radioautographs of sections of the second homograft showed that as in the first graft most of the labeled cells were in the dense mononuclear cell infiltrate in the graft bed; a few labeled cells were seen in the more superficial regions apparently "invading" the still intact epidermis of the graft. This was an isolated finding to which little significance can be attached. As in the previous experiments about 5% of the total injected radioactivity was still present in the lungs at the time the sheep was killed.

Specificity of the Entry of Labeled Cells into Skin Homografts.—It remained to investigate the specificity which governs the entry of the labeled cells into the homografts. The autograft situation is not a satisfactory analogy because there was only a very sparse mononuclear infiltrate in the autografts and the absence of labeled cells in this situation probably follows from this fact. An obvious way of demonstrating that the entry of labeled cells is influenced by immunological specificity would be to use two homografts, each from a different donor. Lymph cells from one site only could then be collected, labeled, and injected back into the animal. The distribution of the labeled cells between the two homografts might be expected to show a difference if true immunological spec-

ificity were involved. This plan was not adopted because of the possibility of the donors sharing transplantation antigens, and because the assay procedures were too inaccurate to detect small differences in the distribution of labeled cells. Instead, the following experiments were performed.

A homograft was applied to the right flank in the usual way but the right prefemoral efferent lymphatic was left intact. Instead the left prefemoral efferent lymphatic was cannulated. 2 days later the left prefemoral node was stimulated antigenically by injecting a suspension of killed *Salmonella typhi* "O" organisms into the left flank. 100 hr later about 10% of the cells in the lymph from the left prefemoral node were basophilic cells characteristic of the immune response and which contained specific antibody (3). These cells were labeled with thymidine-^{3}H and injected; biopsies of the homograft and blood specimens were then collected as in the previous experiments. The results of this experiment were similar to the results of the previous ones in that a maximum of 0.3% of the injected radioactivity was present in the homograft. The distribution of radioactivity in the blood leukocytes, lungs, liver, and spleen was of the usual pattern. Thus in this experiment the labeled cells, which were "coded" to react against a known bacterial antigen and which were presumably irrelevant to the homograft reaction, entered the homograft with a facility at least equal to that of the cells which were "specifically coded" to react with homograft antigens. This experiment was repeated using *Brucella abortus* as the antigen. The same results were obtained.

In these latter experiments radioautographs of sections cut from the biopsies of the homografts showed that the labeled cells were, as usual, randomly scattered throughout the mononuclear cell infiltrate (Fig. 6). In absolute terms the number of labeled cells was small because the response to the bacterial antigens had yielded fewer cells for labeling and injection than the homograft responses in the earlier experiments.

It was concluded, tentatively, that the immunological specificity of a given lymphoid cell plays little part in its selection for entry into a homograft.

DISCUSSION

The types of cells seen in the efferent lymph from the regional node draining the site of a skin homograft were identical with those seen in other types of immune responses. Thus in any type of immune reaction there is an apparent uniformity of the cellular response in the efferent lymph from the node concerned; this phenomenon has been described previously (4). The distinguishing feature of the responses to skin homografts is the timing of the cellular response. Firstly a significant response in the lymph does not become apparent for nearly 200 hr after the homograft is applied, whereas by this time the response to a single injection of conventional antigen would be almost over. An obvious explanation of this "latent period" would be that there is an interval of almost 100 hr between the application of a homograft and the arrival of significant

amounts of potent antigen at the node. This, in turn, would imply that the reestablishment of lymphatic connections between the graft and the host tissue is a prerequisite for the transport of homotransplantation antigens. By using skin grafts containing radioactively labeled serum proteins it has been shown that labeled material reaches the regional node within 24 hr (10). However serum proteins would be capable of diffusing into the host tissue and thus reaching lymphatics relatively quickly. Much larger molecules or pieces of cell debris, which may be the prime movers in the homograft situation in vivo, would not be able to do this. Their transport would depend on the close proximity of intact lymphatic collecting vessels or on mobile phagocytic cells. It was shown, in the experiments on afferent lymph, that in the homograft situation there is, in this lymph an increased number of cells with the morphological features of macrophages. However in absolute terms the number of these cells was very small and also a significant number of cells of this type was present in the lymph immediately after grafting. If these cells do carry a significant amount of antigen it is difficult to see why the response in the node and thus the efferent lymph, takes so long to develop. Furthermore cells of the macrophage type are generally believed to be capable of moving through the tissues and thus although they may be present in lymph they cannot be regarded as being solely dependant on the lymphatic system for their transport. Indeed they are conspicuously absent from intermediate and central lymph (3).

If, in the homograft situation, these macrophage-like cells represent the initial pathway in the route of sensitization it is difficult to account for the survival of homografts in anatomical situations where lymphatic vessels are absent, for example in the brain (11) and the cheek pouch of the hamster (12) but where mobile phagocytes are presumably present. A more acceptable explanation would be that it is the "debris" which is present in afferent lymph and which depends on afferent lymph for its transport, that is responsible for initiating the immune response in the node. From this it follows that the stimulation of the regional node could only take place after the establishment of a direct lymphatic connection between the graft and the node and that the regional node is the primary site of the immunological reaction against the homograft. Considerable evidence for this view has been presented in the classical studies of Billingham, Brent, and Medawar (13), and Mitchison (14, 15). However, the increased percentages of macrophages and the lack of a significant lymphoid cell reaction in the afferent lymph draining from the homografts still await an explanation. It is probably untrue to say that these features are characteristic of homotransplantation reactions in general. Recent experiments have shown that intradermal or subcutaneous injections of homologous lymphocytes are followed by exuberant lymphoid cell reactions of the usual type in both afferent and efferent lymph.[2] From this it is possible to argue that the unique features of afferent

[2] Hall, J. G. 1966. Unpublished observations.

lymph draining from skin homografts may to some extent be the result of the initial disturbance of the local microanatomy and in particular the interruption of the local lymphatic network which must inevitably follow the actual grafting operation.

The other salient feature of the cellular response in efferent lymph to homografts was the fact that the number of activated, basophilic cells only reached a peak after the homograft had been destroyed. A possible explanation for this is that the initial damage to the graft is brought about by a very small number of cells that are released in the early stages of the response. This primary damage might then lead to the release of more antigen which in turn would initiate a more powerful onslaught and so on until the graft was finally destroyed.

It might also be that much of the material released by a degenerating homograft, although antigenic in the sense that it provokes a cellular response, would not necessarily be related to the histocompatability system. In this sense much of the cellular response observed in the efferent lymph may not be concerned with primary graft rejection as such, but might be directed against denatured components of dead cells and be concerned ultimately with encouraging the phagocytosis of debris and the repair and remodeling of the damaged area. In any case it is possible that the response in efferent lymph cannot be regarded as a purely "primary" event. It seems almost certain that antigens from homografts must be continually arriving at the node over a period of many days. The node is thus subjected to repeated stimuli and the later phases of the response would correspond to the vigorous cellular responses that occur in response to secondary challenges with conventional antigens (3). If these interpretations are correct it follows that any investigation of the cellular reactions in lymphoid tissue to homografts will encompass cellular events which for the most part are concerned with the establishment of secondary reactivity and the response to nonspecific antigens rather than the events of primary homograft rejection alone. This might be one of the reasons why, in the experiment described here, so few of the labeled cells entered the bed of the primary graft. However before examining this question in detail it is necessary to take into account what is already known about the transport and distribution of the "activated" lymphoid cells.

It seems certain that immunologically activated cells do appear ultimately in the blood of animals after the application of skin homografts (16, 17). It is also clear that these cells probably depend on an intact lymphatic pathway for their transport from the regional node to the blood (4). Once these cells have entered the blood it is tempting to believe that they "home" onto the homograft and destroy it. Unfortunately in experiments on homografts undergoing adoptive destruction following the injection of labeled donor cells the radioautographs of the homografts did not show significant numbers of donor cells to be present (18, 19). However, a more recent radioautograph study by Prendergast (20)

showed that cells originating from the regional node and labeled in vivo, ultimately invaded the skin homograft in significant numbers. Sometimes as many as 11% of the mononuclear cells in the graft beds were labeled, though usually there was less than this. However these experiments also showed that labeled cells entered grafts from unrelated donors with equal facility.

In general terms then, the experiments on sheep described here confirm Prendergast's results, in that the invasion of homografts by immunologically activated cells from the regional node can be shown to occur, although the phenomenon is nonspecific. None of these experiments show directly that the labeled cells that enter the homografts play a part in the destruction of the grafts.

In Prendergast's experiments the percentage of labeled mononuclear cells in the graft was up to 50 times higher than the percentage of labeled mononuclear cells in the blood indicating that such cells had a special affinity for sites of graft rejection. This result was based solely on the counts of labeled cells in radioautographs and so a considerable error may be involved, particularly as a systematic study of the blood mononuclear cells was not reported by Prendergast. In all the experiments of sheep the specific radioactivity of the DNA of blood leukocytes was of the same order as that of the DNA of the homograft tissue. If this result is correct it suggests an explanation not only of the entry of "nonspecific" labeled cells into the homografts but also of the low number of "specific" cells found in the homografts. As a hypothesis I suggest that entry of mononuclear cells into homografts is, as far as the cells are concerned, a purely random process. It may be that at the sites of homograft rejection, delayed hypersensitivity reactions, or indeed any pathological situation that results in a local accumulation of mononuclear cells, the behavior of the capillary (or venular) endothelium is altered so that mononuclear cells are filtered out of the blood. The composition of the mononuclear cell exudate would then merely reflect the composition of the mononuclear cell population of the blood at the time. In the homograft situation in so far as "activated" cells were present in the blood they would also be present in the mononuclear cell infiltrate in the graft. But by the same token, cells that had been activated and released into the blood by other, unrelated stimuli would also be present, as indeed they were shown to be in the above experiments on sheep. Furthermore the existence of capillaries or venules with a special function in relation to the transmission of lymphoid cells has been demonstrated in the lymph nodes of rats (21). There is also some indirect evidence that in sheep this specialized function may be influenced by the local antigenic environment (22). If this general hypothesis is correct it implies that once sensitization has occurred homograft reactions in vivo may be mediated by the capillary endothelium as much as by the innate potentialities of the lymphocytes as such. Indeed the first damage to the homograft may be inflicted by an interference with the microcirculation which first reduces the blood supply and

then allows large numbers of mononuclear cells to enter the area and complete the destruction of the already damaged donor tissue.

In the above experiments on sheep the actual number of labeled cells in the homografts was small. They never accounted for more than a fraction of a per cent of all the mononuclear cells present. One reason for this might be because many of the cells that were injected were removed from the blood stream and retained by the lungs. The invariable finding that even at the end of the experiments the lungs contained at least 5% of the total amount of radioactivity that had been injected suggested that in the earlier phases of the experiments a large proportion of the labeled cells were removed from the circulation by the lungs and thus prevented from reaching the graft. The injected cells were of course damaged to a greater or lesser degree. It has been my experience that normal lymph cells incubated for 1 hr in vitro and then returned to the sheep are initially retained for many hours in the reticuloendothelial system, even though they may ultimately survive to function normally elsewhere.[2] In the present experiments the cells were also labeled very heavily with thymidine-^{3}H. The high intensity of labeling was necessary to make possible the assay of radioactivity in small biopsy specimens. It seems likely that some of the cells might have been subjected to considerable radiation damage which could well have proved lethal during a later mitosis. Such damage might well have rendered them susceptible to intrapulmonary trapping. Although a considerable proportion of the labeled cells might have been lost in this way, the interpretations of the results are based on relative measurements and are thus still valid. However it is perhaps worthwhile to point out that there is evidence that in normal animals the lungs may play an important part in regulating the numbers of white cells in the blood (23) but unfortunately it is not yet known whether this function of the lungs is immediately concerned with the cellular economy of the systemic immune response.

SUMMARY

Intermediate lymph (efferent from the prefemoral lymph node) was collected for 600 hr from both flanks of each of four sheep that had an autograft of skin on the left flank and a homograft of skin on the right flank.

8 days after the grafts had been applied considerable numbers of large basophilic cells, apparently identical with those that appear during immune responses to conventional antigens, appeared in the lymph draining from the homografts. No such cells appeared in the lymph draining from the autografts. At this time the homografts were already showing signs of rejection and were apparently dead well before the cellular response in the lymph reached a peak, about 350 hr (14–15 days) after the homografts had been applied. During the peak of the response up to 40% of the cells in the lymph were basophilic cells and in one experiment such cells were leaving the lymph node at a rate of 200 million per hr.

Peripheral lymph (afferent to the popliteal lymph node) draining from the sites of homografts of skin was collected from five sheep. This lymph contained few white cells (<1000 per mm^3) and showed only an insignificant lymphoid cell reaction. Although the percentage of macrophage-like cells was increased significantly there were few signs of a lymphoid cell reaction; the lymph also contained much amorphous debris.

Experiments in which the basophilic cells from the efferent lymph were labeled in vitro with thymidine-^3H and returned to the sheep by intravenous injections were carried out in six sheep. The presence of the labeled cells in the grafts, blood, and other tissue was detected by liquid scintillation counting of nucleic acid extracts of biopsy and postmortem material and by radioautography. 2–3 labeled cells out of every 1000 injected entered the homografts but hardly any entered the autografts. However, labeled basophilic cells that had originated in response to bacterial antigens entered the homografts with equal facility. It is thus hard to believe that the immunological specificity of a lymphoid cell endows it with a specific "homing" capability. Furthermore, in all the experiments the specific radioactivities of the nucleic acids extracted from the blood mononuclear cells were approximately of the same order as those of the nucleic acids extracted from the homografts. It was concluded that most of the mononuclear cells that infiltrate homografts represent a random selection from the mononuclear cell population of the blood.

BIBLIOGRAPHY

1. Ehrich, W. E., and T. N. Harris. 1942. The formation of antibodies in the popliteal lymph node in rabbits. *J. Exptl. Med.* **76**:335.
2. Hall, J. G., and B. Morris. 1962. The output of cells in the lymph from the popliteal node of sheep. *Quart. J. Exptl. Physiol.* **47**:360.
3. Hall, J. G., and B. Morris. 1963. The lymph-borne cells of the immune response. *Quart. J. Exptl. Physiol.* **48**:235.
4. Hall, J. G., B. Morris, G. D. Moreno, and M. D. Bessis. 1967. The ultrastructure and function of the cells in lymph following antigenic stimulation. *J. Exptl. Med.* **125**:91.
5. Hall, J. G. 1964. The function of the lymphatic system in immunity. Ph.D. thesis. Australian National University, Canberra, A. C. T., Australia. 138.
6. Rymaszewska, T., K. Włodarski, L. Tomaszewski, M. Saleski, and W. Płonka. 1965. Studies on the lymph from the efferent vessels of regional lymph nodes after heterotransplantation. *Transplantation.* **3**:114.
7. Speirs, R. S., V. Jansen, E. E. Speirs, S. Osada, and L. Dienes. 1962. Tritium in the physical and biological sciences. *Intern. At. Energy Agency, Tech. Rept. Ser. 2.* 301.
8. Schneider, W. C. 1957. Estimation of nucleic acids by pentose analysis. *In* Methods in Enzymology. S. P. Colowick and N. O. Kaplan, editors. Academic Press, New York. **3**:681.
9. Dain, A., and J. G. Hall. 1967. The isolation of white cells from sheep blood by differential lysis in hypotonic saline solutions. *Vox Sanguinis.* In press.

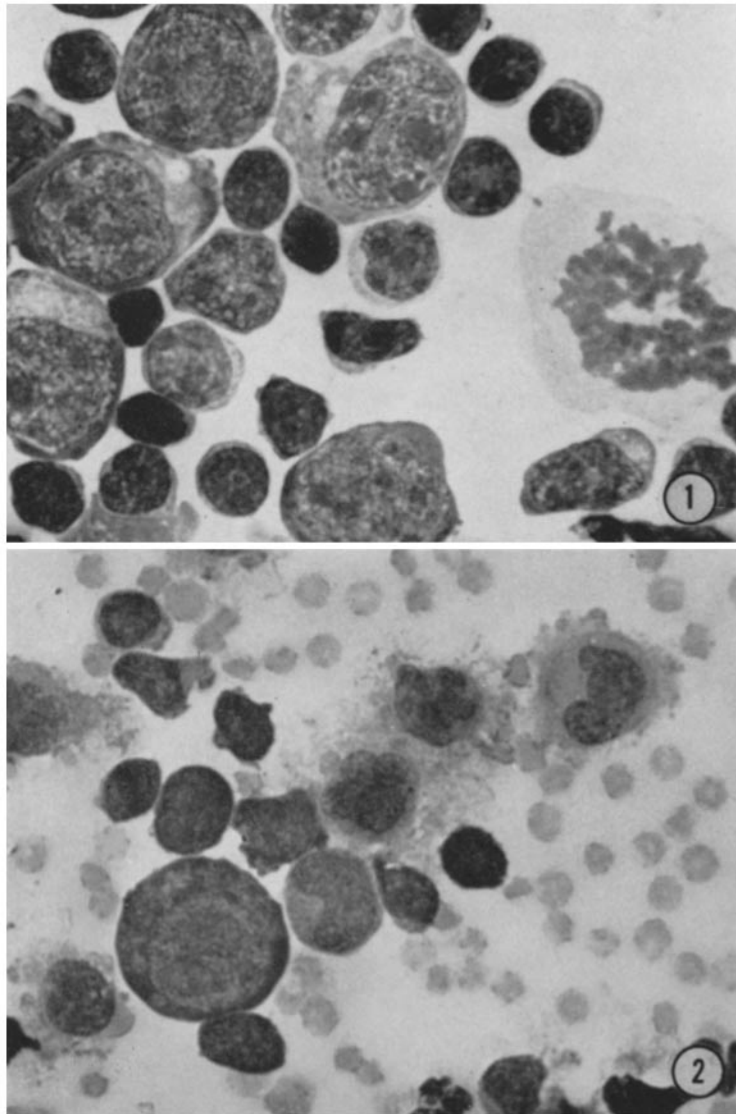
10. Weatherly-White, R. C. A., R. B. Stark, and M. De-Forest. 1961. Physiologic evidence of lymphatic repair after skin homo-transplantation. *Surgery.* **50:**784.
11. Medawar, P. B. 1948. Immunity to homologous grafted skin. III. The fate of skin homografts transplanted to the brain, to the subcutaneous tissue, and to the anterior chamber of the eye. *Brit. J. Exptl. Pathol.* **29:**58.
12. Billingham, R. E. and W. K. Silvers. 1962. Ciba Foundation, Symposium on Transplantation. J. and A. Churchill Ltd., London. 90.
13. Billingham, R. E., L. Brent, and P. B. Medawar. 1954. Quantitative studies on tissue transplantation immunity. II. The origin, strength and duration of actively and adoptively acquired immunity. *Proc. Roy. Soc. London, Ser. B.* **143:**58.
14. Mitchison, N. A. 1954. Passive transfer of transplantation immunity. *Proc. Roy. Soc. London, Ser. B.* **142:**72.
15. Mitchison, N. A. 1955. Studies on the immunological response to foreign tumour transplants in the mouse. *J. Exptl. Med.* **102:**157.
16. Billingham, R. E., K. Silvers, and D. B. Wilson. 1962. The adoptive transfer of transplantation immunity by means of blood-borne cells. *Lancet.* **1:**512.
17. Brent, L., J. B. Brown, and P. B. Medawar. 1962. Quantitative studies on tissue transplantation immunity. VI. Hypersensitivity reactions associated with the rejection of homografts. *Proc. Roy. Soc. London, Ser. B.* **156:**187.
18. Najarian, J. S., and J. D. Feldman. 1962. Passive transfer of transplantation immunity. *J. Exptl. Med.* **115:**1083.
19. Billingham, R. E., K. Silvers, and D. B. Wilson. 1963. Further studies on the adoptive transfer of sensitivity to skin homografts. *J. Exptl. Med.* **118:**397.
20. Prendergast, R. A. 1964. Cellular specificity in the homograft reaction. *J. Exptl. Med.* **119:**377.
21. Marchesi, V. T., and J. L. Gowans. 1964. The migration of lymphocytes through the endothelium of venules in lymph nodes; an electron microscope study. *Proc. Roy. Soc. London, Ser. B.* **159:**257.
22. Hall, J. G., and B. Morris. 1965. The immediate effect of antigens on the cell output of a lymph node. *Brit. J. Exptl. Pathol.* **46:**450.
23. Gillman, T. 1956. Studies on haematological reactions produced in rats by prolonged treatment with trypan blue. I. Quantitative haematological data, Acta Haematol. **15:**364.

EXPLANATION OF PLATES

PLATE 90

FIG. 1. Photomicrograph of a cell film prepared from the efferent lymph of the prefemoral node during the response to a homograft of skin. Note the mitotic figure and large, basophilic cells. May-Grünwald Giemsa, × 1500.

FIG. 2. Photomicrograph of a cell film prepared from peripheral lymph (afferent to the popliteal node) draining from the site of a homograft of skin that was in the process of being rejected. Several macrophage-like cells are present. A large basophilic cell is also shown; cells of this type are very rare in this type of preparation. May-Grünwald Giemsa, × 1000.

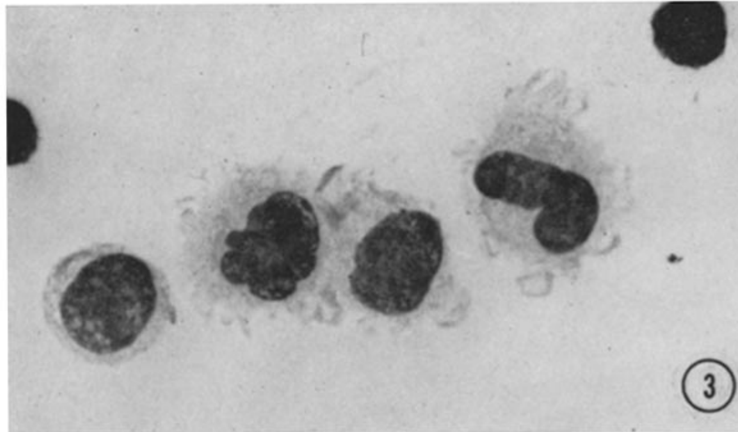


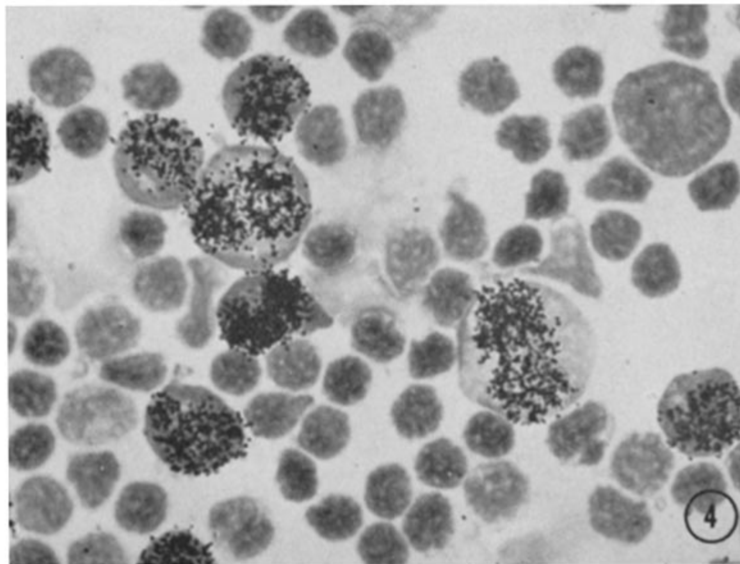
(Hall: Cells in the lymph from homografts)

PLATE 91

FIG. 3. Higher power photomicrograph of three macrophage-like cells similar to the ones shown in Fig. 2 to show the variation in nuclear shape and the irregular borders of the cytoplasm. May-Grünwald Geimsa, × 1500.

FIG. 4. Photomicrograph of a radioautograph of a cell film prepared from efferent lymph collected during the homograft response. The cells have been labeled in vitro with thymidine-^{3}H prior to being returned to the sheep by intravenous injection. 7-day exposure; May-Grünwald Giemsa, × 1000.



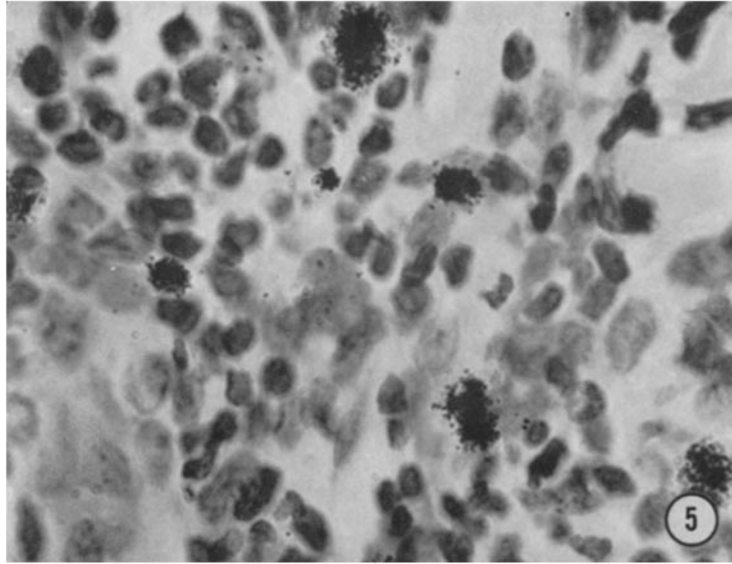


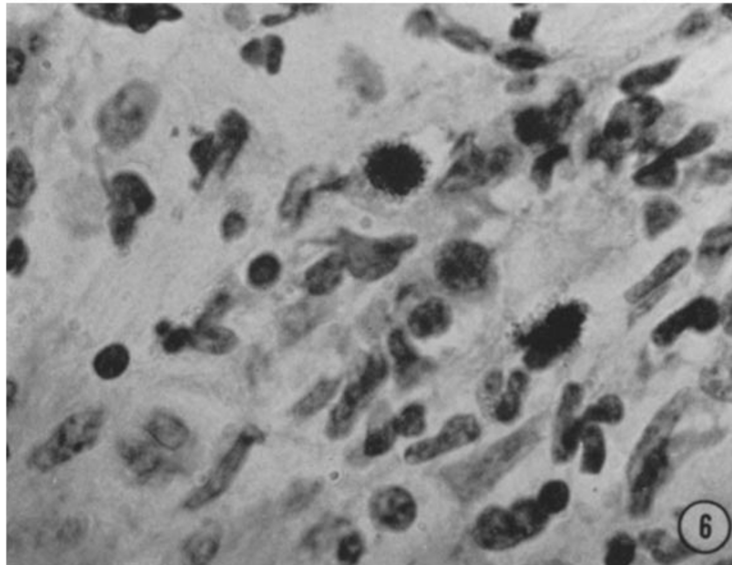
(Hall: Cells in the lymph from homografts)

Plate 92

Fig. 5. Photomicrograph of a radioautograph of a section of a biopsy taken from a homograft of skin following the intravenous injection of lymph cells, which had been labeled in vitro with thymidine-^{3}H. The lymph cells had been collected from the efferent lymph of the regional node draining the site of the skin homograft. The labeled cells can be seen scattered randomly throughout the mononuclear cell infiltrate. 14-day exposure; hematoxylin and eosin, $\times$ 1000.

Fig. 6. As for Fig. 5, but in this case the lymph cells were collected from a lymph node remote from the site of the skin homograft. This node had been stimulated antigenically by a subcutaneous injection of a suspension of killed *Salmonella typhi* "O" organisms 100 hr previously. 14-day exposure; hematoxylin and eosin, $\times$ 1000.





(Hall: Cells in the lymph from homografts)