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Studies of efferent lymph cells from nodes stimulated with oxazolone

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Summary. Efferent lymph from nodes regional to areas of skin that had been treated with solutions of oxazolone in acetone was collected from unanaesthetized sheep. The application of 5% solutions of oxazolone to unsensitized sheep caused no signs of acute inflammation or 'shut-down' of lymphocyte traffic; none the less, normal immune responses ensued so that immunoblasts, some containing immunoglobulin, were discharged into the lymph together with specific humoral antibodies. When previously sensitized sheep were challenged with 2·5% solutions of oxazolone the vigorous secondary responses were heralded by Arthus reactions, induced presumably by pre-existing antibodies, which were mainly of the IgG class. A similar sequence of events occurred in a thymus-deprived sheep which had undergone intra-uterine thymectomy at 60 days of gestation. Repeated applications of oxazolone to normal sheep did not exhaust or inhibit the characteristic changes in the flow and composition of the lymph. When immunoblasts from efferent lymph were radiolabelled with ^{125}I-UdR and returned intravenously to the sheep they showed no significant tendency to localize either specifically or non-specifically in areas of skin that had been treated with contact-sensitizing chemicals.

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INTRODUCTION

Oxazolone (i.e. 4-ethoxymethylene-2-phenyloxazolone) was one of the substances used by Gell, Harington & Rivers (1946) in their investigations of the antigenic functions of simple chemical compounds. Since then, other investigators (Oort & Turk, 1965; Davies, Carter, Leuchars & Wallis, 1969) have used this immunogen in studies of the histology and cytogenetics of immune responses and it is still used as a model for the study of contact sensitivity in the mouse (Asherson, Allwood & Mayhew, 1973). The first attempts to study contact sensitivity in the sheep used fluorodinitrobenzene (FDNB) as the sensitizing agent (Hall & Smith, 1971); unfortunately, the vesicant properties of such compounds caused acute inflammation and granulocyte exudation which complicated the changes in the regional lymphatic system. Skin-painting with modest doses of oxazolone does not cause acute inflammation in unsensitized sheep and we describe here some properties of cells that are discharged into the efferent lymph by the regional nodes.

MATERIALS AND METHODS

Animals and surgical preparations

Cross-bred wethers were obtained at local auctions and time-mated pregnant ewes from the University of Manchester experimental farm. Polyvinyl cannulae were inserted into the efferent ducts of the prefemoral or popliteal nodes as required. After the operations

sheep were placed in individual metabolism cages and the lymph was collected quantitatively into sterile polythene bottles which contained heparin and antibiotics. The bottles were changed at appropriate intervals. The volume and thus the rate of flow of each collection was measured, total and differential white cell counts were performed, and a sample of lymph plasma was stored frozen pending the assay of specific antibody.

Detailed accounts of the anaesthesia, surgical techniques and post-operative care have been published (Hall & Morris, 1962; Hall, 1967a, b). Lambs were thymectomized *in utero* after the methods of Cole & Morris (1971).

Application of oxazolone

4-Ethoxymethylene-2-phenyloxazolone was obtained from BDH Chemicals Ltd, Poole, Dorset and dissolved in 'Analar' grade acetone to the required concentration. The solution was loaded into a polythene syringe fitted with a 27 gauge needle and sprayed on the appropriate area of skin, which had been freshly shorn and wiped with a mixture of equal parts of ethanol and ether to remove the wool fat. When the popliteal lymphatic system was the subject of the experiment, 5 ml of a solution of oxazolone was applied to the lateral aspect of the cannon, i.e. between the fetlock and the hock on the hind leg. In order to stimulate the prefemoral node 15 ml of the solution was sprayed on the appropriate flank just caudal to the costal margin.

In some experiments acetone solutions of DNCB (dinitrochlorobenzene) or picryl chloride, or suspensions of killed *Br. abortus* organisms (Wellcome) were used as control antigens.

Antibody assay

Antibodies to oxazolone were titrated in terms of their ability to agglutinate isologous red cells which had oxazolone attached to their surfaces (Askenase & Asherson, 1972). Doubling dilutions of lymph or blood plasma were made in phosphate-buffered saline (PBS, pH 7·4, containing 1·0% bovine serum albumin, Koch-Light Laboratories, Colnbrook, Bucks.) in 0·2 ml systems in WHO haemagglutination trays. One drop of a 5% solution of sensitized cells was added to each well and the agglutination patterns were inspected after the plates had stood overnight at room temperature. Haemagglutinins for oxazolone-treated SRBC were never detected in the blood or lymph plasma of sheep which had not been deliberately sensitized.

Lymph plasma collected from several immunized sheep at the height of the responses was pooled to yield a standard antiserum with a haemagglutinating titre of about 1 in 256. The ability of oxazolone–protein adducts to inhibit this antisera was used to detect the presence of oxazolone in lymph plasma collected immediately after skin-painting. When lymph plasma suspected of containing oxazolone was collected, doubling dilutions of it were made in the above, standard antiserum; inhibition of haemagglutination for at least the first three wells was taken as indicating that oxazolone residues were present.

Preparation of immunoglobulins from lymph cells

At the peak of an immune response up to half of the cells in the efferent lymph can be immunoblasts, some of which contain immunoglobulin. Although it is possible to extract this immunoglobulin (Ig) by treating pellets of washed lymph cells with non-ionic detergents (Hall, Scollay, Birbeck & Theilen, 1975), the product is difficult to titrate because the detergent lyses the red cells. Instead, the Ig associated with the immunoblasts was obtained by xenogeneic transfer method (Hall, Parry & Smith, 1971). Briefly, washed lymph cells, containing no less than 2×10^8 blasts, were injected subcutaneously into each mouse. Four days later the mice were exsanguinated and the serum containing the Ig products of the immunoblasts in a form suitable for gel diffusion technique and haemagglutination assay was collected.

Immuno-histochemical demonstration of cellular immunoglobulin

For light microscope studies of Ig on small lymphocytes and Ig in immunoblasts immuno-peroxidase reagents were applied, either directly or by using a 'sandwich' technique, to conventional films of washed lymph cells that had been fixed with methanol/ether. The details of the methods including the preparation of anti-$F(ab')_2$ reagents and conjugates have been published (Scollay, Hall & Orlans, 1976; Hall, Hopkins & Orlans, 1977).

In addition, internal Ig was demonstrated in immunoblasts at the ultrastructural level (Hall, Birbeck, Robertson, Peppard & Orlans, 1978).

Total and differential cell counts

The total number of white cells in samples of lymph was counted visually in a haemocytometer. Differential counts were performed by inspecting a drop of lymph directly with a × 100 objective using phase-

contrast optics. Where necessary the results were confirmed by Romanowsky stained cell films (Hall & Morris, 1963) and/or electron microscopy (Hall, Morris, Moreno & Bessis, 1967).

Radiolabelling of lymph cells and radioassay of tissues
In some experiments, the immunoblasts in efferent lymph were labelled by incubating the lymph cells *in vitro* with ^{125}I-deoxyuridine (^{125}I-UdR, Radiochemical Centre, Amersham, Bucks.) at a concentration of 0·1 μCi per ml (Hall *et al.*, 1977). After they had been washed, the cells were returned by intravenous injection to the sheep from which they had been collected. Twenty hours later the recipient was killed and exsanguinated; various tissues including areas of skin that had been treated with contact-sensitizing agents were fixed briefly in 10% formal–saline, blotted dry, weighed and loaded into vials so that their γ-emissions could be assayed in a model 2001 gamma counter (Wilj Electronics, Ashford, Kent).

In these experiments, it was usual to inject at least 10^6 counts per minute (c.p.m.) of cell-associated radioactivity and the selected tissues all contained significant amounts of radioactivity. This was expressed as a specific activity, i.e. c.p.m. per gram weight; however, in order to make the results from different experiments directly comparable, the tissue with the highest specific radioactivity (usually the efferent lymph cells collected after the i.v. injection) was arbitarily given the value of 100, and the other results were converted to correspond accordingly.

Autoradiographs of labelled immunoblasts were prepared as described previously (Birbeck & Hall, 1967).

RESULTS

Efferent lymph was collected from thirty different sheep before and after the application of oxazolone to the regional skin; twenty-five primary and eighteen secondary responses were studied. In six sheep, the preparations functioned long enough for several sequential responses to be studied.

Dose of oxazolone

In the earliest experiments, 10% solutions of oxazolone in acetone were applied to the skin. Even in unprimed animals, this caused neutrophil polymorphonuclear granulocytes to appear in the lymph as well as an acute 'shut-down' (Hall & Morris, 1965) of lymphocyte traffic through the node. Later, it was found that the solutions containing under 7% of oxazolone did not cause any signs of inflammation and it became our practice to use 5% solutions for primary sensitization and 2·5% solutions for secondary challenge. Primary applications were made usually to two different areas of skin in order to prevent a failure of systemic sensitization that might have resulted from the chronic collection of lymph from a single site (Hall *et al.*, 1967).

Nature of primary and secondary responses

The changes in the numbers and types of cells in the efferent lymph from a node draining the site of primary sensitization and secondary challenge are shown diagramatically in Fig. 1. Generally, the responses did not differ from those provoked by conventional

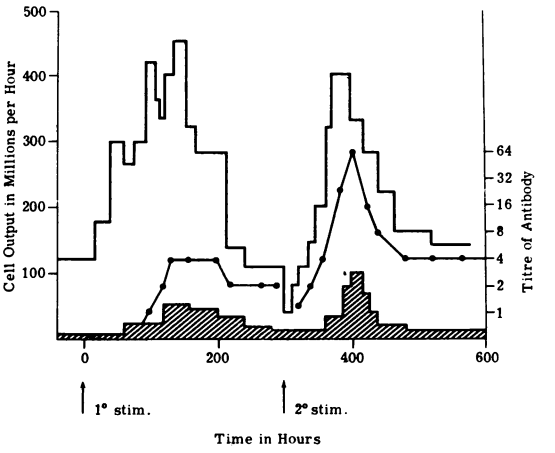

Figure 1. The changes in efferent lymph from a prefemoral node after primary and secondary stimulation with oxazolone, an acetone solution of which was applied to the skin of the appropriate flank at the times indicated by the arrows. The clear area of the histogram denotes the output of small lymphocytes, the shaded area denotes the output of immunoblasts. The titre of antibody to oxazolone in the lymph plasma is shown by the line ●——●. Note that the primary stimulus caused no 'shut-down' of lymphocyte traffic.

antigens (Hall & Morris, 1963; Hall *et al.*, 1967) and need only a brief description here. Before any stimulus was applied, the lymph was populated almost entirely with normal small lymphocytes. The primary stimulus caused the output of these cells to double or treble and between 100 and 200 h substantial numbers of blast cells appeared in the lymph, together with specific

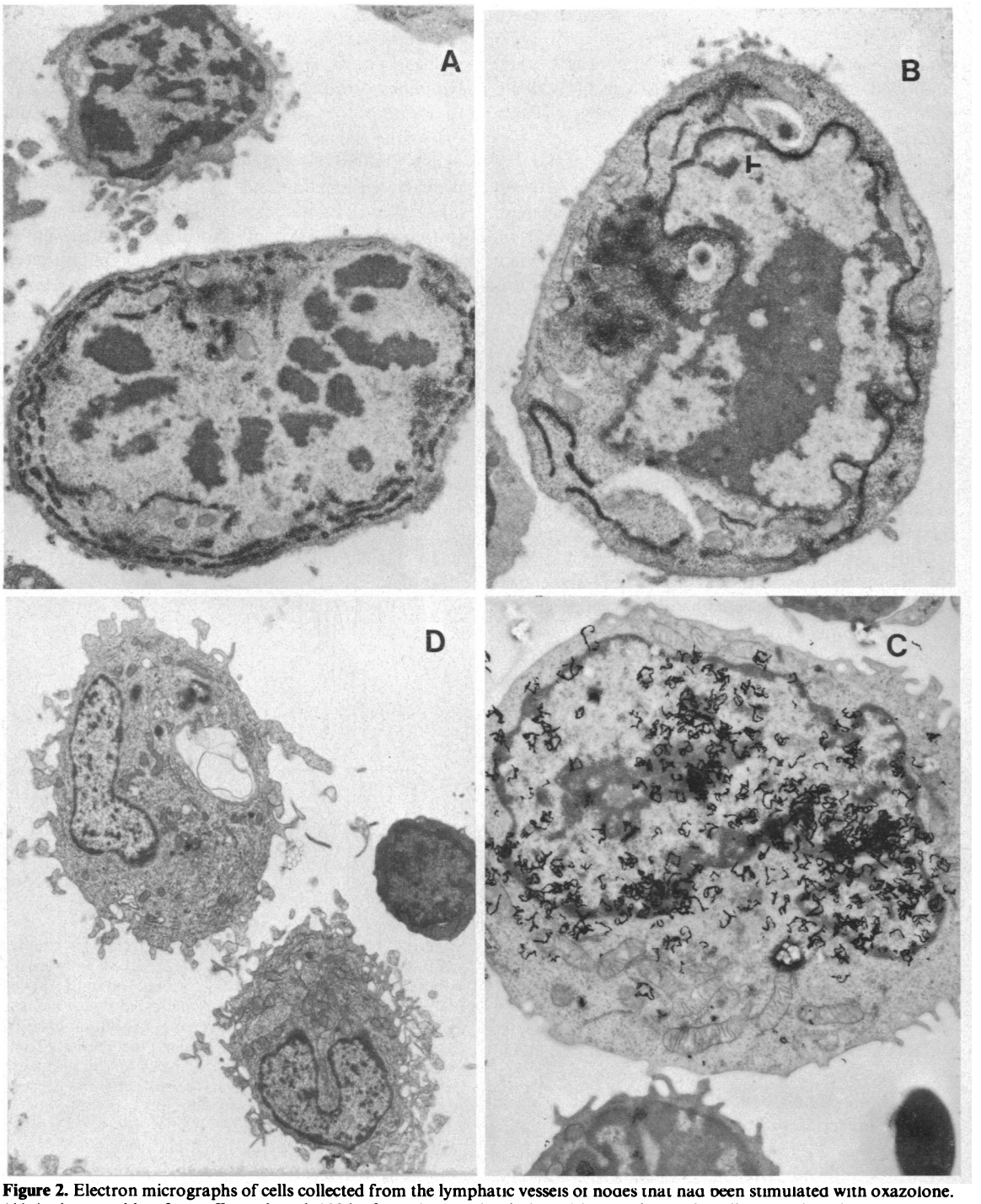

Figure 2. Electron micrographs of cells collected from the lymphatic vessels or nodes that had been stimulated with oxazolone. (A) An immunoblast from efferent lymph 120 h after primary stimulation with oxazolone. The cell was undergoing mitosis and the immunoglobulin in the endoplasmic reticulum (ER) has been demonstrated by an anti-F(ab′)$_2$–peroxidase conjugate (× 7000). (B) As in (A) but the cell is not in mitosis. Immunoglobulin is present in the nuclear membrane and the Golgi region, as well as in the ER (× 7000). (C) Autoradiograph of an immunoblast from efferent lymph that had been incubated *in vitro* with ^{125}I-UdR; for demonstration purposes the radiolabelled thymidine analogue was used at a concentration of 1 μCi per ml. The nucleus of the cell is labelled heavily and the cytoplasm contains little in the way of ER (× 8500). (D) Macrophages in peripheral lymph, afferent to the popliteal node. Although such cells were abundant in afferent lymph they never appeared in the efferent lymph (× 4650).

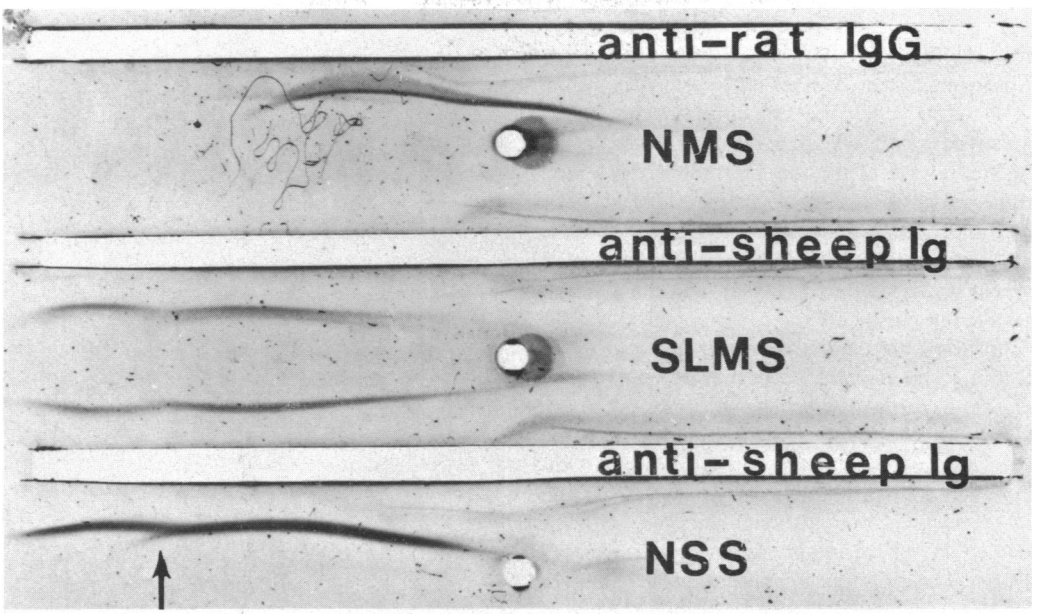

Figure 3. Immunoelectrophoretic patterns formed by normal mouse serum (NMS), normal sheep serum (NSS) and serum from a mouse given washed sheep lymph cells s.c. (SLMS), with antisera to rat IgG and sheep Ig. The anti-rat serum reacted well with the mouse globulin. The anti-sheep Ig distinguished between IgG1 and IgG2 (arrowed) and shows that the sheep immunoblasts generated in response to oxazolone made both these Igs.

antibody. Even in primary responses, up to 30% of these immunoblasts contained intracellular Ig (Fig. 2) and many were seen in mitosis. After a secondary challenge these events were repeated more rapidly so that the content of immunoblasts and specific antibody in the lymph both reached a peak about 100 h after the stimulus was applied. Macrophages, which were abundant in the peripheral lymph afferent to the nodes (Hall, 1979), never appeared in the efferent lymph. Oxazolone was detected in lymph plasma, by haemagglutination-inhibition, for 30–50 h after the primary application. It was not detected unequivocally after secondary challenge, perhaps because of the smaller dose and the presence of specific antibody in sheep with established immunity.

The classes of Ig represented in the specific antibody were not determined precisely. Only at the beginning of the primary responses did treatment with 2-mercaptoethanol have a significant effect on the antibody titre in the lymph plasma and the Igs extracted from the blast cells by the xenogeneic transfer system were of the IgG classes (Fig. 3). Actual antibody activity against oxazolone was detected only in the sera of mice that had received sheep immunoblasts generated in secondary responses; although sheep immunoglobulin was present in the sera of mice that had received immunoblasts generated in primary responses, it was apparently insufficiently avid to agglutinate the specific antigen.

There was one new and noteworthy feature of these responses to oxazolone. This was the absence of a 'shut-down' of the output of lymphocytes in the efferent lymph after the primary local application of a 5% solution of oxazolone. Although 'shut-down' did not occur the subsequent immune response seemed entirely normal. The 'shut-down' phenomenon was always seen after secondary challenge of a sensitized sheep but it was accompanied always by signs of acute inflammation. Polymorphs appeared in the lymph and its flow rate and protein concentration increased significantly (Hall, 1979). The area of painted skin became abnormally warm to the touch and, in cases where the experiment involved the leg, local oedema was obvious. In other words, secondary challenge always provoked an Arthus reaction, presumably because of the presence of pre-existing specific antibody capable of binding complement. This does not mean that a state of cutaneous, delayed-type hypersensitivity did not co-exist, but if it did it was always obscured by the florid, immediate hypersensitivity reaction.

Effects of repeated stimuli

It has been suggested (Alexander, Bensted, Delorme, Hall & Hodgett, 1969) that the continual bombardment of a lymph node with (e.g. tumour) antigens may prevent the release of immunoblasts into the lymph and thus promote a state of systemic anergy. The repeated injection of suspensions of bacteria, etc., is not feasible in unanaesthetized large animals and it was hoped (Hall & Smith, 1971) that the application of skin-sensitizing chemicals would overcome this difficulty and allow an experimental test of the original proposition. The vesicant properties of FDNB prevented this experiment but, at the doses we used, oxazolone does not have this disadvantage and the results of giving six sequential applications of oxazolone at 48 h intervals are shown diagramatically in Fig. 4. It can be seen that although antigen must be assumed to have been impinging on the node for 12 consecutive days this did not prevent the development of a response that was vigorous both in terms of antibody production and the release of immunoblasts.

Similarly, in another sheep the cannula in the effer-

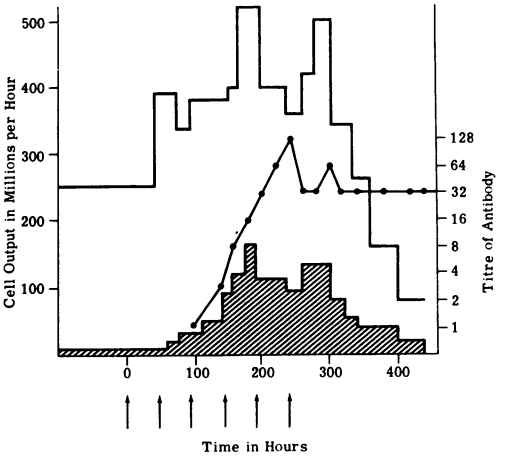

Figure 4. The changes in efferent lymph from a prefemoral node caused by six sequential cutaneous applications (arrowed) of a solution of oxazolone in acetone. The clear area of the histogram denotes the output of small lymphocytes; the shaded area that of immunoblasts, and the line ●——● shows the titre of antibody in the lymph plasma.

Table 1. The radioactivities of contact-sensitized ears of sheep 20 h after an i.v. injection of autochthonous, lymph-borne immunoblasts labelled with ^{125}I-UdR

Expt No.	Details of labelled efferent lymph cells that were injected i.v.		Radioactivity in left ear (treated with oxazolone)	Radioactivity in right ear (control)	Radioactivity in normal skin
	Number	Time of collection			
1	$1{\cdot}4 \times 10^9$ 15% blasts 5×10^6 c.p.m.	138–148 h after 1° stim. with oxazolone	Sp. Act. = 19·5 Recovery = 0·05%	Treated with acetone Sp. Act. = 1·0 Recovery = 0·003%	Sp. Act. = 0·4
2	5×10^8 10% blasts 10^6 c.p.m.	137–144 h after 1° stim. with oxazolone	Sp. Act. = 11·9 Recovery = 0·20%	Treated with DNCB Sp. Act. = 20·8 Recovery = 0·34	Sp. Act. = 0·4
3	$3{\cdot}5 \times 10^9$ 12% blasts 7×10^7 c.p.m.	126–145 h after 1° stim. with oxazolone	Sp. Act. = 8·6 Recovery = 0·015%	Treated with DNCB Sp. Act. = 20·8 Recovery 0·038	Sp. Act. = 1·1
4	2×10^9 10% blasts 6×10^6 c.p.m.	91–96 h after 1° stim. with oxazolone	Sp. Act. = 25·1 Recovery = 0·31%	Treated with picryl chloride Sp. Act. = 2·7 Recovery = 0·034%	Sp. Act. = 0·2
5	6×10^9 17% blasts $1{\cdot}6 \times 10^7$ c.p.m.	73–91 h after 2° stim. with *Br. abortus*	Sp. Act. = 22·2 Recovery = 0·15%	Treated with DNCB Sp. Act. = 5·9 Recovery = 0·039%	Sp. Act. = 0·2

One day before the lymph cells were collected for radiolabelling *in vitro* the sheep's ears were treated with acetone solutions of contact sensitizers, as indicated above. Thus about 40 h elapsed between challenge and radioassay of the treated skin. The specific activities were derived from measurements of c.p.m. per g wet weight as described in the Methods section.

ent duct of one popliteal node continued to function for over 3 months and we were able to observe the responses to six sequential applications of oxazolone given at fortnightly intervals. Even at the end of this period the responses in the efferent lymph were of normal vigour.

Effect of thymectomy

One of a pair of twin lambs was thymectomized *in utero* on the 60th day of gestation. After it was born, the thymectomized sheep thrived and, apart from the operation scar, the only detectable abnormality was an alteration in the ratio of surface Ig-negative:surface Ig-positive small lymphocytes in the recirculating pool. In the thymectomized lamb the usual ratio of 70–80:30–20 was reversed, so that the bulk of the circulating small lymphocytes exhibited Ig on their surfaces.

When the thymectomized sheep was fully grown the efferent duct of one prefemoral node was cannulated and the skin of the corresponding flank treated with oxazolone. Primary and secondary responses similar to those shown in Fig. 1 were observed. With the advantage of hindsight it might be possible to say that numbers of cells involved in these responses were on the lower side of the normal range but without recourse to the results of staining for surface Ig it would be impossible to state that this sheep's responses to oxazolone were abnormal in any way.

Distribution of labelled immunoblasts *in vivo*

In order to find out whether the immunoblasts generated in response to oxazolone would 'home' preferentially to the sites of application of contact sensitizing chemicals, the following experiments were carried out. A day before the immunoblast response in the efferent lymph was due to reach a maximum, the sheep's left ear was sprayed with a 2·5% solution of oxazolone, and the right ear treated with a control solution. At the peak of the immunoblast response, the lymph was collected and incubated with ^{125}I-UdR as described. Twenty hours after the washed, labelled cells had been injected intravenously, the sheep was killed and the ears and normal skin assayed for radioactivity. The results of five such experiments are shown in Table 1. The salient finding was that over 99·5% of the injected cells did not enter the sites of application of skin sensitizing agents, and those few that did were not selected on the basis of immunological specificity.

DISCUSSION

The responses of regional lymph nodes to the local application of oxazolone provoked cellular and humoral changes in the efferent lymph similar to those that occur after challenge with conventional antigens, and lend no support to the notion that oxazolone stimulates preferentially the cell-mediated component of immunity. Even in primary responses many of the immunoblasts that were generated were engaged unambiguously in the synthesis of immunoglobulin, although the doses of oxazolone used were, relative to body weight, less than those customarily given to mice.

Similarly, the ability of a thymus-deprived sheep to respond well to oxazolone argues against a fundamental role for T cells in all aspects of the response (see Morris, 1973). The reasons why oxazolone produces expansion of the traffic areas of the regional node have been discussed elsewhere (Hall, 1979).

The failure of immunogenic, but sub-inflammatory doses of oxazolone to cause 'shut-down' probably follows from the fact that unsensitized sheep had no pre-existing antibody to this chemical. In immunized sheep, where antibody was present, complement was bound and, as in the case of high primary doses of oxazolone or FDNB, polymorphs were attracted and 'shut-down' occurred. This suggests that some pharmacologically active products of acute anaphylaxis are the prime movers in the 'shut-down' reaction. Indeed, recent work (McConnell, Hopkins & Lachmann, 1979) has implicated prostaglandins in the mediation of the phenomenon; it is entirely credible that such substances would be formed when antigens, antibodies and complement interact in proximity with the endothelium of lymphatic or blood vessels. However, the findings suggest that 'shut-down' is an epiphenomenon of the induction of immunity rather than an essential prerequisite.

No evidence was found to support the theory that the continual bombardment of a lymph node with antigen inhibits the release of immunoblasts into the lymph. Although the unavailability of radiolabelled oxazolone prevented a direct measure of the uptake by the regional lymphatic system of oxazolone applied locally to the skin, it seems reasonable to assume that it would follow the same kinetics as ^{3}H-FDNB (Hall & Smith, 1971) and the haemagglutination inhibition results support this. Such experiments showed that oxazolone was detectable in the lymph for up to 48 h after primary skin-painting. We conclude, therefore, that by repeating the skin-painting at intervals of 48 h,

the regional node was effectively perfused with antigen and yet was able to respond normally. Similarly, the release of immunoblasts by sheep lymph nodes regional to growing tumours was unimpaired (Hall *et al.*, 1975).

Hay & Morris (1976) concluded that it is rather difficult to deplete an individual sheep of specifically responsive cells by the quantal collection of lymph from a node undergoing repeated stimuli, and our results are consistent with this view, though contrary claims exist (McConnell, Lachmann & Hobart, 1974).

The experiments with radiolabelled immunoblasts represent yet another instance of failure in the attempt to show antigen specific 'homing' by this class of cell (Hall, 1976b; Moore & Hall, 1973; Rose, Parrott & Bruce, 1976). After a dozen years of negative results it seems proper to conclude that immunoblasts are not generally capable of localizing in foci of specific antigen. Their occasional entry into such areas seems to be a random event, conditioned probably by non-specific mediators of the inflammatory process (Asherson & Allwood, 1972; Asherson *et al.*, 1973). Most immunoblasts become distributed in other lymphoid tissue in the lymph nodes and spleen (Hall *et al.*, 1977), and there is evidence that it is primarily in such tissues that they express their diverse potentials (Fahy, Gerber, Morris, Trevella and Zukoski, 1979).

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