Studies on the Afferent and Efferent Lymph of Lymph Nodes Draining the Site of Application of Fluorodinitrobenzene (FDNB)

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Summary. Peripheral lymph (afferent to the popliteal node) or intermediate lymph (efferent from the popliteal or prefemoral node) was collected from unanaesthetized sheep before and after painting the skin of the drainage area with a 10 per cent solution of fluorodinitrobenzene (FDNB) in acetone. In some experiments FDNB labelled with tritium ([^{3}H]FDNB) was used.

The changes in the cell population of efferent lymph from nodes thus stimulated were generally similar to those which occur following stimulation with conventional antigens, i.e., between 90–120 hours later many large basophilic lymphoid blast cells (immunoblasts) appeared in the lymph and specific antibody appeared in the lymph plasma.

Studies with [^{3}H]FDNB showed that although some of it appeared in afferent lymph almost immediately after application, substantial amounts were not present usually until 20 hours or so later. All of the FDNB in the afferent lymph was bound to soluble proteins in the plasma and none was found in association with the lymph cells. Apparently, this protein bound FDNB was inefficiently phagocytosed in the regional node because much of it passed through the node so that it could be recovered in the efferent lymph for 100 hours or more following the original application.

It was concluded that skin sensitizing chemicals of the FDNB class are transported to the node after they have combined with soluble proteins that enter the lymph; in the combined form they behave like other soluble protein antigens and provoke similar cellular responses in the regional tissue.

INTRODUCTION

It has often been suggested that the cellular responses in regional lymph nodes to antigens, such as skin sensitizing chemicals which lead to a state of cutaneous delayed type hypersensitivity (DTH) are qualitatively different from those responses which are followed by the appearance of circulating antibodies (e.g. Oort and Turk, 1965). The important cell type in lymph nodes mediating DTH reactions is believed to include the large pyroninophilic cells similar to those described by Scothorne and McGregor (1955) in lymph nodes draining the site of skin homografts. A further anology with the skin homograft situation has been suggested by the observations of Macher and Chase (1969) who concluded that the induction of this contact sensitivity to chemicals depends on lymphoid

cells which have become sensitized by contact with the chemical in the peripheral tissues, rather than on the direct entry of antigen into the node. This hypothetical concept of 'peripheral sensitization' was originally applied by Medawar (1965) to account for the induction of the response to homografts of skin.

However, in spite of these findings and speculations there is still enough uncertainty about the details of these processes to warrant a more direct investigation into the transport of skin-sensitizing chemicals and the type of cellular response that they provoke. For this reason fluorodinitrobenzene (FDNB) was applied to the skin of sheep and the cellular responses in the afferent and efferent lymph of the regional node were studied. By using [³H]FDNB it was possible to measure by scintillation counting the occurrence and distribution of the chemical in various samples of lymph and lymph cells.

MATERIALS AND METHODS

Sheep

Cross-bred, 1–2 year-old wethers were used. Under general anaesthesia a cannula was inserted into either a popliteal afferent duct or the efferent duct of either the popliteal or prefemoral lymph node. After the sheep had recovered the lymph was collected quantitatively into sterile bottles which contained a buffered salt solution which included heparin and antibiotics. The bottles were changed at appropriate intervals so that the volume of lymph could be measured and the rate of flow calculated. A total and differential cell count were performed and a sample of lymph plasma was stored frozen pending the assay of antibody, radioactivity and protein concentration. These techniques have been described previously (Hall and Morris, 1962; Hall, 1967a).

Application of FDNB

A 10 per cent solution of FDNB (Analar BDH) was prepared in 'Analar' acetone. The skin to which the chemical was applied was shorn and cleaned thoroughly with a mixture of equal parts of ethanol and ether to remove the wool fat or 'yolk'. A convenient site, drained by the popliteal lymphatic system, is the external surface of the cannon, just below the hock. To stimulate the prefemoral lymphatic system an area on the flank, 15 cm in front of the anterior border of the thigh was chosen. In either case an area approx. 10 × 2 cm was used. The solution of FDNB was slowly dripped on to the area from a graduated glass syringe. Usually about 3 ml was applied, though precise measurement was difficult in the case of frisky animals.

In some experiments [³H]FDNB was used. In these cases 100 μCi of the labelled compound (1-fluoro-2,4, dinitrobenzene 3,5,6-T, 5 Ci/mM, Radiochemical Centre, Amersham) was added to 5 ml of the standard 10 per cent solution which acted as a carrier. A measured portion was retained for radio-assay.

Antibody assay

Antibodies to DNP in efferent lymph plasma were titrated by the agglutination of sensitized red cells, as follows.

DNP residues from FDNB were conjugated to isologous serum albumin by the method of Eisen, Orris and Belman (1952). The DNP-albumin was attached to washed isologous red cells by diazotization (Gordon, Rose and Sehon, 1958). The ability of lymph plasma to agglutinate the sensitized red cells was titrated in doubling dilutions of saline in WHO

haemagglutination trays. The haemagglutination caused by positive samples could be inhibited by mixing excess DNP-albumin with the lymph prior to carrying out the titration.

Preparation and iodination of bacterial extracts

In some experiments it was necessary to use a radioactively labelled bacterial antigen for purposes of comparison. An extract of *M. radiodurans* was prepared as described previously (Hall, Smith, Edwards and Shooter, 1969). This material was then labelled with ^{125}I (using carrier free Iodide, Radio-chemical Centre, Amersham) by the method of Webster, Laver and Fazekas de St Groth (1962). The labelled extract was stored in a dialysis sac, contained in a large volume of buffered saline, until required for use.

Assay of radioactivity

The amount of radioactivity in material from experiments in which [^{3}H]FDNB had been used was assayed by counting the β emission in a toluene–methoxyethanol BBOT liquid scintillation system. Up to 0·25 ml of lymph plasma could be dissolved in 15 ml of scintillation fluid. In parallel with the direct counting of lymph plasma, samples of the precipitated lymph protein were prepared by adding 1 ml of lymph to 9 volumes of ethanol. The precipitate was then redissolved in 1 ml N NaOH and counted.

Cells from a known volume of lymph were deposited by centrifugation, washed twice in normal saline and dissolved in 1 N NaOH before being added to the scintillation fluid. In all cases internal standards were employed and a quenching correction applied. In some experiments autoradiographs of cell smears were prepared using standard techniques.

The radioactivity of material from experiments in which antigens labelled with ^{125}I had been used was assayed by counting the γ-emission of 2-ml samples of lymph plasma in a well-type crystal scintillation counter.

Total and differential cell counts

Counts of the total white cell population in samples of lymph were determined by haemacytometer counts in the usual way. The classification of lymph cells was carried out following light microscopy of Romanowsky stained cell smears (Hall and Morris, 1963), high power phase contrast microscopy of living cells and electron microscopy of araldite sections prepared from pellets of lymph cells fixed in buffered glutaraldehyde and post-fixed in Osmium tetroxide (Hall, Morris, Moreno and Bessis, 1967).

Protein estimations

The total protein content of samples of lymph plasma was determined by the biuret reaction (Gornall, Bardawill and David, 1949).

RESULTS

THE EFFECT OF FDNB ON THE SKIN OF SHEEP

The intensity of the local reaction varied considerably between individuals but followed the same general pattern. Within a few minutes of applying the chemical signs of acute inflammation appeared and within $\frac{1}{2}$–1 hour oedema was obvious. In some cases this was little more than localized 'peau d'orange' but usually there was gross, pitting oedema which gravitated to dependent parts. The oedema subsided in 1–2 days leaving a bright

yellow, indurated area which gradually discoloured as the epidermis died and was converted into a hard eschar. Because of the intensity of the initial inflammation and the later discoloration it was not possible to observe whether or not there was a secondary DTH 'flare' between the 5–10th day; at these times the gross oedema had subsided but the area was still indurated and felt unusually warm. During the next 2–3 weeks the superficial eschar peeled away to reveal an area of new, wool-bearing skin which apart from being rather pink seemed perfectly normal and was formed without any scarring or loss of mobility. The desquamative process was always completely dry and at no stage was there any 'weeping' or exudation. In general the picture was one of a first degree chemical burn associated initially with a vigorous inflammatory reaction and followed by dry desquamation. Although the upper layer of the epidermis was killed it regenerated quickly from below with no sign of damage to deeper tissues.
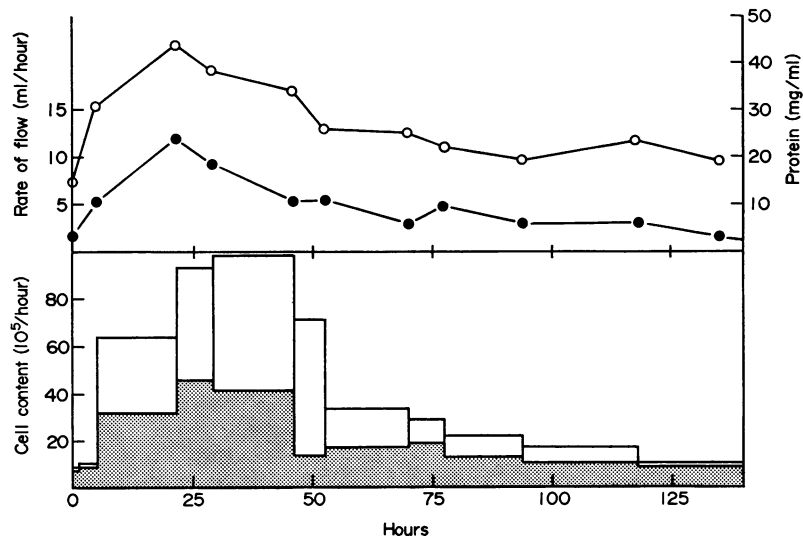

FIG. 1. The changes in peripheral lymph, afferent to the popliteal node, following the local application of FDNB to the skin at time zero. The upper graph shows the protein concentration O and flow rate ● of the lymph. The histograms of the lower graph represent the output of cells in the lymph. The clear areas denote the output of neutrophil polymorphonuclear granulocytes and the blocked areas the output of mononuclear cells.

AFFERENT LYMPH

Afferent lymph was collected from five sheep before and after the application of FDNB.

Normally this lymph flows at 1–3 ml/hr; the total protein concentration of the lymph is about 20 mg/ml; and it contains only 200–500 white cells per mm.^{3} About three quarters of these cells are small lymphocytes and the remainder are macrophages plus occasional neutrophil and eosinophil polymorphonuclear granulocytes.

The main events that followed the local application of FDNB are shown graphically in Fig. 1. Within 24 hours of the application the flow rates and protein concentrations doubled or trebled and did not return to normal for 2 or 3 days. Within 2–5 hours of the application the output of white cells started to increase and by 24–48 hours had increased up to

ten-fold. Most of this increase could be accounted for by the appearance of large numbers of neutrophil polymorphs but the output of mononuclear cells usually doubled or trebled as well. These changes gradually subsided but significant numbers of neutrophil polymorphs remained present for 100 hours or more. At this time lymphoid blast cells appeared but although they occasionally displayed mitotic figures their numbers were low and they accounted for less than 1 per cent of the total white cells. In summary, then, the changes observed in afferent lymph following the local application of FDNB, were those of acute inflammation. The initial hyperaemia and increased capillary permeability were reflected in increases in the rate of formation and protein content of the lymph, together
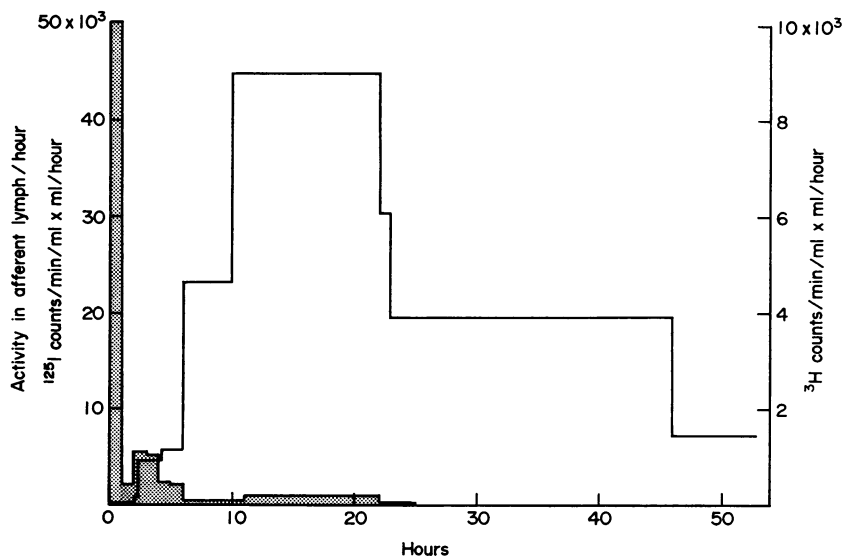

FIG. 2. The recovery of radioactivity in peripheral lymph afferent to the popliteal node following the s/c injection of a radioactively labelled bacterial antigen and the application of radioactive FDNB to the skin. The results of two separate experiments are shown on the same graph. The blocked area shows the recovery of radioactivity following the s/c injection of an extract of *M. radiodurans* labelled with [125]I. The clear areas show the recovery of radioactivity following the local application of [³H]FDNB to the skin. Both materials were given a time zero.

with an influx of neutrophil polymorphs. At later times there was only equivocal evidence in terms of blast formation, of the local immune reaction that can for example, follow the subcutaneous injection of conventional antigens (Hall and Morris, 1963).

RECOVERY OF DISTRIBUTION OF RADIOACTIVITY IN AFFERENT LYMPH

The presence of radioactivity in afferent lymph following the local application of [³H]FDNB is shown graphically in Fig. 2. For purposes of comparison an extract of *M. radiodurans* labelled with ^{125}I was injected subcutaneously, into one sheep preparation. It can be seen that this material entered the lymph immediately after injection so that after 30 minutes only trivial amounts were recovered. In contrast, [³H]FDNB, applied to the skin, behaved quite differently. Detectable radioactivity did not begin to appear in the lymph until an hour had passed and substantial amounts were not present for 6 hours or so. Thereafter the level of radioactivity increased and, in the experiment illustrated, a

maximum value was established 10 hours after painting the chemical on the skin, and this level was maintained for a further 12 hours and was still substantial 50 hours later. In other words the absorption and transport of skin sensitizing chemicals is very much slower than that of conventional antigens which are injected subcutaneously. Indeed, in one experiment the radioactivity in the lymph did not reach a maximum value until 30 hours after the [^{3}H]FDNB had been applied.

All the radioactivity in the lymph plasma was bound to protein, in so much as it could be recovered quantitatively in the material which was precipitated by ethanol. No significant amount of radioactivity was ever detected in the washed lymph cells by scintillation counting and, similarly, autoradiographs of the lymph cells (which had been exposed for a 100 days before being developed) showed no trace of activity in any class of leucocyte.
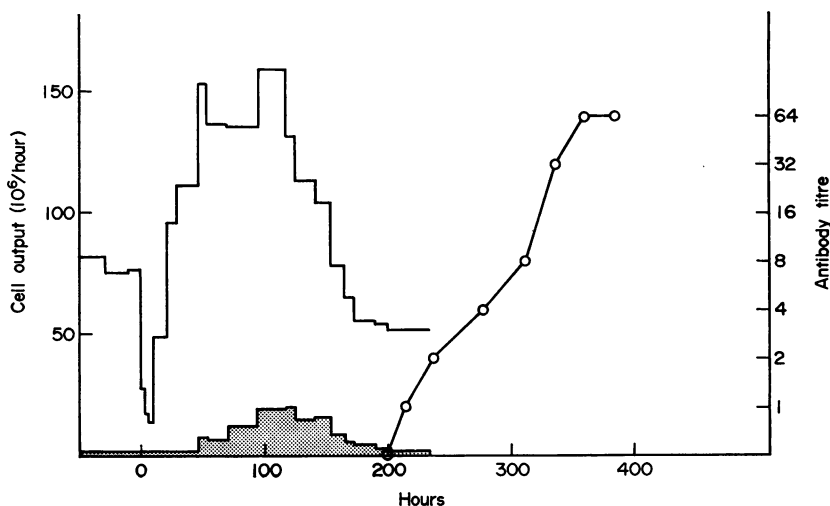

FIG. 3. The changes in efferent lymph from the prefemoral node following the application of FDNB to the skin of the flank at zero time. The clear histogram denotes the output of small lymphocytes, the blocked area denotes the output of basophilic immunoblasts. The titre of antibody in the lymph plasma to DNP-ovine albumin conjugate is shown by the line O———O.

The actual recovery of radioactivity in the lymph was difficult to calculate precisely because of the difficulty of applying an accurately measured amount to the skin. In the five experiments the percentage recovery varied between approximately 1 and 10 per cent of the amount applied.

EFFERENT LYMPH

Efferent lymph, from either the popliteal or the prefemoral node, was collected from five sheep before and after the application of FDNB to the drainage area. The results of one such experiment are shown graphically in Fig. 3 and are very similar to those obtained following stimulation with heterologous serum proteins or bacterial antigens (cf. Hall and Morris, 1963).

The basal cell output of the preparation was about 75×10^6/hr, most of the cells being normal lymphocytes with 1–2 per cent of large blast cells. Within 1½ hours of the applica-

tion of FDNB the lymphocyte output started to fall precipitously even though the flow rate of the lymph was increasing. This acute response of the cell output has been observed following the administration of any antigenic, though not necessarily noxious, material and is considered to represent the first response of a lymph node to the arrival of antigenic material (Hall and Morris, 1965). In the present case it took rather longer to start than after the injection of soluble antigens and this time lag presumably reflects the time taken for significant amounts of the FDNB to penetrate the skin, combine with proteins, enter the afferent lymph and reach the regional node. After this acute episode the output of lymphocytes recovered and was soon double the resting level. At this stage some 5 per cent of neutrophil polymorphs were present but they disappeared after 2 days. About 50 hours after the FDNB had been applied increased numbers of immunoblasts appeared in the lymph and between 100–120 hours they accounted for 15 per cent of all the lymphoid cells present; thereafter their numbers declined and between 200–250 hours the cell picture had returned virtually to normal. On the basis of their appearance in light and electron microscope studies the immunoblasts were judged to be identical to those seen during responses to conventional antigens; the morphology and ultrastructural characteristics of such cells have been described exhaustively elsewhere (Hall and Morris, 1963; Hall *et al.*, 1967) and will not be recapitulated here. However, such structural identity does not necessarily exclude significant biochemical differences.

Specific antibodies to DNP–protein conjugates were first detected in the lymph plasma 200 hours after the application of FDNB. This is rather later than the time at which antibodies to conventional antigens first appear; for example, specific antibodies to bacterial antigens can be detected in the lymph within 100 hours of primary challenge (Hall and Morris, 1963). This delayed appearance of detectable antibodies may reflect the technical problems of titrating anti-hapten antibody but, more probably, could result from the presence in the lymph of an excess of antigen. Support for the latter view came from an experiment in which [³H]FDNB was used.

THE RECOVERY AND DISTRIBUTION OF RADIOACTIVITY IN EFFERENT LYMPH

In this experiment [³H]FDNB was applied to the flank of a sheep in which the efferent duct of the prefemoral node of the same side had been cannulated. Because such preparations produce a greater volume of lymph than the afferent preparations the specific radioactivity of the [³H]FDNB was increased by adding double (i.e., 200 μCi) the amount of labelled material. The result of the experiment is shown in Fig. 4. Radioactivity appeared in the lymph rather more quickly than usual and maximum amounts were present within an hour or two of application. The efferent lymph had a very high counting rate for the first 50 hours but substantial amounts were present from 50–150 hours and radioactivity was still unequivocally present until 200 hours. In other words some antigen was present in the efferent lymph throughout the period of immunoblast release and specific antibody was not detected until most of it had disappeared. As in the experiments on afferent lymph all of this radioactivity was protein-bound and none was detected in association with the washed lymph cells. The radioactivity recovered in the lymph between 0 and 200 hours accounted for approximately 8 per cent of the dose applied to the skin. This was as high as the amounts usually recovered from the afferent preparations and suggests strongly that only a small percentage could have been retained by the lymph node.
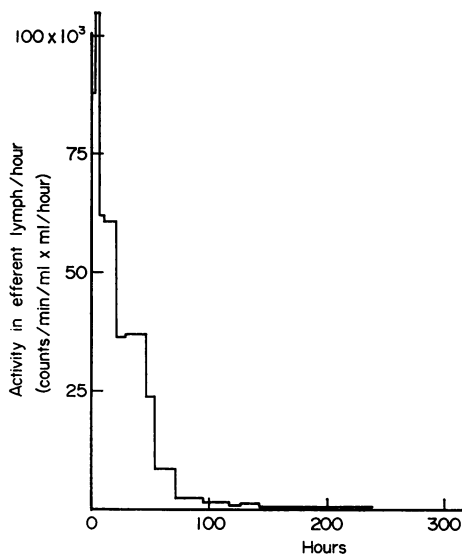
FIG. 4. The output of radioactivity in the efferent lymph of the prefemoral node following the application of [³H]FDNB to the skin of the flank at zero time.

DISCUSSION

The results show that, under the conditions described, skin sensitizing chemicals of the DNP class are transported to the regional node in combination with proteins in the afferent lymph plasma. It is not known whether they combine with a specific class of protein, lipoprotein or perhaps even a soluble carrier protein that is released from the damaged skin. However, the fact that the radioactivity in the lymph plasma could be precipitated with ethanol suggests that little FDNB was simply dissolved in lipid components. There was no evidence to suggest that either the mononuclear or granulocytic leucocytes in afferent lymph played any part in the transport of antigen. However, because of the relatively low specific radioactivity of the [³H]FDNB used for skin painting the possibility that some molecules of antigen were, in fact, conveyed by these cells cannot be excluded. Nonetheless, the amount transported in this way by the small number of cells available can only have been an infinitesimal fraction of the amount transported in combination with protein. Furthermore, the lymphoid cells in afferent lymph showed only occasional morphological signs of immunological activation.

There is already much evidence to suggest that an intact lymphatic pathway between the site of application and the regional node is a prerequisite for the development of contact sensitivity, and also that the regional node is the site of the immunological reaction (Simon, 1936; Landsteiner and Chase, 1939; Frey and Wenk, 1957; Turk and Stone, 1963). The main area of uncertainty concerns the nature of the antigenic stimulus which is transported up the afferent lymphatics. Macher and Chase (1965) concluded that lymphocytes sensitized by direct contact with the chemical are all important but, as Parker and Turk (1970) have pointed out, there are serious objections to the experimental model on which this conclusion is based. There is little doubt that peripheral sensitization of lymphocytes to transplantation antigens can occur in renal allografts (Strober and Gowans,

1965) where vast numbers of blood-borne host lymphocytes can react directly with the endothelium of the blood vessels of the graft and where the activity of the regional lymph nodes is irrelevant (Pedersen and Morris, 1970). However, the situation in small allografts of skin (Hall, 1967b) seems to be similar to that in the present experiments on sheep. In both cases the traffic of lymphocytes through the antigen depot is infinitely smaller than that through renal allografts and probably quite insufficient to provide the wherewithal for rapid peripheral sensitization.

In general, the changes that occurred in efferent lymph following the application of FDNB were almost identical to those which follow stimulation of the node with conventional antigens; however, it should be remembered that even conventional antibody responses may often be associated with a delayed hypersensitivity component (Turk and Heather, 1965). This apparent similarity between the cellular responses to contact sensitization and conventional antigenic stimulation is at variance with the results of histological studies on the lymph nodes of small laboratory animals (e.g., Oort and Turk, 1954). It has been found usually that contact sensitization is followed by a dramatic enlargement of the paracortical areas, which become replete with large pyroninophilic cells, whereas the response to e.g., pneumococcal polysaccharide is characterized more by the appearance of large numbers of plasma cells in the medullary cords and germinal centres in the cortex. However, there may be important quantitative factors which invalidate the direct comparison of results from large and small experimental animals. For example, the application of a skin sensitizing chemical to the ear of a mouse results in a situation where the effluent of nearly 1 cm^2 of sensitized skin drains to a node weighing perhaps 1 mg, whereas, in the above experiments on sheep, the effluent from 20 cm^2 of sensitized skin drained to a node weighing at least 1000 mg. These ratios differ by a factor of 50. Possibly, the relatively large dose of antigen which must be used on small animals in order to induce hypersensitivity (Parker and Turk, 1970) may paralyse temporarily the release of immunoblasts into the lymph and lead to their accumulation in the paracortical areas, in a manner analogous to that which is alleged to occur within lymph nodes draining large tumours (Alexander, Bensted, Delorme, Hall and Hodgett, 1969). Certainly, on the one occasion when we applied FDNB to 200 cm^2 of sheep skin the immunoblast response in the efferent lymph of the regional node, although ultimately vigorous, did not begin for over 100 hours, after the application of the chemical. In any case, it is open to question whether it is valid to compare directly the cellular response to a very small amount of a highly immunogenic (in terms of antibody production) bacterial antigen, to the response to relatively large amounts of a noxious and unnatural chemical which is presented in combination with native proteins over a longer period of time against a background of acute inflammatory disturbances in the local micro-circulation.

The present experiments did not provide the opportunity for measuring precisely the amount of DNP that was retained in the lymph node but clearly this amount could only be a small fraction of the amount applied. Parker and Turk (1970) found that only about 0·04 per cent of the applied dose could be recovered from the regional lymph nodes. It seems, then, that the barrier function of the regional node operates very inefficiently in the case of DNP-protein conjugates, just as it does in the case of heterologous serum proteins (Hall and Morris, 1963). It seems likely that in any dose of foreign serum protein only a small percentage of the molecules are denatured to the extent that they are readily phagocytosed and thus immediately immunogenic; the remainder escape phagocytosis, become widely disseminated and, although they possess an intact antigenic determinant,

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are only feebly immunogenic. This view is supported by the finding that the immunogenicity of soluble protein antigens may be markedly decreased by ultracentrifugation. The precipitate of denatured protein is highly immunogenic whereas the supernatant is much less so and may induce tolerance if given in large enough doses (Dresser, 1962; Golub and Weigle, 1969). Taken together the above observations suggest that in the case of contact sensitization a considerable amount of hapten–protein conjugate may be formed locally but that only a small amount of this is denatured enough to be phagocytosed by the local node and initiate a specific immune response. Most such material, however, is not phagocytosed immediately and is therefore only feebly immunogenic and free to pass through the regional node and become widely disseminated. Such a process offers an explanation for the frequent finding in studies of contact sensitivity that although the regional node is the main site of the immunological response, significant amounts of the inducing chemical may be found in other nodes (e.g., Turk, 1967; Parker and Turk, 1970). This seems to us to be, at the moment, the most straightforward explanation of the available facts, and one that avoids the need to invoke peripheral sensitization as a major component of the afferent limb of the primary allergic response to skin sensitizing chemicals.

ACKNOWLEDGMENTS

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