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Mechanisms of immunity to leishmaniasis. IV. Significance of lymphatic drainage from the site of infection

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SUMMARY

The course of *Leishmania tropica* infection in BALB/c and B_6D_2 mice has been followed after injection of the parasite at different sites. The effect of interruption of lymphatic drainage from the inoculation site has also been examined. In both strains of mice, more severe disease resulted from infection induced in the shaved rump, as compared to infection in the footpad. The removal of the popliteal lymph node prior to footpad infection, caused a considerable exacerbation of the disease. Such increased severity was associated with an initial inhibition of the induction of delayed-type hypersensitivity and a delay in the emergence of acquired resistance. Lymph node removal did not however compromise the effector arm of the acquired immune response, nor prevent the eventual suppression of delayed hypersensitivity that has been shown to occur in the BALB/c, during leishmanial infection.

INTRODUCTION

Current experimental evidence now leaves no doubt that the progressive non-healing forms of leishmaniasis result from a suppression of cell-mediated immune mechanisms (for reviews see Poulter, 1980; 1981). More specifically, it has now been clearly shown (Howard, Hale & Liew, 1980), that the high susceptibility of the BALB/c mouse to *L. tropica* infection, is associated with the emergence of a suppressor T cell population which inhibits the induction of delayed-type hypersensitivity (DTH). The underlying cause of the emergence of this powerful suppressor mechanism has yet to be determined. As high levels of antigen have been demonstrated to induce T suppressor cell activity (Gefford & Orbach-Arbouys, 1976), it seems possible that heavy infections perhaps due to genetically determined macrophage disfunction, (Bradley, 1977), could be responsible. Some weight is added to this argument by the fact that the BALB/c mouse appears unique in its susceptibility to *L. tropica* infection. With the exception of the nude mouse (K. P. Chang, 1980; personal communication), and the hairless mouse (Packchanian, 1979), no other mouse has been found that is anywhere near as susceptible to this pathogen than is the BALB/c mouse.

Progressive diffuse cutaneous leichmaniasis (DCL) can also be produced in the guinea-pig, by infection with *L. enriettii* provided that relatively large inocula are injected into the ear (Bryceson, Bray & Dumonde, 1974; Poulter & Pearce, 1980). The reason for this site dependancy is unclear, although other observations relating severity of disease to route of leishmanial infection have made (Wilson, Dieckmann & Childs, 1979).

Kadivar & Soulsby (1975), clearly demonstrated that interruption of the lymphatic drainage of the injection site predisposed the host to develop DCL. It was felt possible therefore that variations

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in clinical disease resulting from differing routes of infection could be a reflection of variations in lymphatic drainage from these sites.

This hypothesis has been tested in susceptible BALB/c and resistant B_6D_2 mice, by altering the route of infection and deliberately interrupting lymphatic drainage.

MATERIALS AND METHODS

Animals. BALB/c and B_6D_2 (DBA/2 × C57/Bl) F1 mice were bred at the Trudeau Institute. Males 8–10 weeks old were used in all experiments.

Infection. The strain of *L. tropica major* used, was kindly donated to the institute by Dr Adeleh Ebrahimzadeh of Cornell University, New York Medical Centre. Leishmania were maintained by repeated passage in the footpads of BALB/c mice. Organisms used for experiments were cultured for at least 48 hr and not more than 7 days in Grace's insect medium supplemented with 30% inactivated fetal calf serum (FCS) and antibiotics as described previously (Poulter, 1979). Animals were infected with 10^7 promastigotes suspended in 0·05–0·1 ml of sterile saline by subcutaneous injection into the footpad or the shaved rump.

Quantitation of organisms. Viable numbers of *L. tropica* in the footpad were quantitated by teasing all tissues of the examined feet away from the bone and ligaments and then lightly grinding the tissue debris in a glass homogeniser. Aliquotes of the resulting suspensions were then placed in supplemented Grace's medium and viable counts performed after an appropriate time of culture. This method has been described in full for *L. enriettii* (Poulter, 1979). The doubling time of the *L. tropica* was found to be 8·6 hr.

Induction of DTH. Purified soluble antigen (PSA) of *L. tropica* was prepared by the method of Bryceson *et al.* (1970). To detect DTH, 50 μl of PSA (2 mg/ml) was injected into the footpads of mice. The thickness of the foot was measured with a skin caliper gauge immediately before PSA injection and 24 hr after. The DTH reaction is expressed as the increase in footpad thickness in millimetres.

Excision of lymph nodes. Mice were anaesthetized with ether, the inner side of the left hind leg was shaved and the skin swabbed with 20% alcohol. A small incision was made through the skin with a scalpel and the popliteal node plucked from the tissue with fine forceps. The incision was closed by painting with collodian.

Sham operated mice were anaesthetized, the incision made and the forceps introduced between the leg mucles before closing.

RESULTS

The significance of the injection site

Within 8 weeks of the injection of 10^7 *L. tropica* into the shaved rump of multiple groups of BALB/c mice (> 50 animals) all animals were developing metastatic foci of infection on the feet, nose and lips. When an equivalent number of animals were injected with 10^7 *L. tropica* in the footpad no metastatic foci were seen, although the local lesion on the foot grew progressively to encompass the whole leg, which, if the infection was allowed to proceed was eventually lost.

The effect of lymph node removal on the course of infection

To try and determine whether lymphatic drainage played any significant role in limiting the extent of infection following footpad injection, further groups of BALB/c mice were infected at this site after surgical removal of the popliteal lymph node (XPLN). The course of infection was monitored by measuring the size of the foot lesion, and observing the appearance of metastatic lesions. Figs 1 & 2 demonstrate that the removal of the popliteal lymph node causes a more rapidly developing and severe *L. tropica* infection in both the susceptible BALB/c and resistent B_6D_2 mice. By week 8 a majority of the XPLN BALB/c mice had developed metastatic lesions while infection in intact mice remained localized. Although all infected B_6D_2 animals recovered, significantly larger, and more persistent, lesions developed in the XPLN animals.

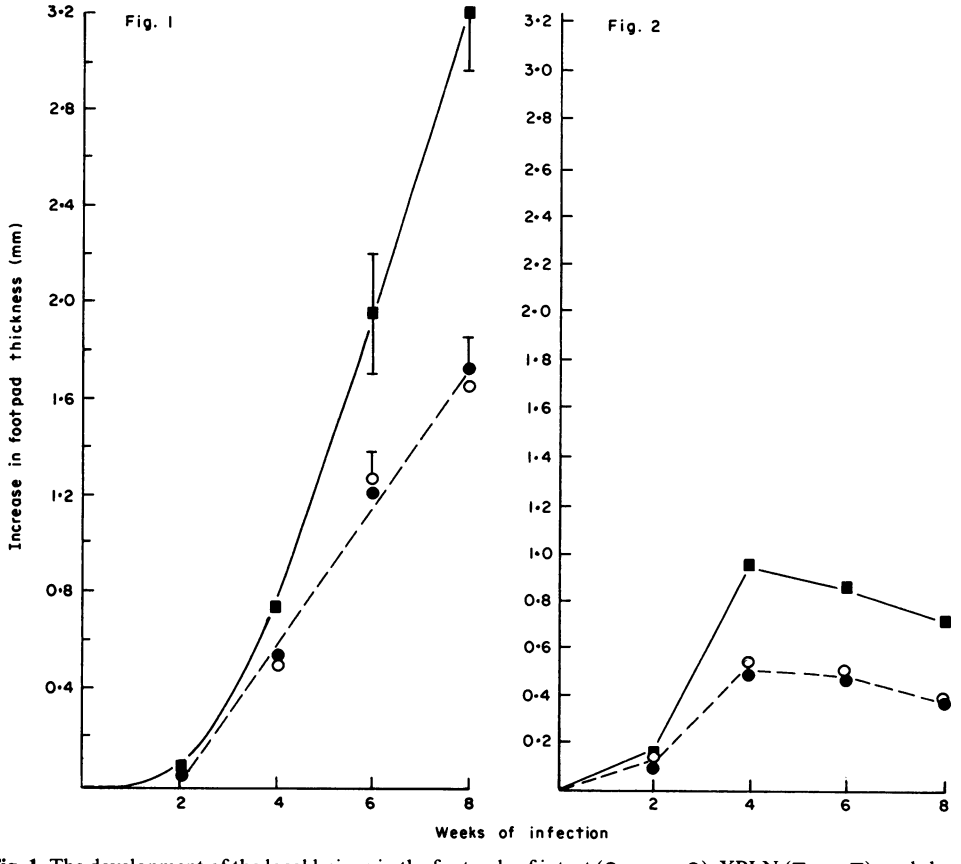

Fig. 1. The development of the local lesions in the footpads of intact (● - - - - - ●); XPLN (■——■); and sham operated (○——○), BALB/c mice infected with 10^7 *L. tropica*. (Mean of five mice.) As sham operated animals always responded as normals such data is omitted from Figs 3–7. In this experiment four of the five XPLN animals had developed metastatic lesions by week 8 while none of the others had.

Fig. 2. The development of the local lesion in the footpads of intact (● - - - - - ●); XPLN (■——■) and sham operated (○) B_6D_2 mice infected with 10^7 *L. tropica*. No animals in any group developed metastatic disease. Means of five mice at each point.

When infection was monitored directly by quantitating the numbers of *L. tropica* in the infected feet, data showed that the increased size of the lesions in the B_6D_2 mice was indeed reflected in increased numbers of organisms (Fig. 3).

In the BALB/c a more rapid multiplication of the parasite was observed in the XPLN animals, but by week 8 the numbers of leishmania in the feet of both XPLN and intact animals was the same (Fig. 3).

The development of DTH

Even the highly susceptible BALB/c mice develop DTH, albeit transiently (Howard *et al.*, 1980). The effect of route of infection and lymph node removal on the development of this correlate of cell-mediated immunological reactivity was therefore tested. BALB/c mice infected in the footpad expressed a higher level of DTH than equivalent mice infected in the shaved rump (Fig. 4). When XPLN BALB/c animals were tested however it was found that the absence of the draining node prevented the emergence of any significant DTH. Despite the higher levels of DTH developing after footpad infections (compared to the shaved rump route), this reactivity still declined after 4 weeks

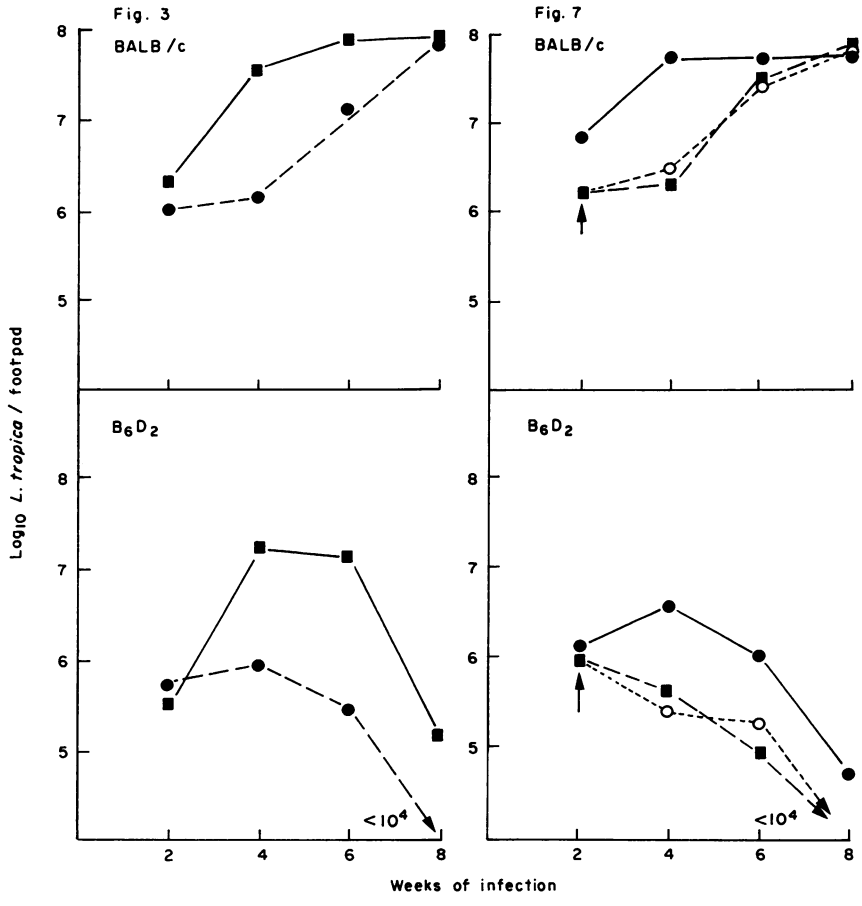

Fig. 3. The course of *L. tropica* infection in the footpads of intact (● - - - - - ●) and XPLN (■——■) BALB/c and B_6D_2 mice as determined by the growth of the organisms. Means of triplicate cultures from three mice at each time point.

Fig. 7. The progression of *L. tropica* infection in the LHFP of BALB/c and B_6D_2 mice, intact (■ - - - - - ■); XPLN left side 24 hr prior to infection, (●——●), and XPLN left side 2 weeks after infection (○ - - - - - ○). All animals received 10^7 *L. tropica*. Mean of triplicate cultures from three animals at each point. The arrows signify the time at which the politeal lymph nodes were removed.

of infection. In the resistent B_6D_2 animals the induction of DTH was slower in animals infected in the shaved rump than in animals infected in the left hind footpad (Fig. 5). When XPLN B_6D_2 mice were infected in the footpad the emergence of DTH was further retarded yet reached equivalent levels to that in intact animals by week 8.

The development of resistance to challenge

Groups of BALB/c and B_6D_2 animals were infected in the shaved rump or in the footpad. Further groups of XPLN animals also received footpad infections and resistance was tested in all groups 3 and 6 weeks after infection.

After 3 weeks the B_6D_2 mice expressed resistance to a standard challenge both when primary infection had been in the shaved rump or in the footpad (Table 1). The XPLN B_6D_2 mice however had failed to establish resistance to challenge by this time.

Results with the BALB/c mice revealed that only intact animals, receiving the primary infection in the footpad developed acquired resistance (Table 1). XPLN mice and mice infected in the shaved rump expressed no immunity.

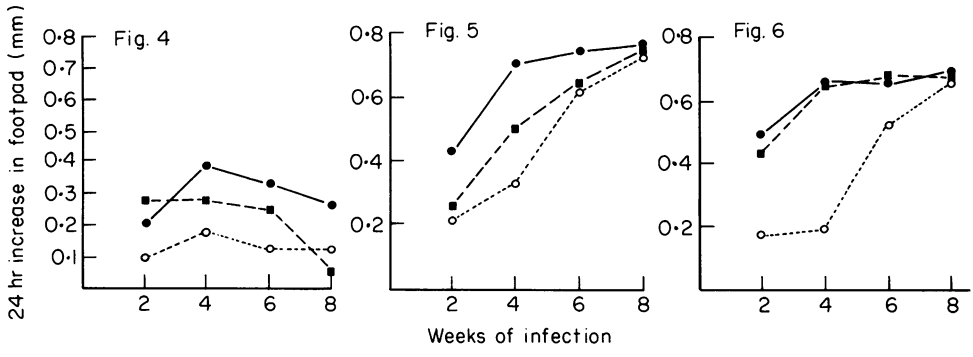

Fig. 4. Delayed-type hypersensitivity to PSA as expressed by intact (●——●) and XPLN (○ - - - - - ○) BALB/c mice at progressive times after footpad infection with 10^7 *L. tropica*. The level of DTH in animals infected in the shaved rump is also shown (■ - - - - - ■). DTH is measured as the increase in right hind footpad thickness 24 hr after antigen injection. Mean of five animals at each point.

Fig. 5. Delayed-type hypersensitivity to PSA in groups of B_6D_2 mice. Symbols and experimental conditions identical to those described for Fig. 4.

Fig. 6. Delayed-type hypersensitivity to PSA expressed as the increase in LHFP thickness measured at progressive times after infection with 10^7 *L. tropica* in the RHFP; three groups of animals were tested; intact (●——●); XPLN left side (■ - - - - - ■); XPLN right, (○ - - - - - ○). Means of five mice at each point.

Table 1. The effect of injection site and lymphatic drainage on resistance to *L. tropica* 3 weeks after infection

Animal	Status	Challenge	Log_{10} 2 week survival of challenge (Mean ± s.d.)
B_6D_2	Normal	1×10^7 RHFP*	5·84 ± 0·54
B_6D_2	Infected with 10^7 *L. tropica* LHFP*	1×10^7 RHFP	2·12 ± 0·31
B_6D_2	Infected with 10^7 *L. tropica* shaved rump	1×10^7 RHFP	3·6 ± 0·83
B_6D_2	Infected with 10^7 *L. tropica* LHFP. XPLN†	1×10^7 RHFP	5·2 ± 0·47
BALB/c	Normal	1×10^7 RHFP	5·6 ± 0·72
BALB/c	Infected with 10^7 *L. tropica* LHFP	1×10^7 RHFP	2·4 ± 0·18
BALB/c	Infected with 10^7 *L. tropica* shaved rump	1×10^7 RHFP	4·6 ± 0·53
BALB/c	Infected with 10^7 *L. tropica* LHFP. XPLN	1×10^7 RHFP	6·1 ± 0·66

* RHLP = Right hind footpad; LHFP = Left hind footpad.
† XPLN = Left popliteal lymph node removed 24 hr prior to infection.

Six weeks after infection all infected B_6D_2 animals were resistent to challenge irrespective of their status.

In contrast, no groups of BALB/c mice could resist the challenge inoculum (Table 2).

The role of the draining lymph node on the expression of DTH

To determine whether the draining lymph node was required for the expression of DTH the experimental situation was reversed in that animals whose left popliteal lymph node had been removed were infected in the contralateral foot. The expression of DTH was then tested at progressive times in the left footpad. The results of this experiment showed that the levels of DTH expressed were not affected by the absence of the popliteal node (Fig. 6). This result clearly indicated that although the absence of a draining lymph node suppressed the induction of DTH, it had no effect on the expression of this reactivity. The conclusion was substantiated in an experiment where

Table 2. The effect of injection site and lymphatic drainage on resistance to *L. tropica* 6 weeks after infection

Animal	Status	Challenge	Log_{10} 2 week survival of challenge (Mean ± s.d.)
B_6D_2	Normal	1×10^7 RHFP*	5·84 ± 0·54
B_6D_2	Infected with 10^7 *L. tropica* LHFP*	1×10^7 RHFP	$< 10^2$
B_6D_2	Infected with 10^7 *L. tropica* shaved rump	1×10^7 RHFP	$< 10^2$
B_6D_2	Infected with 10^7 *L. tropica* LHFP. XPLN†	1×10^7 RHFP	$< 10^2$
BALB/c	Normal	1×10^7 RHFP	5·6 ± 0·72
BALB/c	Infected with 10^7 *L. tropica* LHFP	1×10^7 RHFP	4·2 ± 0·36
BALB/c	Infected with 10^7 *L. tropica* shaved rump	1×10^7 RHFP	6·78 ± 0·78
BALB/c	Infected with 10^7 *L. tropica* LHFP. XPLN	1×10^7 RHFP	6·4 ± 0·59

* RHFP = Right hind footpad; LHFP = Left hind footpad.
† XPLN = Left popliteal lymph node removed 24 hr prior to infection.

intact animals were infected, in the left hind footpad, and the left popliteal node removed 2 weeks after initiation of infection. Monitoring the effect of lymph node removed by quantitating the multiplication of the *L. tropica*; it was found that the loss of the draining node failed to influence the subsequent course of infection (Fig. 7). It was concluded therefore that after 2 weeks of infection the acquired response had been induced and the course of the infection was thus predetermined.

DISCUSSION

It has been known for some time that the induction of a cell-mediated immune response by antigen introduced topically, or within the skin, requires intact lymphatic drainage from the site, (Turk & Stone, 1963; Barker & Billingham, 1968). As a successful acquired immune response to infection requires the induction of similar host mechanisms, it would not be surprising to find that intact lymphatic drainage from the site of infection was similarly necessary. This was indeed shown to be the case when Kadivar & Soulsby (1975) demonstrated that DCL could be induced by interrupting the lymphatics draining the site of leishmaniasis infection.

Recent reports have described variations in the course of leishmania infection following inoculation by different routes (Wilson *et al.*, 1979; Poulter, 1980; Poulter & Pierce, 1980). These observations, together with the severe diffuse disease associated uniquely with the rump infection of the BALB/c mouse led us to explore more closely the relationship between lymphatic drainage and an acquired cell-mediated immune response.

The results clearly demonstrated that the interruption of lymphatic drainage from a site of infection considerably delayed the induction of delayed-type hypersensitivity and resistance to infection. There was, however, no effect on the effector arm of this response, once induction had occurred. Such observations complement the findings of others (Turk & Stone, 1963; Balfour *et al.*, 1974) who have reported similar results from experiments using chemical contact sensitizers.

The considerable differences in the course of infection in intact animals resulting from different routes of infection were similar to those produced by interrupting lymphatic drainage. Whether such variations reflect differences in the lymphatic system at these sites remains to be tested directly.

The results obtained using XPLN BALB/c mice were of added interest as DTH has been shown to decay in this animal due to an active T suppressor cell mechanism (Howard *et al.*, 1980). Our results demonstrated that the removal of the draining lymph node prior to infection blocked the initial induction of DTH. However, such reactivity later emerged in the resistant B_6D_2 animals while no emergence occurred in the XPLN BALB/c. This result implied, that while inhibiting the induction of DTH, it did not inhibit the emergence of the suppressor mechanisms responsible for the eventual loss of cell-mediated immunity in the susceptible animals.

How significant the site of initial infection might be in man, is unknown but these results demonstrate that the immune response to infection may be affected by parameters hitherto neglected in relation to infectious diseases.

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