Cutaneous leishmaniasis: immune complex formation and necrosis in the acute phase

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Summary. Twenty biopsies of lesions of cutaneous leishmaniasis were classified according to the mechanism of parasite elimination, on the basis of macrophage activation (five cases) or macrophage lysis (15 cases). The immunoperoxidase technique was used to demonstrate free *Leishmania* antigen, immunoglobulins, complement, lysozyme, C-reactive protein, β-lipoprotein, α_1-antitrypsin, α_2-macroglobulin, plasminogen and factor VIII, which were quantitated and comparatively assessed. The fall in the parasite load during the course of the infection was associated with rising levels of IgG, IgM and IgE, and of the complement components of the classical pathway. Macrophage lysis supervened when there was an approximate equivalence of antigen and antibody, and was associated with the deposition of immune complex components. Lysis of the acute focal type (C response) was accompanied by a massive liberation of free *Leishmania* antigen, followed by a fall indicative of parasite elimination. The lysis of small numbers of macrophages scattered diffusely in the lesion, which was slow to reach completion (B response), was less effective and immunologically closer to the non-lytic (A) response. A terminal fall of the immunological factors other than the globulins, suggestive of resolution, was observed mainly in the C response. Lymphocytes may be important in macrophage activation associated with the macrophage A response and in the later stage of the B and C responses. However immunologically induced host-cell lysis is more important than macrophage activation for the elimination of *Leishmania* in the acute stage of most skin lesions. It is associated with, and may be caused by, the formation *in situ* of immune complexes of *Leishmania* antigen and antibody at an appropriate ratio.

Keywords: leishmaniasis, immune complex formation, immunoperoxidase

Cutaneous leishmaniasis (CL) is a diverse, often self-limiting infection, which serves as a model for the immunopathological mechanisisms found in more serious forms leishmaniasis. It is of interest in that the elimination of the intracellular organisms in the acute stage commonly results from immunologically induced lysis rather than activation of the host macrophages (Ridley 1979; Ridley & Ridley 1983a). The following variants of the histological response occur. In some endemic areas, notably Ethiopia, there are cases without macrophage lysis; parasite elimination is effected slowly following activation (A response). Elsewhere in the majority of cases there is lysis of macrophages which may be either patchy and incomplete (B response), or focused at the

centre of a macrophage granuloma (C response), which is usually associated with many lymphocytes and plasma cells. In all three forms of response, tuberculoid granulomas may develop before resolution (Ridley & Ridley 1983a). This classification may have some prognostic correlation, and patients with a B response, though they respond better than those with an A response, tend to relapse (Ridley 1980). Further study to elucidate the mechanisms involved in parasite elimination are indicated.

Early experimental studies of *L. enriettii* in guinea-pigs suggested that lysis of parasitized macrophages might be brought about by sensitized lymphocytes (Bryceson *et al.* 1970). However in-vitro evidence for lysis could not be repeated (Mauel *et al.* 1975), but in-vivo confirmation of a lytic mechanism was obtained by Monroy *et al.* (1980), who also noted an initial increase of plasma cell–macrophage ratio and later of lymphocytes in the guinea-pig lesion. Circulating antibody was detected by Radwanski *et al.* (1974), who also noted different localization of intracellular amastigotes, γ globulin and complement in the guinea-pig lesion which led them to dismiss immune complex formation as a pathogenetic entity.

Our recent study of CL, with its three forms of response possibly arising from a common immunological mechanism, prompted this immunoperoxidase study. Its purpose is, first, to evaluate the comparative levels and distribution of antigen, antibody and of complement; and secondly of certain other factors associated with inflammation at the site of the lesion.

Patients and methods

Biopsies. The number of samples available was restricted by the need to have a standard fixative procedure. There were 20 biopsies, five for the A response, eight for the B response and seven for the C response. They were received from most of the main endemic areas of the world (Ethiopia, the Mediter-ranean, Middle East and the Orient, and from Belize). None of the patients had any muco-cutaneous involvement.

Tissue processing. The biopsies were fixed in a formol-mercuric chloride, acetic acid fixative (10 ml formalin, 2 g mercuric chloride, 3 ml acetic acid in 100 ml water) for a period of 1.5–3 h and then transferred to 70% alcohol before routine processing. This is the fixative of choice to meet the requirements of Curran & Gregory (1980) for the immunoperoxidase technique. It preserves intracytoplasmic and some cell surface components and has been in routine use for leprosy. Excessive heat was avoided at all stages of preparation. Perfectly flat serial sections cut at 5 μm were obtained. One section was stained by haematoxylin and eosin for classification and examination for parasites.

Parasite index. The parasite index (PI) was a numerical assessment of the number of amastigotes on a logarithmic scale ranging from $1+ = \geqslant 1$ amastigotes per section to $6+ = \geqslant 10^5$ amastigotes per section (Ridley & Ridley 1983a). In the present study the highest PI obtained was $5+$.

Immunoperoxidase method. The technique has been described in detail (Ridley 1983).

Antisera. The antisera were raised without Freund's complete adjuvant and were obtained from DAKO (Mercia Brocades, Watford). Their specificity has been described (Mason *et al.* 1980). Included in this study were antisera to immunoglobulins IgG, IgM, IgE and IgA (diluted 1/100), complement components C3, C4 (1/50), C1q and C3d (1/100), lysozyme 1/200, coagulation protein plasminogen (1/200), and factor VIII (1/20), protease inhibitors α_1-antitrypsin 1/200 and α_2-macroglobulin and plasma acute phase reactants, C-reactive protein (CRP, 1/200 was a gift from Dr M.B. Pepys, London) and apoβ-lipoprotein (LDL 1/100 Miles-Yeda, Israel). In addition anti-*Leish-mania* antiserum (*L. tropica*, *L. major* and *L.*

mexicana) was a gift from Dr M.L. Chance, Liverpool, prepared according to the method of Sells & Burton (1981). 0.05 M Tris-buffered saline, pH 7.4, was used as the diluent and for washing the sections.

Controls. Many haem-containing molecules, including haemoglobin and catalase, oxidize diaminobenzidene (DAB) to form the insoluble polymer. This reagent varies with fixation, incubation time and pH. There is a need therefore for strict control procedures. One section was stained for endogenous peroxidase by direct application of diaminobenzidene-tetrahydrochloride plus hydrogen peroxide. Control slides included one section stained for endogenous peroxidase after blocking for this enzyme. A second control was to use normal rabbit serum in place of the anti-serum and a third control was obtained after absorption of the antibody by purified antigen. This was possible with IgG, α_1-antitrypsin, lysozyme (Sigma) and CRP (Dr M.B. Pepys) and with *L. tropica*. The antigens were diluted 1/10 with Tris buffer and equal volumes of antigen and dilute antiserum were incubated at room temperature for 20 min, centrifuged, mixed, filtered and used in place of the antiserum.

Analysis of results. A semi-quantitative method of scoring immunoperoxidase-positive staining cells and exudate was used. By this means 3+ represented $\geqslant 50$ cells per field ($\times 16$ objective); 2+ = 20–50 per field; 1+ = 10–20 per field, $\frac{1}{2}+$ = a few per section. The extracellular exudate was assessed as a percentage of the granuloma where 1+ = 5%, 2+ = 25%, and 3+ = 50%. The final analysis was the total intra- and extracellular components. Two independent assessors scored the findings, the mean result of which was plotted on a graph.

Results

Control sections using normal rabbit serum in place of the first antiserum and those in which the antiserum was absorbed by puri-

fied antigen were negative. Endogenous peroxidase was present in acute lesions with polymorphs, (C response). It was seen as a patchy deposit in the area of necrosis. It was blocked successfully before the immunoperoxidase technique was applied. Immunological factors plotted against high, medium and low parasite indices for the A, B and C responses are shown in Fig. 1 in which certain trends are apparent. Individually these points are based on small numbers of biopsies so that minor differences are probably insignificant.

The most striking result is seen in the rise of immunoglobulins, particularly IgG (Fig. 1c), but also IgM (Fig. 1d) and IgE (Fig. 1e), as the parasite load falls to zero. This applies to all three types of responses. Similarly, high levels of complement are observed in all three responses although the level falls notably after necrosis in C (Fig. 1b). The tendency of other mediators, C-reactive protein (Fig. 1g), α_1-antitrypsin (Fig. 1f) and β-lipoprotein (Fig. 1h), as well as of the macrophage-constitutive secretory product lysozyme (Fig. 1. f), to fall after the necrotic episode in the C response is also marked. It is accompanied by a reduction in *Leishmania* antigen (Fig. 1), both extracellular and in parasite numbers.

Distribution of immunological factors

Leishmania antigen. Amastigotes stained by anti-*L. tropica*, anti-*L. major* and anti-*L. mexicana* antiserum showed no difference in staining pattern. Extracellular organisms stained more darkly than intact amastigotes contained in macrophages (Fig. 2), and aggregated or diffuse antigen could be detected when no organisms were seen by the haematoxylin and eosin stain (PI = 0). There was pale staining of amastigotes in some macrophages but no marked intracellular deposits of antigen were found in the parasite-laden activated macrophages of the A response. By contrast following macrophage lysis in the C response, there were conspicuous darkly stained extracellular deposits in the exudate, together with intra-
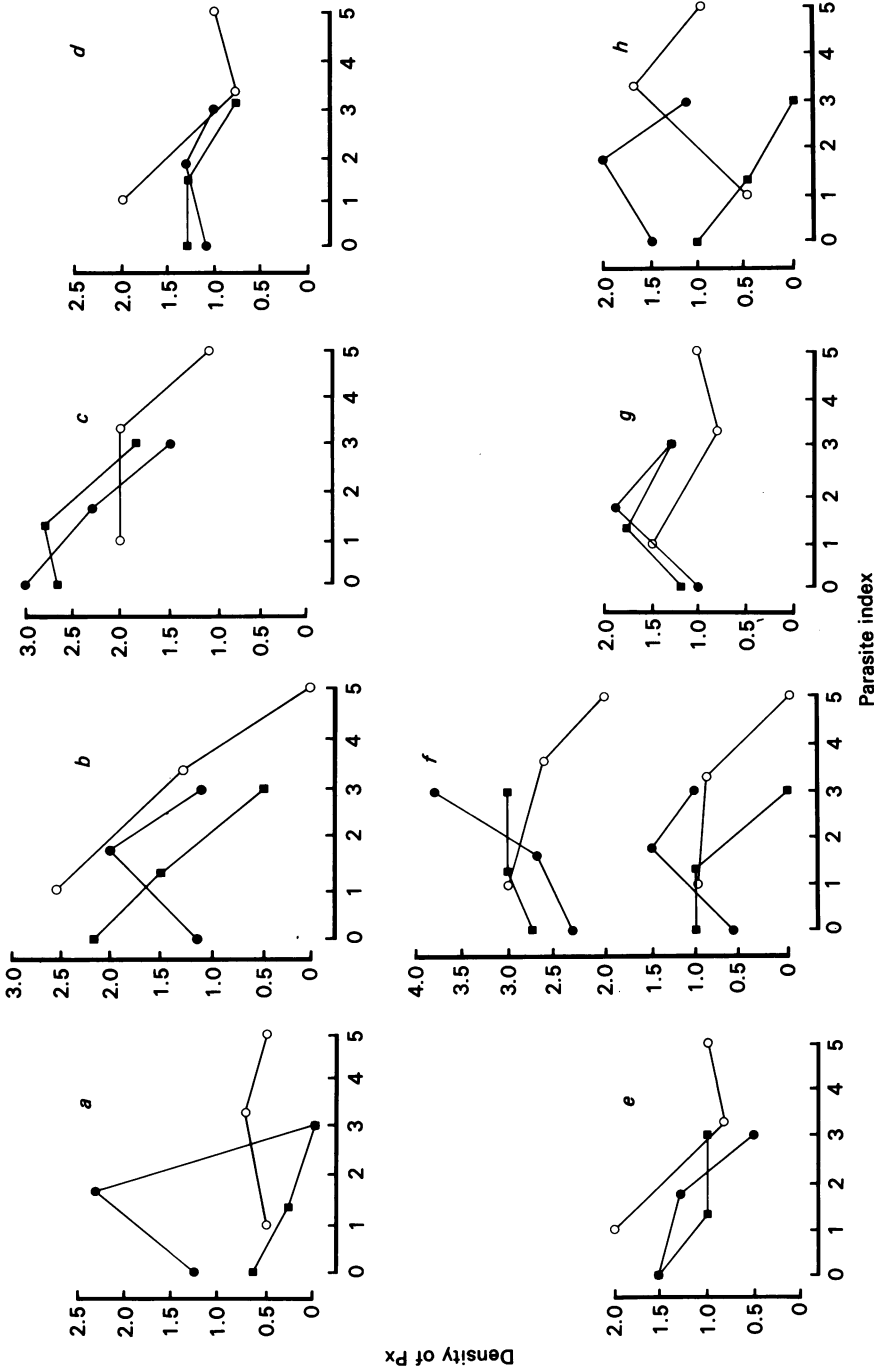
Fig. 1. *a* Extracellular leishmanial antigen. Intracellular antigen contained in macrophages corresponds to the parasitic index of each response. *b* Anti-C3. Complement levels rise sharply as the PI falls. In the C response necrosis is followed by a reduction in complement secretion. *c* Anti-IgG. Immunoglobulin IgG rises as the PI falls in all three responses. *d* Anti-IgM. Immunoglobulin IgM reaches a moderately high level in the A response as the PI falls. *e* Anti-IgE. Immunoglobulin IgE is moderately increased in the three responses as the PI falls. *f* Anti-lysozyme (top) and anti-α_1-anti-trypsin (bottom) Lysozyme is sharply elevated in the three responses. In A it is related to activation of macrophages; in B and C the levels fall slightly following lysis of macrophages. Polymorphs in B and C are strongly positive for lysozyme. α_1-anti-trypsin appears to be closely bound to extracellular leishmanial antigen. *g* Anti-CRP. C-reactive protein rises sharply following lysis of macrophages in B and C. Both responses show polymorph infiltration. *h* Anti-LDL. Apoβ-lipoprotein (LDL) is found extracellularly. O = A response; ■ = B response; ● = C response.
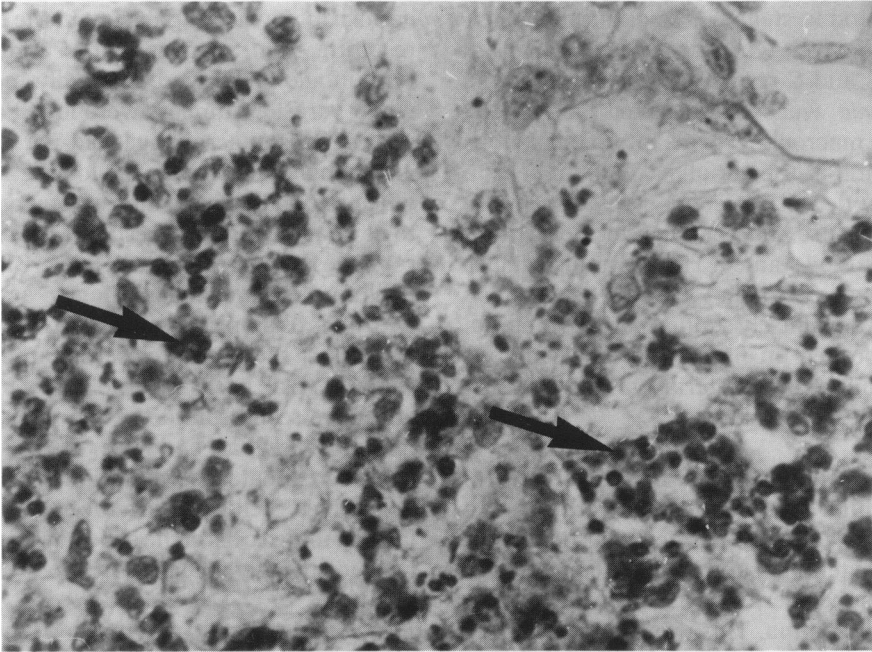
Fig. 2. The C response shows increased extracellular aggregated and diffuse, soluble leishmanial antigen (arrows) some of which also appears in polymorphs and young macrophages, at the basement membrane. Anti-leish. × 500.
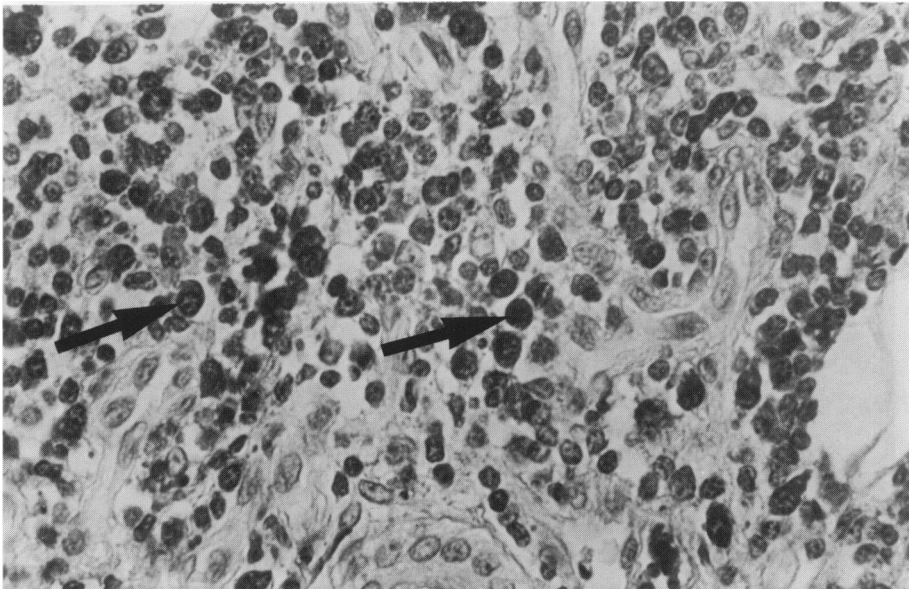

Fig. 3. A response. Plasma cells are strongly positive for IgG (arrows). anti-IgG. × 500.

cellular deposits in polymorphs, macrophages and in stellate cells of the subepidermal basal layer, which was heavily involved in the B and C responses (Fig. 2). Debris of *Leishmania* was also detected in spindle-shaped macrophages in inter-collagenous spaces and at the periphery of giant cells in the deep dermis. In some cases, notably in the B response, macrophage cell membranes were diffusely stained. There was also diffuse staining of perithelial cells of small blood vessels and diffusely stained positive material was deposited on fibres of the oedematous basal lamina. This material was most persistent in the B response.

Immunoglobulins. IgA was not demonstrated. Intracellular IgG, IgM and IgE were found in plasma cells (Fig. 3). Extracellular IgG and to a lesser extent IgM and IgE were conspicuous in the subepidermal basement membrane and basal lamina. This was seen in all three responses, most pronouned in B and C (Fig. 4). IgG was also seen bound to connective

tissue and degenerating elastic fibres of the dermis but it was insignificant in the exudate resulting from necrosis in the C response. Immunoglobulins were markedly increased when parasite numbers were low. Parasites contained in macrophages did not stain for immunoglobulins except following necrosis when antibody-coated amastigotes appeared to be phagocytosed by freshly recruited macrophages. All three forms of Ig were found in polymorphs and macrophages associated with necrosis, in particular in the B and C responses; these cells closely bound to the subepidermal basal layer of the epidermis and were seen around the zone of necrosis. IgM appeared as a diffuse deposit in the necrotic exudate, in which IgE was seen as aggregates. Stellate cells in the epidermis and large rounded cells of the subepidermal layer, stained strongly for IgG.

Lysozyme. Lysozyme was marked in all three responses. It occurred in small macrophages and in large activated macrophages that
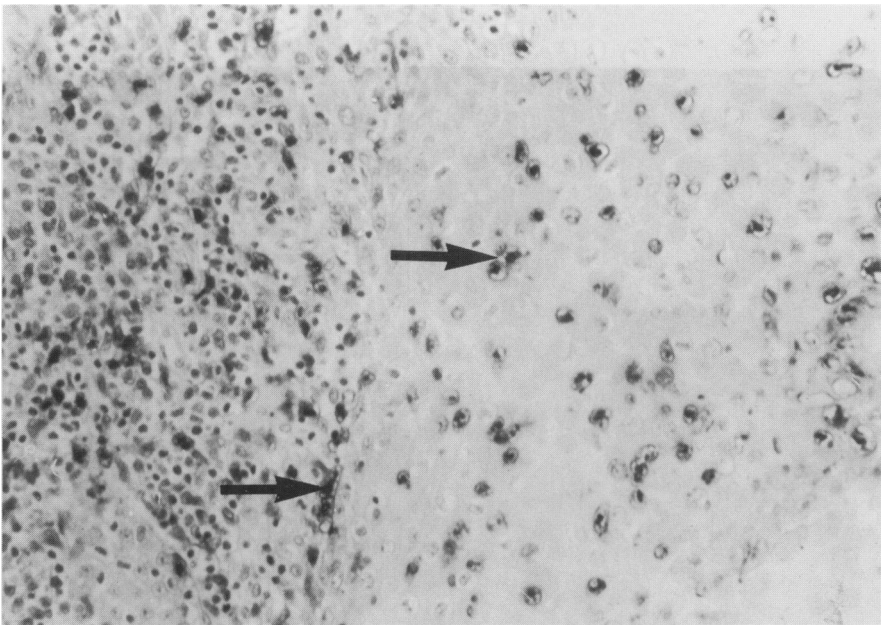

Fig. 4. B response. IgG is marked in polymorphs and young macrophage with amastigotes, accumulated at the basement membrane and which infiltrate the epidermis (arrows). anti-IgG. × 250.

contained few or no parsites. Parsite-laden cells stained weakly or not at all. Lysozyme was notable in the exudate associated with necrosis (mainly in C response), in which it was associated with polymorphs and scanty eosinophils more than with macrophages. Cells of the granulation tissue formed during resolution of the C response were devoid of lysozyme or had small amounts of the enzyme located in granules close to the nucleus of some cells. In the B response small macrophages and polymorphs which stained densely for lysozyme were accumulated at the basal layer of the epidermis and some of these cells infiltrated through the epidermis.

Other factors. α_1-Antitrypsin closely followed the distribution of extracellular leishmanial antigen, being bound to degenerate extracellular parasites, although staining was weaker than with anti-*Leishmania* antiserum. It was not seen in macrophages without parasites. C-reactive protein was elevated in the necrotic reactions of B and C responses, coinciding with the influx of polymorphs. Much of it was extracellular or in macrophages and polymorphs containing aggregated antigen. In the A response CRP appeared as a diffuse exudate, notably at low PI where it was bound to connective tissue. β-lipoprotein was always extracellular around blood vessels and in inter-collagenous spaces. Plasminogen was present only in insignificant amounts. α_2-Macroglobulin was elevated slightly in the extracellular spaces in the C response. Factor VIII was not seen in the granuloma, although endothelial cells in all 3 responses were conspicuously stained. However, endothelial cells of the granulation tissue in the C response were negative, as were similar cells in the acute B and C responses.

Complement. C3, C1q and C3d were present at high levels extracellularly following necrosis in the B and C response in which C4 was minimal. C3 and C3d were also detected in polymorphs associated with the subepidermal reaction and connective tissue fibrils

of the basal lamina stained strongly. Small macrophages at the periphery of the lesion were positive for C3 and in the A response there were fine granular deposits of C3 in activated macrophages without parasites.

Discussion

The findings can be summarized briefly by saying first that immunoglobulins and complement components in the lesion of cutaneous leishmaniasis tend to increase during the course of the infection as the parasite index falls. This implies that these immunological factors may be participating in some effector mechanism which may be common to the three forms of response. Secondly, the increase in many of the inflammatory factors studied is sharpest in the C response, characterized by acute lysis of macrophages. In this response lysis supervenes when immunological factors are at peak levels and it is associated with massive liberation of leishmanial antigen. Following this episode the quantities of antigen, complement, CRP, α_1-antitrypsin and β-lipoprotein all fall. By contrast in the A and B responses these factors usually remain constant or continue to rise as the PI approaches zero.

The results do not give a direct measure of antigen–antibody ratios. Nevertheless at PI = 4 and 5, Ig levels are low and there appears to be an antigen excess. At PI = 0 or 1, Ig levels are high and there is apparently an antibody excess. At the onset of necrosis in the B and C responses the PI of 2 or 3 is associated with moderate Ig levels which are probably near to equivalence. Complement (of the classical pathway) is also present in moderate amounts. Antigen, Ig and complement are found in the same situations, in the extracellular spaces and in the area of necrosis and especially in the exudate and polymorphs associated with necrosis. All these results parallel those of an immunoperoxidase study of experimental BCG infection (Ridley *et al.* 1983). A condition in which necrosis was found to be induced by the

injection of preformed complexes at equivalence but not at any other ratios (Ridley *et al.* 1982*a*). Similarly in leprosy, necrosis in erythema nodosum leprosum is associated with immune complex formation at an appropriate antigen–antibody ratio (Ridley & Ridley 1983*b*). We conclude that immune complexes are produced at the site of the lesion in cutaneous leishmaniasis and that the antigen–antibody ratio is associated in some way with macrophage lysis.

There was no increase in circulating complement levels, and circulating immune complexes were not found in a Brazilian study (Moriearty *et al.* 1982), which makes the local presence of these factors all the more striking. Radwanski *et al.* (1974), using a relatively insensitive fluorescence technique, detected essentially similar results to ours for antibody and complement in their study of guinea-pig lesions. Their failure to find antigen, antibody and complement at the same site of parasitized-macrophage accumulation may be explained by electron microscopic evidence of early disintegration of macrophage membrane (Monroy *et al.* 1980), and the fact that in infection C3 is quickly denatured whereas C3d persists (Charlesworth *et al.* 1974). Moreover, necrosis in the guinea-pig lesion, unlike CL, is not accompanied by polymorph infiltration, so that immune complexes may not be involved in quite the same way as in man.

It is evident that *Leishmania* antigen is present in different forms. Parasites both live and dead stain with anti-*Leishmania* antibody, but the strongest uptake is by the degenerate residues of the amastigotes, either extracellular or intracellular. This staining is specific because it can be blocked by absorption of the antiserum with leishmanial antigen. The density of staining may reflect exposure of antigenic material by the 'fit' of the vacuolar membrane surrounding it (Chang & Dwyer 1978; D'Arcy Hart & Young 1979). Although the immunoperoxidase technique used here is not suitable for demonstrating immunological factors bound to cell surface membranes, leishmanial

antigen has been demonstrated *in vitro* on the surface of macrophages in man (Berman & Dwyer 1980) and mice (Farah *et al.* 1975; Handman *et al.* 1979) and it is modulated by antibody (Dwyer 1976).

Immunoglobulins, particularly IgG, were found here to be raised at the site of the lesion, which may be fundamentally important. An inverse correlation between the lympho-plasmacytic infiltrate and the number of *Leishmania* within the lesion has been noted by a number of authors and the distribution also may be significant. In the A and B responses the infiltrate is diffuse; in C it is dense and peripheral to the granuloma. Despite conflicting evidence immune serum probably enhances protective immunity in mice (Preston & Dumonde 1976) and guinea-pigs (Poulter 1980*a*). In human CL, circulating immunoglobulin is at a low level except in severe infections and it appears not to be protective (Menzel & Bienzle 1978). However low circulating levels are linked with a lack of polyclonal activation of B cells by dermotrophic leishmanias (Campos-Neto & Bunn-Moreno 1982), and they do not correlate with intra-lesional levels (Moriearty *et al.* 1982). IgE levels in the lesions of our study were unexpectedly high, and Peterson *et al.* (1982) reported raised circulating levels. The rising levels, expecially in the A response, might suggest a connection with delayed-type hypersensitivity which is the opposite of the result of Lynch *et al.* (1982). Mast cells, which may be implicated with DTH (Askensase & Loverin 1983), were few. Possibly the IgE may bind to the macrophage, and in so doing form an antigen complex without complement (Capron *et al.* 1980); but further study is needed.

The A response, in which there is no necrosis, depends entirely on macrophage activation (a process also seen in the terminal phase of all three responses). It might be expected, therefore, that the A response would comprise mechanisms similar to those observed in leprosy, and this is to some extent the case. In CL, the immunoglobulins and lysozyme rise as the PI falls just as they

do at the high resistant tuberculoid (TT) pole in leprosy (Ridley *et al.* 1982*b*). Similar also is the absence of significant change in CRP and α_1-antitrypsin, although plasminogen is negligible in CL. Lysozyme, whose secretion is said to be independent of phagocytic stimuli (Gordon 1980), was found at high levels in activated macrophages and in small recently recruited macrophages without ingested organisms; and parasitized cells appeared to synthesize little or no lysozyme. By contrast, in necrotizing lesions most of the lysozyme was in active, mature polymorphs, and the level fell sharply following necrosis. Lysozyme is to be the subject of more detailed discussion in a separate communication.

In conclusion, all the cellular and molecular components of macrophages, T lymphocytes, antigen, antibody, complement and immune complexes are present at the site of the lesion of CL. Without overlooking the importance of T lymphocyte factors, especially in the A response, this study emphasizes the fundamental role of lysis of parasitized macrophages in the elimination of organisms in the acute phase, with immune complex formation at an appropriate antigen–antibody ratio. Activation of macrophages at antibody excess follows, as demostrated in other systems (Capron *et al.* 1977; Walker 1977). The aspects of immunity which determine resistance to challenge with *Leishmania* and inhibition of metastasis in experimental animals are separate issues—see reviews by Poulter (1980*b*) and Mauel & Behin (1982).

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