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Monocyte recruitment, antigen degradation and localization in cutaneous leishmaniasis

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Summary. The relationship between the destruction of *Leishmania*, the recruitment of monocytes and macrophage activity in the lesions of cutaneous leishmaniasis (CL) was studied in 53 biopsies representing the phases of evolution of the infection. Lysozyme, amastigotes and their degradation products were located by their specific antibodies. A rising level of monocyte influx was found to correlate with the degradation and solubilization of antigen, a falling level with final clearance. Differences in the results supported the previous concept of macrophage activation and macrophage lysis as alternative mechanisms for the elimination of *Leishmania*. Macrophage activation appeared to coincide with the re-phagocytosis of externalized antigenic products of different type and origin. Macrophage lysis was a fully effective mechanism only when the antigen was contained within a focalized granuloma before mass lysis. Failing this, degradation and clearance of antigen were incomplete, and residues were sequestered on the periphery of the lesion where they bound to collagen and epidermis with consequential tissue damage. Antigen was demonstrated on the surface of lightly parasitized macrophages but not heavily infected ones. Other cells bound antigen without ingesting it, a process which might allow antigen presentation though it would also favour survival of parasites within the cell.

Keywords: leishmaniasis, macrophage–parasite interaction, lysozyme, monocytes

Cutaneous leishmaniasis (CL) is a complex model of immunopathogenic mechanisms and homeostasis which usually succeed in limiting the infection. Recovery leads to acquired immunity and often to skin test positivity to leishmanin (Garnham & Humphrey 1969). A few cases develop diffuse cutaneous disease or destructive metastasizing mucocutaneous lesions.

Control of the many species and subspecies of *Leishmania*, which infect cells of the mononuclear phagocyte series, involves two processes: (a) activation of the macrophage by T lymphocytes, and (b) lysis of the host macrophage. Evidence for both methods of control comes from experimental data (Bryceson *et al.* 1970; Preston & Dumonde 1976; Louis *et al.* 1982; Andrade *et al.* 1984). In humans also a corresponding double-stranded immuno-histological host response has been demonstrated (Ridley 1979; Ridley & Ridley 1983). We have designated macrophage activation the A response, host cell lysis the B and C responses; lysis is either sporadic, involving small groups of macrophages or isolated cells (B), or compact involving focalized granuloma (C). Each response evolves through a weak tuberculoid granuloma towards resolution; strong tuberculoid granulomas occur only in a minority of cases

(Ridley & Ridley 1984*a*). The parasite load is initially highest in the A response in most cases.

Recent research on the immune response focuses on the central role of the macrophage in presenting antigen to T lymphocytes (Unanue 1981). To investigate further monocyte migration and macrophage activity in CL we made use of lysozyme, an antimicrobial agent synthesized and secreted by macrophages for which it is a marker (Gordon *et al.* 1974). The appearance of monocytes and their derivative cell types was studied in relation to the stages of degradation of *Leishmania* amastigotes in the various forms and phases of the infection. Both the failure of some individuals to achieve resolution despite an apparently successful granulomatous response, and poor granuloma development leading to chronicity of the disease or relapse in others, require further explanation.

Material and methods

Patients. 53 biopsies from 45 untreated patients with solitary lesions of CL, including two sequential biopsies from each of eight patients were studied. The patients came from Ethiopia, Belize, Guyana, the Middle East, India, Pakistan and the Mediterranean. Of those that were skin tested intradermally with 100 μl of an autoclaved suspension of 10^6 promastigotes/ml saline, the majority were positive. There was no evidence of secondary infection clinically, or by Gram and acid-fast staining in the lesions examined.

Classification. Each biopsy was classified according to the system described previously (Ridley & Ridley 1983); the A, B and C lesional responses are coupled with an a, b or c to represent the stage of evolution of the lesion, and with the parasite index. Thus Aa6 is a pure macrophage response with maximum parasite load, and Cc0 is a weak tuberculoid granuloma with no detectable organisms in the section. The D response denotes a strong tuberculoid granuloma. Our cases comprised 11 A, 22 B, 17 C and 3 D lesions. Sequential biopsies were obtained from four patients classified as A, 3 as B and 1 as C. The time scale of the three responses was evaluated in the first instance by evolution of the granuloma from a passive macrophage to a tuberculoid form, with a concurrent fall in the parasite index. The results were checked by the known time intervals between sequential biopsies where these were available.

Tissue processing. Biopsies for histology were fixed in 'FMA' (10 ml formalin, 2 g mercuric chloride, 3 ml acetic acid in 100 ml distilled water) which is optimal for the immunoperoxidase technique (Curran & Gregory 1980). After 2 h fixation, biopsies were placed in 70% alcohol for processing. Some (12 B and 7 C) were fixed for 12 h in FMA.

Staining. Sections were stained in haematoxylin-celestine blue-eosin for histology and indexing of parasites.

Parasite index. The parasites were counted in the sections using a × 100 objective, and the index estimated on a log scale of 1+ to 6+: 1+ = one or more organisms per section; 2+ = 10 or more per section; 6+ was estimated as 100 000. For very small or large sections the score was adjusted to that of a standard section occupying 3 fields with a × 10 objective (Ridley & Ridley 1983).

Immunoperoxidase Technique. The unlabelled antibody peroxidase–antiperoxidase (PAP) method was used. Deparaffinized sections were brought to water and incubated in 1% H_2O_2 in methanol to block endogenous peroxidase activity, and then in normal swine serum to reduce non-specific binding by the conjugate. Rabbit antiserum to antigen bound in tissue was then applied at optimal dilution, followed by swine anti-rabbit Ig as a bridge to link the primary antibody to the second antibody-PAP complex raised in the rabbit (Dakopatts, Den-

mark). Reaction product was revealed with DAB-peroxide (Sigma). Tris/saline buffer (0.05 M) was the washing buffer. After prolonged fixation in FMA (12 h), initial immersion in trypsinizing solution (0.05 g trypsin + 0.05 g $CaCl_2$ + 100 ml M_2O, pH 7.2) at 37°C for 5 min increased the sensitivity. Incubation in DAB-peroxide was used to detect endogenous peroxidase.

Antiserum. Monospecific rabbit anti-human lysozyme IgG was obtained from Dakopatts. It was raised without Freunds complete adjuvant. Rabbit anti-*Leishmani* species IgG was a gift from Dr M.L. Chance, Liverpool; it was prepared from promastigotes injected i.v. into rabbits. The IgG fraction was purified through a protein A-sepharose CL-3B column and dialysed (Sells & Burton 1981). No difference in staining was obtained with antisera to *L. tropica*, *L. major* or *L. mexicana*. The results given are those obtained with antiserum to *L. tropica*. The optimal dilution of anti-lysozyme was 1/100 and anti-*Leishmania* 1/200.

Controls. 1. Absorbed anti-*L. tropica* rabbit IgG and absorbed anti-lysozyme rabbit IgG were substituted for the antibody. In each case 50 μl antiserum was mixed with a pellet of 10^6 promastigotes or 100 μg lysozyme (Difco), and the suspension incubated at room temperature for 30 min before centrifugation, filtration and use. 2. Normal rabbit serum replaced the primary antibody. 3. One section was stained with DAB-peroxide after blocking the endogenous peroxidase activity. The specificity of staining was shown by a negative reaction in each case.

Analysis. The number of positively stained cells was assessed using a ×40 objective and a ×8 eyepiece. At this magnification the granuloma occupied about six fields, each with about 500 cells. Peripheral infiltrate was not included. The number of reactive cells per cent was estimated from the total number of granuloma cells. Those in ulcerated or necrotic areas were omitted from the final count. The mean results were tabulated. Extracellular parasitic residue was measured separately: + + + marked excess, + + a moderate amount, + a little per section.

Additional studies. Tissues from selected biopsies of all groups were divided into three parts; one was fixed for histology, one was snap-frozen in liquid nitrogen for cryostat sections, and one was fixed for electron microscopy.

Adjacent cryostat sections were stained for endogenous peroxidase, acid phosphatase and non-specific esterase; also with OKM1 monoclonal antibody (Ortho Diagnostics, USA), using an indirect fluorescense technique to identify monocytes and cells of the mononuclear phagocyte series (MPS) and null cells. Fluorescence was observed with FITC-conjugated rabbit anti-mouse Ig (Dakopatts).

Results

The histology of the single biopsies and of the sequential biopsies that represented the evolution of the lesion were consistent and are considered together. There was an A response to 11 cases (3 Aa, 5 Ab, 3 Ac); a B

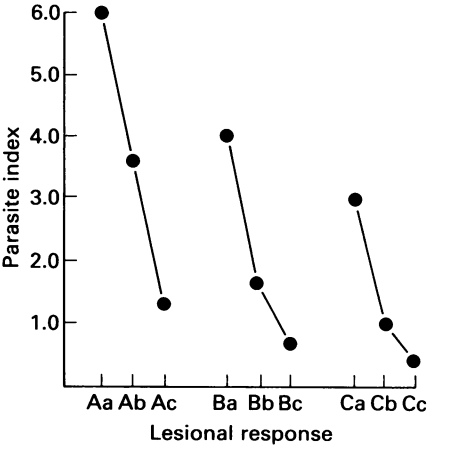

Fig. 1. The mean parasite index of the A, B and C responses during their evolution. The level in A is raised compared to those in B and C.

response to 22 cases (Ba, 10 Bb, 7 Bc); and a C response in 17 cases (3 Ca, 9 Cb, 5 Cc). Three D responses were also obtained. The mean parasite index for each group is given in Fig. 1.

Monocytes

These cells and their derivatives were identified by examining similar cells in cryostat sections from the same lesion. They were strongly positive for acid-phosphatase and non-specific esterase, and they reacted to OKM1 antibody. Many cells containing few ingested organisms had strong endogenous peroxidase activity (which was blocked successfully during the PAP technique).

Lysozyme

Among the cells reactive for lysozyme were cells of the MPS, neutrophil polymorphs and mast cells. Intense staining with lysozyme suggested synthesis, and active secretion was inferred from additional interstitial deposits. Discrete lysozyme-positive (LYS+) granules close to the hof region of the nucleus were a feature of stromal histiocytes. Lysozyme antibody bound to extracellular degenerate parasitic products, but not to ingested amastigotes. The mean scores for LYS+ cells are shown in Fig. 2.

A response. The number of LYS+ monocytes increased as the parasite load declined, and large, activated, densely-stained macrophages appeared at the centre of the lesion. Lysozyme activity was not demonstrated in heavily parasitized cells (20+), but lightly parasitized macrophages (>3) stained densely. In later lesions when parasites were hard to detect, strong LYS+ monocytes and activated macrophages dominated the lesion, with marked intravacuolar depositions of lysozyme in some (Fig. 3). Some weakly LYS+ cells appeared to have internalized large numbers of amastigotes below the cell membrane. These parasites, unlike the others, were usually LYS+, suggesting that they had been previously externalised. The final clearance of residual parasitized LYS− macrophages appeared to be after total envelopment with lysozyme, or in close association with LYS+ giant cells. Neutrophils were rare in these lesions.

B response. Monocyte influx was high and sustained in the middle and late phases of the response (Bb and Bc, Fig. 4). In the early stage, LYS+ neutrophils were the main cells in the areas of parasite and tissue destruction in the dermal–epidermal zone, and more remotely between collagen bundles. Neutrophil invasion fell sharply as parasites and macrophages disappeared during resolution.

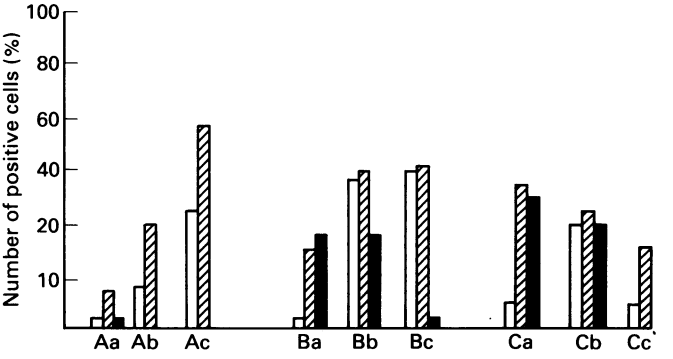

Fig. 2. The mean scores for cells positive to lysozyme. The increase of monocytes and MPS cells in the A response correlates with a fall in the parasite index, the falling levels in C with necrosis and parasite eradication. ▨, lysozyme in MPS; □, monocytes; ■, Pmns.

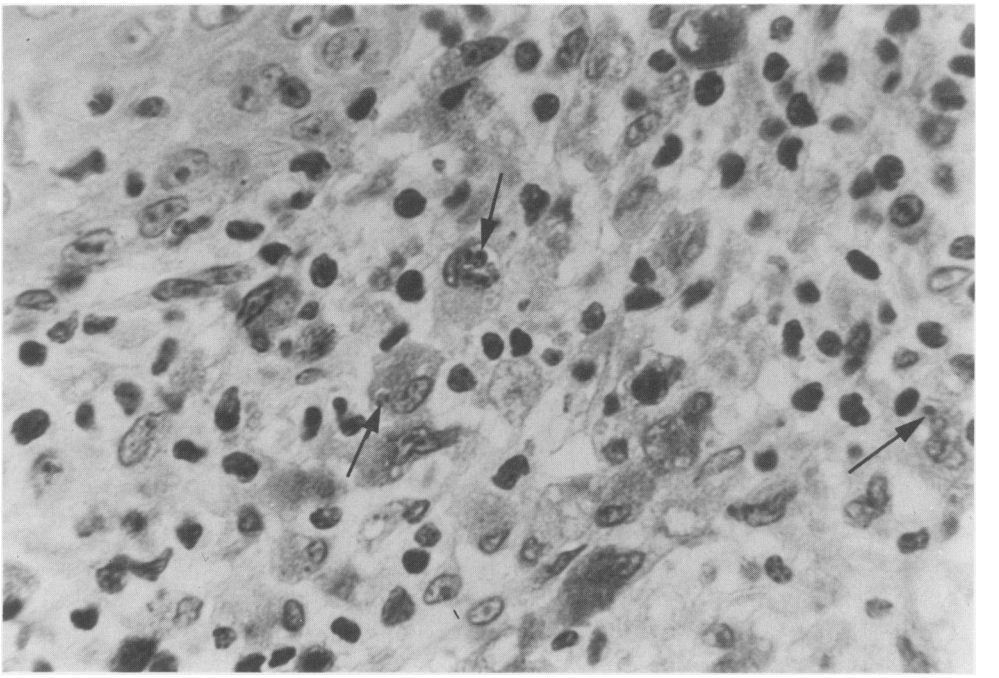

Fig. 3. Response Ab. Activated macrophages are strongly positive, some vacuolated. Lysozyme reactive amastigotes suggest a previous external existence prior to reingestion (arrow) Ipx anti-LYS × 500.

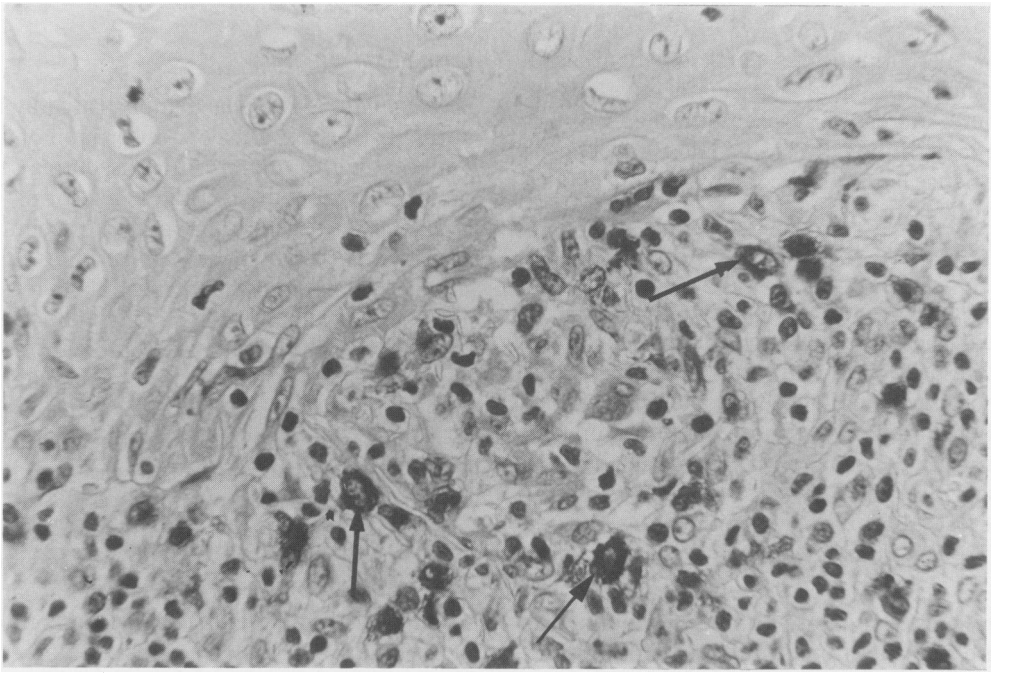

Fig. 4. Response Bb. Monocyte influx is raised (arrow). Subepidermal zone macrophage proliferation is marked. Ipx anti-LYS × 500.

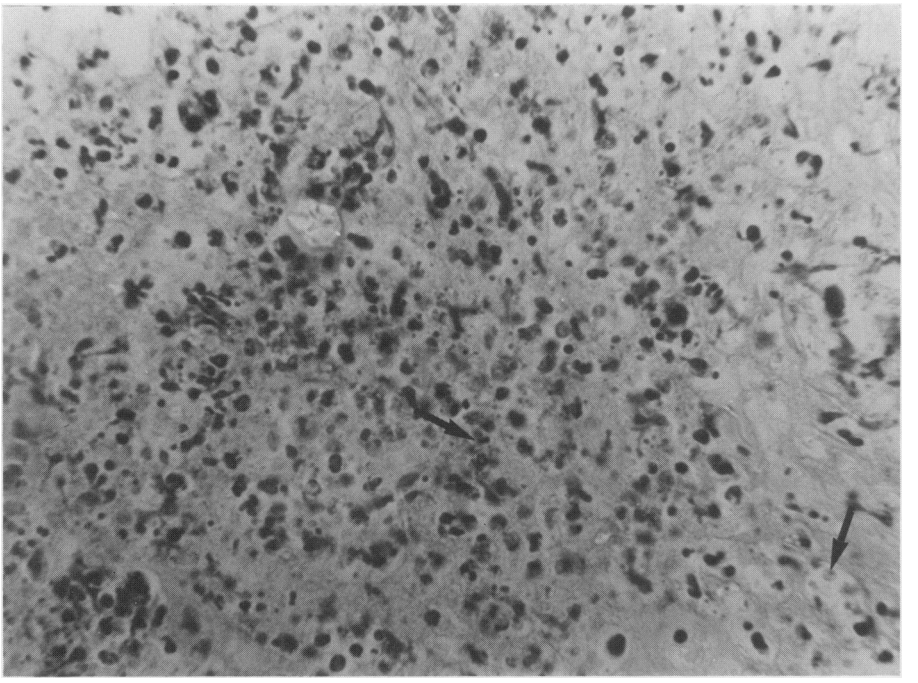

Fig. 5. Response Ca. Central area of necrosis shows neutrohils and macrophages with parasite destruction (arrow). Abundant lysozyme is deposited. Ipx anti-LYS × 250.

However, monocytes persisted in areas of fibrinoid or damaged connective tissue where increased lysozyme was deposited. Some keratinocytes stained densely. Among the dense lymphocytic infiltrate large dendritic cells were isolated some of which were weakly LYS+, while others were negative.

C response. Very few monocytes were seen in the earliest phase. The main cell type of the granuloma was a strongly LYS+ activated macrophage with few endocytozed parasites. Other features were necrosis and damaged connective tissue with heavy deposits of lysozyme (Fig. 5). After resolution of the necrosis the number of monocytes fell and LYS+ macrophages reappeared.

D response. Moderate numbers of monocytes were present, randomly sited among lymphocytes. The granuloma cells formed compact tubercles, all strongly LYS+.

Leishmania antigen

Leishmania antigen was demonstrated by its antibody (but not after pre-absorption of the antibody with antigen). In consecutive sections the antibody identified all forms of *Leishmania* antigen: intra- and extracellular amastigotes, their degraded residues and soluble components. Intracellular intact organisms stained less intensely than their LYS+ degraded products. Soluble components of parasite degradation appeared as a diffuse LYS− deposit. Intact amastigotes were sometimes strongly positive for acid-phosphatase. Other parasites were negative as were the degraded residues.

In the A response, in lightly parasitized cells there was abundant antigen associated with all forms of intact amastigotes, coarse residues and finely diffused soluble components (Fig. 6). In heavily parasitized macrophages the amastigotes stained weakly and the cells had no surface antigen and no

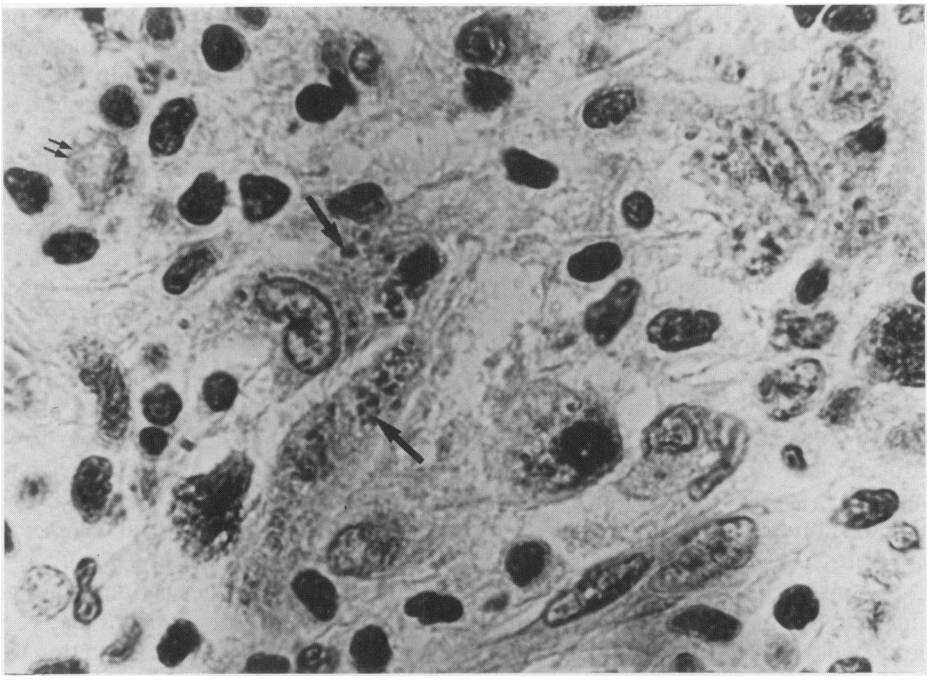

Fig. 6. Response A. Intact amastigotes are reactive to leishmania antibody. Soluble components are seen at the periphery of some cells (double arrow). Ipx anti-LSH. × 1000.

soluble products in the phagocytic vacuoles. Soluble components increased markedly in some cells of later lesions, where residual macrophages packed with ingested parasites still survived.

B response lesions had larger amounts of extracellular parasitic debris, both aggregated and diffuse, which persisted into the late-stage. Soluble components were seen in some epidermal dendritic cells, and aggregated residues in keratinocytes. Lymphatic vessels were packed with densely staining cells.

C response lesions had mainly soluble components localized in the area of necrosis, on damaged connective tissue and epidermal basement membrane, and in giant cells, dermal histiocytes and vascular endothelium. *Leishmania* antigen disappeared after the necrotic episode.

In the D response, minimal deposits of diffuse and aggregated *Leishmania* antigen were present in a few macrophages at the periphery of the tubercles in one section only.

Discussion

In these experiments lysozyme was used to monitor monocyte recruitment and macrophage activity; intact amastigotes were unreactive for lysozyme (LYS−) as were soluble components, but externalized parasites and their degraded residues bound lysozyme (LYS+), as also did some parasites thought to have been re-ingested. *Leishmania* antibody identified all forms of *Leishmania* antigen. It is thus possible to construct the probable cycle of events involving both parasites and their host cells.

The results support the distinction between three types of response in CL (Ridley & Ridley 1983). In general lysozyme levels and the influx of monocytes rose progressively during the A (intact macrophage) re-

sponse, fell in the C (focal necrosis) response, and plateaued at an intermediate level in the B (incomplete lysis) response. Concomitantly there was a reduction in the levels of *Leishmania* amastigotes in all three responses, though with persistent high levels of aggregated debris in B and soluble components in C.

In the A response, it appeared that the death of parasites and host cells, followed by the re-phagocytosis of externalized products, stimulated macrophages and led to their improved cytological differentiation, lysozyme synthesis and accelerated intracellular elimination of amastigotes. The late stage reproduced tuberculoid leprosy, with increased LYS+ cells (Ridley *et al.* 1985).

The alternative method of parasite eradication by host cell lysis was best illustrated in the C response. The lesion developed early from large LYS+ macrophages, which were evidently well differentiated despite a low level of monocyte recruitment. The key to this type of response is the micro-anatomical focalization of these granuloma macrophages and their parasitic content. Necrosis proceeded from the centre where soluble *Leishmania* antigen was abundant. The peripheral lymphoplasmacytic infiltrate may have acted as a barrier. It remains to be shown whether C response individuals are immunologically privileged and why (Rook & Stanford 1979).

The intermediate B response originated in relatively undifferentiated LYS− macrophages in poorly demarcated granulomas, in which the density of lymphocytes and plasma cells could have impeded the interaction of macrophages with other cells outside their immediate vicinity. Monocyte influx was heavy and sustained, but macrophages did not become activated (as judged by lysozyme synthesis) until the parasites had been eliminated. Elimination appeared to be by piecemeal lysis of small clusters of macrophages, followed by the release of degraded aggregates of antigen. The sporadic nature of this process suggests that the immune mechanism in the B response is weak.

When antigen was released at the periphery of the lesion, mainly in the B response, or from stromal histiocytes in the dermis, it bound to collagen and elastin, which probably explains the destruction. Superficially there was epidermal lysis or ulceration. Deeper, widespread damage to the connective tissue of the dermis was confirmed by electron microscopy (Ridley & Wells 1986), and this was more widespread in the B than in the C response. Connective tissue-binding probably explains the very destructive scarring that is a feature of mucocutaneous leishmaniasis (Kerdel-Vegas & Essenfeld-Yahr 1966; Marsden & Nonata 1975).

It has been suggested that development of end stage tuberculoid granuloma, and persistence of the related but more reactive recidivans lesion (Pettit 1962), are associated with the sequestration of antigen on the periphery of the lesion (Ridley & Ridley 1984*a*). The above findings relating to the B response support this view. The widespread dispersal or sequestration of antigen, and the unexplained failure of macrophage activation in the B response, even though monocyte recruitment is rapid, need further investigation.

Leishmania antigen was not observed at the surface of heavily parasitized macrophages. These cells were negative for lysozyme indicating that protein synthetic activity is low (Gordon *et al.* 1974); under such conditions cells are unlikely to be actively engaged in killing and digesting amastigotes, hence the absence of surface antigen. However, surface antigen was detected on lightly parasitized macrophages and has been demonstrated also in *in vitro* systems (Farah *et al.* 1975; Handmann *et al.* 1979; Berman & Dwyer 1981). Our results indicate that surface antigen may vary in origin and composition. It may be derived from the killing of amastigotes, from the breakdown of reingested organisms weakened by a period of externalization, or from degraded residues. Once externalized, antigen may become complexed with exogenous protein or antibody (Ridley & Ridley 1984*b*), which could

modify its handling. It is unlikely that substances excreted by the parasite (Slulsky *et al.* 1979) were detected in our system, since heavily parasitized cells lacked detectable surface antigen. Parasitic residues probably remain immunogenic; sonicated products are reportedly as immunogenic as whole amastigotes (Louis *et al.* 1979). In this case the detection of soluble specific leishmanial antigen in Langerhans cells of the epidermis may be important.

Although lysozyme did not appear to lyse amastigotes and does not restrict their growth (Passwell *et al.* 1984), it could play a subsidiary role in the absorption and neutralization of dead *Leishmania* antigen. This is supported by the fact that such parasites had no acid-phosphatase activity. The internalization of parasites by cells without ingestion into vacuoles is also interesting for it could affect the survival of organisms by allowing them to evade cellular oxidative mechanisms (Wilson *et al.* 1980).

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