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HISTOLOGICAL STUDIES OF THE ELIMINATION OF *LEISHMANIA ENRIETTII* FROM SKIN LESIONS IN THE GUINEA-PIG

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Summary.—Nineteen guinea-pigs were each inoculated intradermally with 10^6 amastigotes of *Leishmania enriettii*, and the development of the lesions was followed from Weeks 4 to 10 with a view to elucidating the histological mechanisms involved with the elimination of parasites. Electron microscopic observations were made in 1 animal.

Extensive necrosis of the parasite-laden macrophages was observed in 7 out of 7 animals at 4 and 5 weeks. In the ulcerated core of the lesion at 4 weeks no intact macrophages could be identified. Very many amastigotes were extracellular. Others were present in the cytoplasm of residual macrophages the cell walls of which had disintegrated. Necrosis was less marked at 8 weeks and absent in the resolving lesions at 10 weeks.

Signs of stimulation or maturation of macrophages were only apparent when parasites were few. At 4 weeks macrophages were almost all of the non-stimulated form, but cytological evidence of activation became progressively more definite and widespread from 5 to 8 weeks, starting at the periphery of the lesion.

Ultrastructural observations of amastigotes suggested that there might be more than one mechanism of degradation. It appeared that the majority of parasites were released through necrosis and discharged through the ulcer, and that intracellular degradation of the remaining parasites was important mainly in the later phase before resolution. The first phase was associated mainly with plasma-cell production, the second mainly with lymphocytes.

THE IMMUNOLOGICAL MECHANISMS involved in the pathogenesis and healing of cutaneous leishmaniasis have been shown to be complex and to involve delayed hypersensitivity besides other cell-mediated responses and antibody (Heyneman, 1971; Turk and Bryceson, 1971; Bryceson, 1972; Preston and Dumonde, 1976). Not enough is known about the histological criteria whereby the patterns of abnormal immunity are linked, nor is it yet possible to link up satisfactorily experimental studies with cutaneous leishmaniasis as it is seen in man (Preston and Dumonde, 1976). Recent histological studies of human infections suggest that a necrotizing mechanism is the most potent means of eliminating parasites (Ridley, 1979), and its operation produces the best prognosis for the patient (Ridley *et al.*, 1980). The concept of an immunologically induced necrosis was first put forward by Bryceson *et al.* (1970) in respect of *Leishmania enriettii* infection in guinea-pigs. However, Mauel *et al.* (1975) failed to repeat the experiments of Bryceson *et al.*, and in the human studies there were apparently other immune mechanisms in operation. It was not clear whether their multiplicity was inherent in the host

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responses to *Leishmania* or to the multiplicity of species or strains (Gardener, 1977), which produce correspondingly varied histological responses in mice and hamsters (Coutinho and Coelho, 1972). The need is for further studies with single strains of parasite, which can only be obtained in laboratory animals.

The most widely used animal model has been *L. enriettii* in the guinea-pig, which has already been studied histologically after inoculation intradermally (Bryceson *et al.*, 1970), s.c. (Rezai, Haghighi and Ardehali, 1972), in the nose tip (Sordat and Behin, 1977) and i.v. (Kanan, 1975). None of these workers has commented on necrosis (other than epidermal ulceration) as an important factor in the elimination of parasites, and none has demonstrated convincingly that elimination in non-immunized animals is associated with any appreciable activation of the host macrophages or epithelioid-cell transformation. On the other hand there is evidence of limited intracellular digestion of parasites (Sordat and Behin, 1977), and their elimination is associated with the development of skin-test hypersensitivity of the delayed type.

Despite the number of previous reports, we decided to undertake a further study of *L. enriettii* in the guinea-pig with a view to elucidating the principal histological mechanisms at work.

MATERIALS AND METHODS

Organisms and antigenic extracts.—*Leishmania enriettii* was maintained by passage in guinea-pigs every 4 weeks by injecting intradermally 10^7 amastigotes into the tip of the nose. The amastigotes were obtained from the infected nose which was dissected out, washed, teased apart in Medium 199 and lightly ground in a glass tissue grinder. At the same time the organisms were grown in conventional Novy, MacNeal and Nicolle's (NNN) medium. Purified soluble antigen (PSA) was prepared from *L. enriettii* by hypertonic urea extraction from sonicated promastigotes, as described by Bryceson *et al.* (1970).

Experimental infection.—Eighteen outbred guinea-pigs weighing about 400 g were infected by intradermal injection of 10^6 organisms in 0·05 ml Medium 199 on the dorsal surface of each ear. The animals were divided into 3 groups for routine histological examination at 5, 8 and 10 weeks from the date of infection. In the light of these results another guinea-pig was inoculated for examination by light and electron microscopy at 4 weeks.

Histology and electron microscopy.—The whole ear lesions were excised. In the 18 animals the whole specimen, and in the last animal half of it, were fixed in buffered formalin and embedded in paraffin for routine histological examination. Multiple sections were examined.

In the last animal the other half of the lesion was divided into small pieces which were fixed in 3% glutaraldehyde buffered with 0·1M cacodylate, pH 7·3. After extensive washing with 0·1M cacodylate buffer, they were post-fixed with 1% OsO_4 and embedded in Araldite. Sections were cut at approximately 50 nm on a Reichert ultra-microtome using glass knives, stained with uranyl acetate and lead citrate and examined in a Philips 201 S TEM microscope using 60 kV accelerating voltage.

Immunological investigations.—The lymph nodes draining the ear lesions were taken for histological examination at the same time as the lesions. Intradermal skin tests were carried out at 4, 7 and 9 weeks using PSA at a dose of 60 μg as previously described. Serum antibodies against *Leishmania* were taken to titre, using the standard indirect immunofluorescent-antibody technique. The results, referred to here briefly, will be the subject of a further more detailed report.

RESULTS

Within 1–2 weeks of inoculation a nodule developed at the inoculation site on the ear. It grew and the overlying skin became keratotic. At 4 weeks the lesion ulcerated and was covered with a crust. Within 6 or 7 weeks it reached its maximum size, about 2 cm in diameter and about 4 mm thick. Healing began at 8 weeks and in some animals was complete by 10 weeks.

Histologically the lesions in the acute stage were characterized by a central ulcer surrounded by a zone of heavily parasitized macrophages and, peripheral to this, more lightly parasitized macrophages which later showed signs of activation. This lesion extended down to the cartilage of the ear, but on the extreme periphery there were chronic inflammatory cells and, later, fibroblasts.

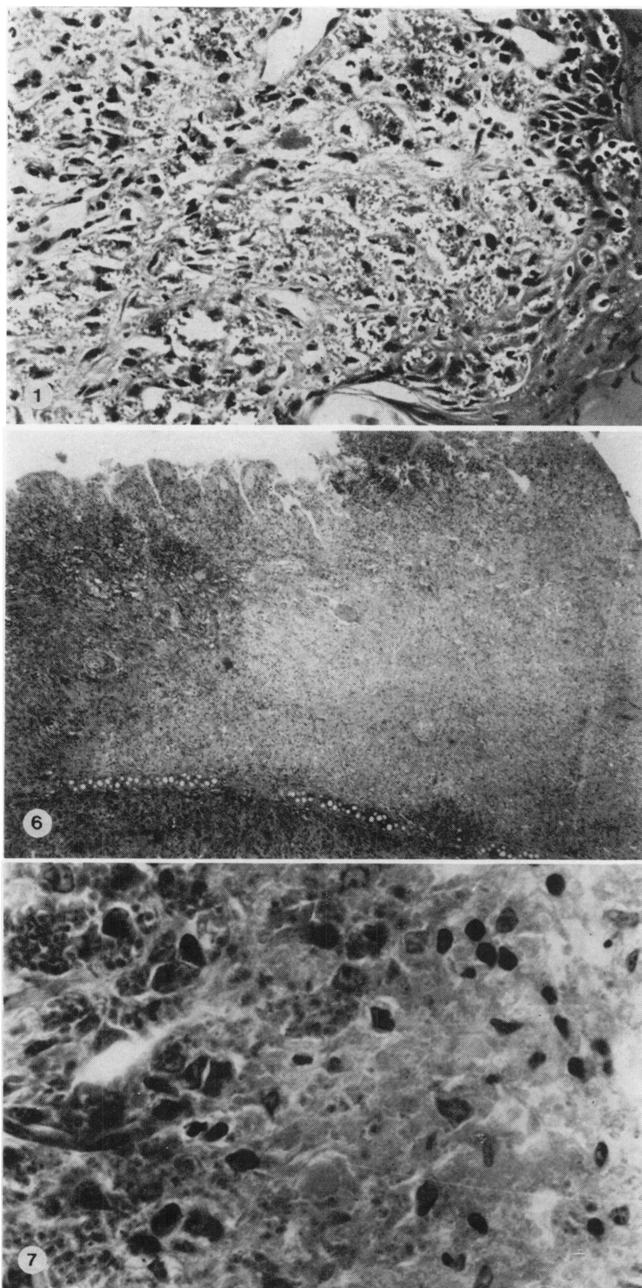

FIG. 1.—4 weeks. Heavily parasitized cells or structures throughout the dermis and epidermis adjacent to the ulcer. H. & E. × 175.

FIG. 6.—5 weeks. Sharply demarcated, completely necrotic core of ulcer extending down to the cartilage. H. & E. × 11.

FIG. 7.—5 weeks. High-power view of periphery of necrotic zone in Fig. 6: parasitized macrophages left, necrosis right. H. & E. × 350.

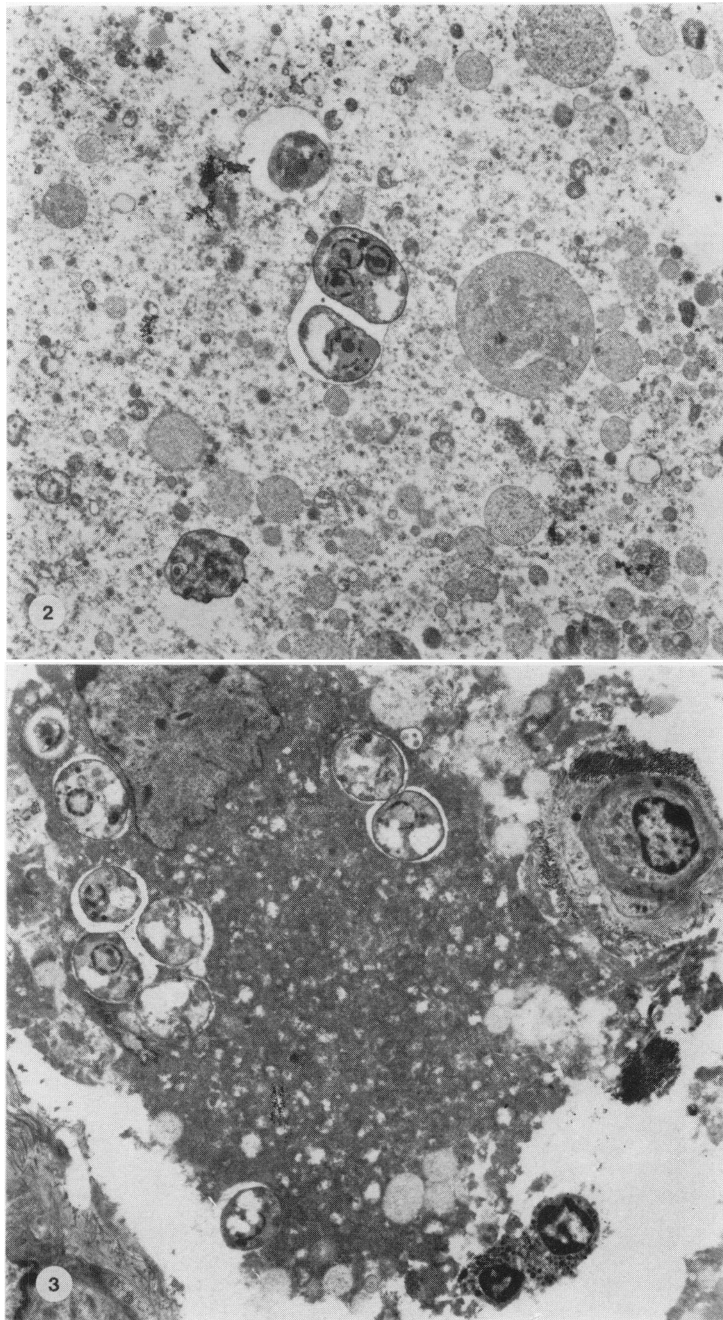

FIG. 2.—4 weeks. Cell debris and amastigotes in the central area of the lesion. × 1800.

FIG. 3.—4 weeks. Heavily parasitized macrophage with intact nucleus but the cell wall has been destroyed. Central area of the lesion. × 1800.

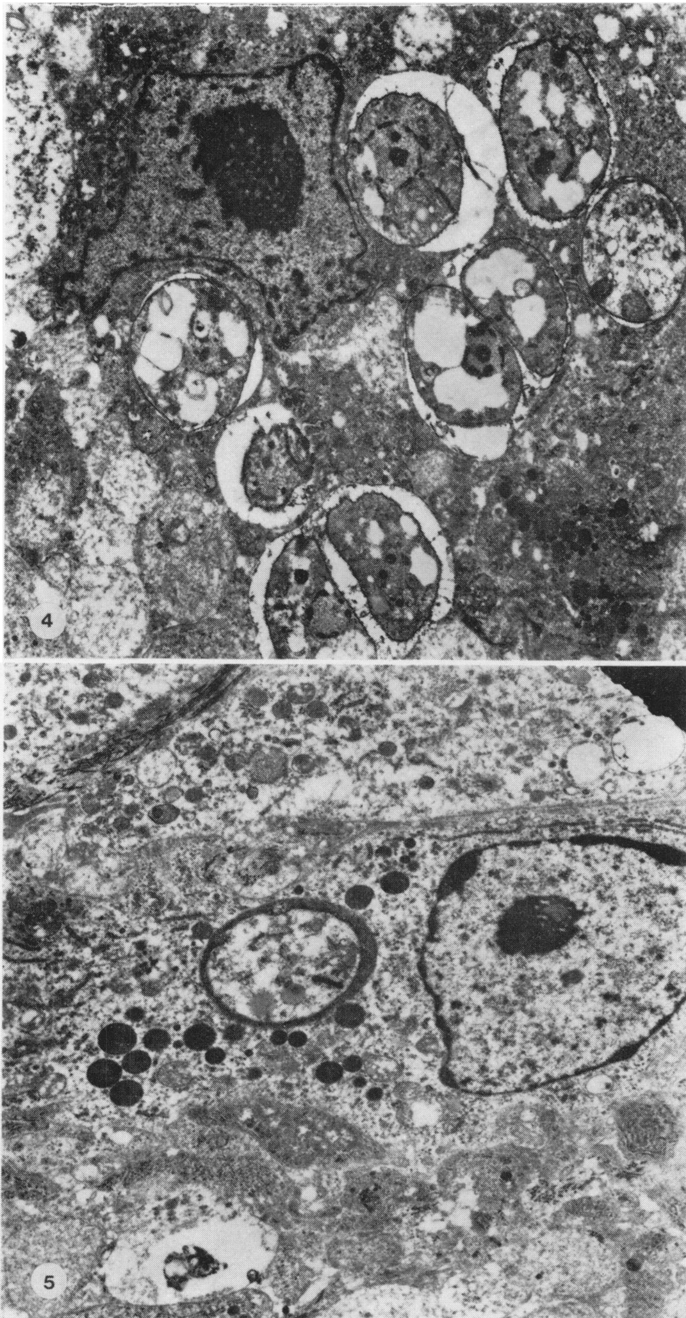

FIG. 4.—4 weeks. Heavily parasitized intact macrophage adjacent to the central area. Most of the amastigotes are structurally similar to those in Figs. 2 and 3. × 5400.

FIG. 5.—4 weeks. Lightly parasitized intact macrophage adjacent to the central area. The amastigote, surrounded by an electron-dense zone, shows signs of structural disintegration. × 5400.

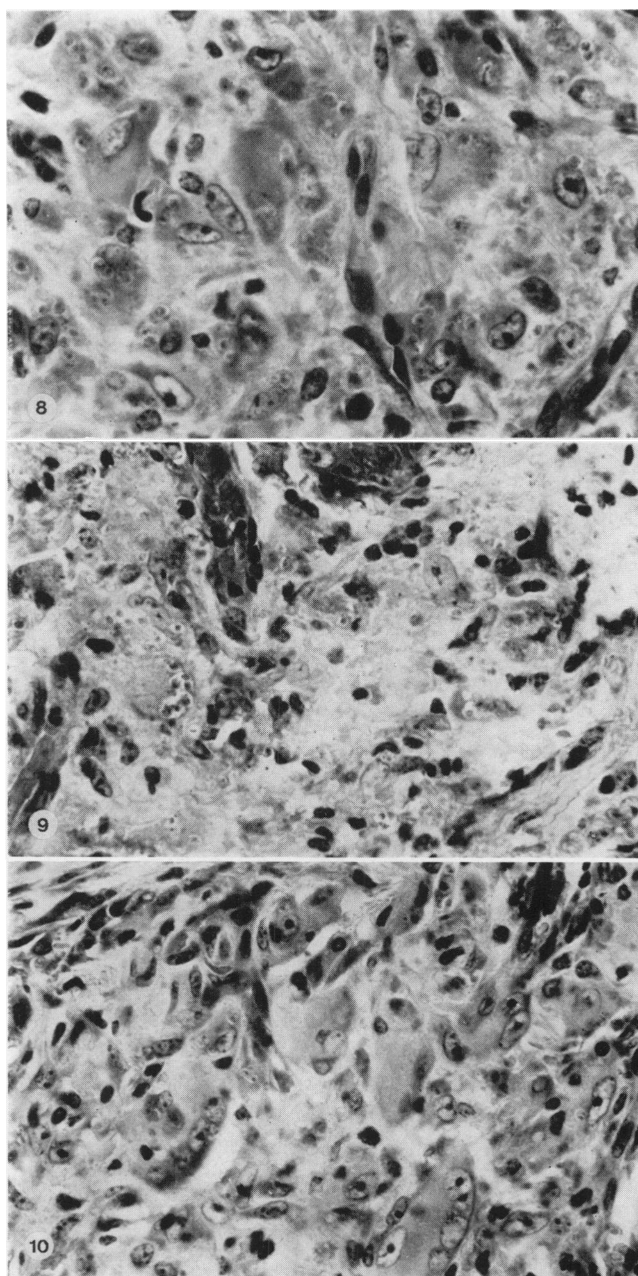

FIG. 8.—5 weeks. Stimulated macrophages amongst parasitized cells on the periphery of the lesion. H. & E. × 350.

FIG. 9.—8 weeks. The parasite load adjacent to the ulcer is diminished but still appreciable. Cell debris still present. H. & E. × 350.

FIG. 10.—8 weeks. The periphery of the lesion shown in Fig. 6. Macrophages and giant cells showing signs of strong stimulation. Parasites are scanty. H. & E. × 350.

Lesion at 4 weeks

Light microscopy (1 animal).—The lesion was already ulcerated. In the central area there were few cells but much cell debris and very many extracellular parasites. It was impossible to be sure whether surviving macrophages were intact. Just peripheral to the ulcer there was considerable parasitization of all layers of the epidermis, and in the subepidermal region cyst-like structures, presumably derived from macrophages, heavily engorged with parasites (Fig. 1). On the periphery of the lesion the parasite load was lower and mostly intracellular. None of the macrophages showed evidence of activation. Beyond the region of macrophages and parasites there was a quite dense localized infiltrate of plasma cells but few lymphocytes.

Electron microscopy (1 animal).—In the centre of the lesion no intact macrophages could be found. There was much cell debris (Fig. 2) and in places many extracellular amastigotes. Many other organisms were found in macrophage residues which had lost their cell membranes (Fig. 3). It was not clear how many of the amastigotes were viable. Beyond the central area there were more of these dead cells and also a few intact macrophages, most of which carried a heavy load of parasites which were structurally similar to the extracellular forms (Fig. 4). In two macrophages with a lower parasite load the amastigotes were surrounded by electron-dense material and showed loss of structural integrity (Fig. 5). None of the macrophages showed structural evidence of stimulation except for one cell with endoplasmic reticulum and a solitary intracellular amastigote.

Lesion at 5 weeks

Light microscopy (6 animals).—Centrally in the floor of the ulcer parasites, some extracellular, were fewer than at 4 weeks or scanty. Cell debris was still present in all animals, and in one there was a sharply demarcated necrotic core which extended down to the cartilage, 2 mm in diameter (Figs 6 and 7). Peripheral to this in the superficial dermis the macrophages were heavily parasitized and lacked signs of stimulation. In the deep zone and further out in the periphery, however, where the parasite load was lower, there were extensive areas where many of the macrophages had developed somewhat (Fig. 8). These cells had larger nuclei with prominent nucleoli and some homogeneous pink-staining cytoplasm. In all parts of the lesion there were primitive giant cells with about 3 nuclei which were structurally similar to those of the macrophages. Plasma cells were similar to those in the 4-week animal or scanty. Lymphocytes were scanty. The epidermis peripheral to the ulcer was still parasitized in all layers.

Lesion at 8 weeks

Light microscopy (6 animals).—The number of parasites was appreciably fewer than at 5 weeks, though still considerable in the superficial zone peripheral to the ulcer. Evidence of necrosis of individual macrophages was still present, but there was no mass necrosis even under the ulcer. In the superficial zone there were few cells with any evidence of stimulation (Fig. 9). However, in the deep zone and periphery of the lesion there were granulomatous areas composed of large macrophages, with large nuclei and prominent nucleoli which were now eosinophilic, and much pale pink homogeneous cytoplasm (Fig. 10). These cells contained few parasites or none. The plasma cell infiltrate was similar to that at 5 weeks. Lymphocytes had increased in number.

Lesion at 10 weeks

Light microscopy (6 animals).—Signs of resolution were present and parasites had been eliminated altogether in 5 animals. The area of the ulcer, when it was still present, was occupied by a heavy infiltrate of lymphocytes, plasma cells, mononuclear cells and young macrophages. Fibroblasts were present in small numbers at the periphery.

No involvement of cartilage in the infective process was evident at any stage of the lesion.

Lymph nodes

At 5 weeks there was a strong preponderance of plasma cells in the medulla, which in some cases extended into the paracortical areas, lymphocytes then being confined to the subcapsular zone and the afferent sinus. Follicles were small except in 1 animal, and in this the plasma cells were mostly immature. At 8 weeks there were some follicles, mainly of the B-cell type, in all animals and the whole cortex and paracortical areas were occupied by lymphocytes. At 10 weeks it was possible to distinguish small peripheral follicles typical of T-lymphocyte production and large active follicles with prominent germinal centres in the deep cortex or medulla, typical of B-lymphocyte production. Plasma cells by this time were scanty except in 1 animal.

Macrophages were found in the subcapsular region at 5 and 8 weeks but had disappeared in 5 of the 6 animals at 10 weeks. Parasites were found in lymph-node macrophages only at 5 weeks.

Skin test

The skin test was positive in all animals at 4 weeks and had increased in intensity appreciably at 7 weeks. In all animals the peak was at 24 h, and by 48 h the skin thickness had decreased to 30% of the peak measurement. At 9 weeks the intensity of the 24h reaction had partially subsided but was better sustained at 48 h.

Serum antibody

Immunofluorescent-antibody titres reached a peak at 6 weeks and thereafter declined.

DISCUSSION

The present study is a repetition of work carried out by the previous workers already quoted, though not all employed electron microscopy. All have used approximately the same inoculum (10^6 amastigotes). The course of the infection, the development of skin-test positivity to leishmanin which reaches a peak at 7 weeks, and the histology of the lymph nodes are not in dispute. Our own results in these respects are in general agreement with the previous studies. The question at issue is the histological mechanisms by which parasites are eliminated.

The evidence of the present study was that there was a massive necrosis of macrophages in the heavily parasitized central area of the lesion at 4 weeks, which persisted with slight diminution at 5 weeks, but had partly resolved at 8 weeks. Although the presence of cell debris in the acute stage of the lesion was clear by light microscopy, the disintegrated state of many of the remaining macrophages could only be ascertained by electron microscopy. This indicated that the great majority of parasites in the 4-week lesion were either rendered extracellular by the complete disintegration of their host macrophages, or they were retained in the cytoplasm of macrophages the cell membranes of which had been destroyed. Since the epidermal cells on the periphery of the ulcer were heavily parasitized, as noted by previous workers, it is possible that destruction of the epidermis in the central area was brought about by the same necrotizing mechanism that caused the destruction of the macrophages. It appeared that the majority of parasites, whether or not they were killed during the necrotizing process, were extruded through the ulcer together with the dead macrophages and cell debris.

Evidence of activation of macrophages was not apparent in the most heavily parasitized parts of the lesion, and at 4 weeks it was barely perceptible. With diminution of the load at 5 weeks, macrophages in the peripheral or deep zone appeared to be somewhat stimulated, and at 8 weeks there were granulomatous areas which by the same criteria (Adams, 1974) would be graded as highly mature macrophages or immature epithelioid cells. Such cells are no doubt capable of intracellular

degradation of parasites, as demonstrated by Sordat and Behin (1977). The beginning of this process was seen by us even at 4 weeks. However, numerically the parasites that could have been destroyed in this way were outweighed many times by those involved in the necrotizing process.

Since necrosis of macrophages was demonstrable in all our 4- and 5-week lesions it may be questioned why, apart from the fact of ulceration, it has not been reported by previous workers. Without the confirmation of electron microscopy, even in a single case, we should have been less confident in claiming necrosis by routine histology. Material needs to be taken for processing from the core of the lesion at the right time. In one of our lesions (taken at 6 weeks for electron microscopy and not reported) the centre could not be found. The intracellular amastigotes of Sordat and Behin were apparently viable at 4 weeks, while the degradation which they demonstrated at 7 weeks is consistent with our own results.

The process of macrophage necrosis in these lesions differed from the normal process of cell deletion called apoptosis (Kerr, Wyllie and Currie, 1972). The loss of the cell walls in the first stage of the necrotic process in the *Leishmania* lesions could simply be caused by bursting due to the heavy parasite load, though this would not explain why the lesion was not perpetuated by a fresh influx and multiplication of macrophages, as in anergic cutaneous leishmaniasis. The loss of cell walls would also be consistent with the hypothesis of Bryceson *et al.* (1970), of an immunogenic necrosis of macrophages which, it was surmised, had become coated with antigen. In our study this necrotizing process developed as the serum antibody level was approaching its peak, and it was followed by a decline. Radwanski *et al.* (1974) found that at 2 weeks a few parasitized macrophages were coated with antibody, which did not penetrate the cell to coat the parasites. By 4 weeks there was only faint diffuse staining for γ-globulin in the central area. This gives some support in the light of our results to the possibility that coating of macrophages by antibody is a preliminary to the necrotizing process, but however it comes about the process appears not to be dependent on complement (Radwanski *et al.*, 1974). According to Bryceson *et al.* (1970) it is initiated by lymphocytes, but it is enhanced by antibody (Preston and Dumonde, 1976).

It is known that macrophages can be stimulated to kill intracellular *L. enriettii* (Preston and Dumonde, 1976). The activation of macrophages in our study coincided with the replacement of plasma cells by lymphocytes in the draining lymph nodes, and to some extent in the skin lesions. It coincided also with the peak of the 24h skin-test reaction. This reaction in leishmaniasis has been universally regarded as being of the delayed hypersensitivity type, though according to the criteria of Turk, Polak and Parker (1976) the 70% decline in skin thickness between 24 and 48 h would seem to be more typical of a Jones–Mote reaction. Such reactions, though equated with cutaneous basophil hypersensitivity, do not necessarily contain many basophils (Yoshida, Nomoto and Himeno, 1979). At the time of healing at 10 weeks the reaction was rather more typical of delayed hypersensitivity but slightly diminished.

Whatever the immunological mechanisms, the results suggested that multiplication of organisms was halted and the majority extruded as a result of the necrotizing process. The extent of this necrosis in the guinea-pig is the main histological point of difference from any of the classes of human oriental sore (Ridley, 1980), and it is probably the reason why the guinea-pig lesions are all quite rapidly self-limiting. Nevertheless the lesions appeared to be brought to the point of healing not by the necrotizing process but by the activation of macrophages which followed it as the parasite load was reduced.

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