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VISCERAL SPREADING DEPLETION OF THYMUS-DEPENDENT REGIONS AND AMYLOIDOSIS IN MICE AND HAMSTERS INFECTED INTRADERMALLY WITH *LEISHMANIA* ISOLATED FROM SUDANESE CUTANEOUS LEISHMANIASIS

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Summary.—Eighteen outbred mice and 21 golden hamsters were each inoculated intradermally with 2×10^6 *Leishmania* amastigotes obtained from 1 case of Sudanese cutaneous leishmaniasis. The skin lesions, spleen, lymph nodes, liver and kidney were examined by light-, polarizing-, and electron microscope at 5, 9 and 18 weeks after inoculation. The aim of the investigations was to follow the development of the inflammatory reaction and the change of the morphology of the lymphoid organs during the infection.

In all the mice and in the majority of the hamsters visceral leishmaniasis developed which was characterized by a "noncure" type of cellular reaction, a selective T-cell depletion in the lymph nodes and the spleen, and the development of a reactive, systemic amyloidosis. These findings point to the failure of the acquired resistance against *Leishmania* to develop. In some of the hamsters the response was of the "cure" type without the development of amyloidosis.

At the site of the inoculation the lesions healed suggesting the positive role of necrosis and the elimination of the parasites through the ulcer in the healing process.

Electron microscopy showed erythrophagocytosis in the spleen of the 2 mice examined presenting an experimental evidence of the destruction of the red blood cells, which is a common feature of human kala azar.

THE ROLE of genetic control in the course of leishmaniasis in various inbred strains of mice has become known in the past decade, leading to the recognition of resistant and susceptible strains (Bradley, 1974; Preston and Dumonde, 1976; Bradley and Kirkley, 1977; Behin, Mauel and Sordat, 1979; Handman, Ceredig and Mitchell, 1979; Howard, Hale and Chan-Liew, 1980; Rezai, Farrell and Soalsby, 1980; DeTolla, Scott and Farrell, 1981). Recent experiments have also shown that after infection with the "dermatotropic" *L. tropica* or *L. mexicana* visceral leishmaniasis can develop in non-resistant strains of mice (Perez, Labrador and Torrealba, 1979; Leclerc *et al.*, 1981, 1982), which also showed a significant decrease of T-cells in the spleen (Djoko-Tamnou *et al.*, 1981). These experiments suggest that the development of the various clinical forms of leishmaniasis could be host-dependent.

In the Sudan 3 distinct varieties of human leishmaniasis occur: visceral, mucosal and cutaneous (Sati, 1958; Milosev *et al.*, 1969; Abdalla *et al.*, 1973) Earlier histomorphological investigations of 20 fatal cases of human visceral leishmaniasis revealed a selective depletion of thymus-dependent regions in the spleen and lymph nodes (Veress *et al.*, 1977). Our previous electron-microscopic and morphometric analysis of *Leishmania* amastigotes obtained from all 3 forms of human leishmaniasis from different parts

of the Sudan showed a similar number of subpellicular microtubules, suggesting that these organisms belonged to the same type of *Leishmania*, namely *L. donovani* (Veress, Abdalla and El Hassan, 1980). However, recent investigations showed that *L. major* is the common cause of the cutaneous disease, without excluding the possibility that *L. donovani* is the cause of some cases (Abdalla, 1982). The parasites obtained from human cutaneous lesions produced visceral infection in outbred mice and hamsters (Abdalla, 1982). We thought, therefore, it is worthwhile to investigate the visceral disease in these animals by observing the morphological alterations of the lymphoid system during their infection.

The results of these experiments have shown that the parasites caused visceral involvement with an inflammatory reaction which was of the "noncure" type and it was accompanied by the depletion of the thymus-dependent regions in the spleen and the lymph nodes and reactive, systemic amyloidosis in nearly all of the animals. The different morphological response of the "cure" type seen in some of the hamsters can possibly be explained by genetic differences. In spite of the visceral disease the local skin lesions healed suggesting the role of local factors (necrosis, ulceration) in the healing.

MATERIALS AND METHODS

Parasites.—*Leishmania* amastigotes were isolated from the skin lesion of a patient suffering from cutaneous leishmaniasis by homogenization of a part of the biospy specimen. This material was injected intradermally into the tip of the nose of golden hamsters. The lesions of the nose were excised and homogenized in Hanks' balanced solution.

Experimental infection.—Eighteen outbred mice weighing about 150 g were infected by intradermal injection of 2×10^6 amastigotes in 0·05 ml Hanks' balanced solution on the back and 21 hamsters weighing about 400 g were given the same dose interdermally into the tip of the nose. The animals were divided into 3 groups (6 mice and 7 hamsters in each group) and killed at 5, 9 and 18 weeks after the inoculation. Five mice and 5 hamsters served as controls having been given only 0·05 ml Hanks' solution intradermally at similar localization and killed at 18 weeks after the inoculation.

Light and electron microscopy.—The skin lesions, visible lymph nodes and part of the spleen, kidney and liver were fixed in 4% formaldehyde in phosphate buffer pH 7·4 rendered isotonic with sucrose for 24 h at 4° and then embedded in paraffin for light microscopy. The sections were stained with haematoxylin and eosin, Giemsa, methylgreen-pyronin and PAS. For polarization microscopy the sections were stained by Congo-red and covered with gum arabic with or without a preceding performiat treatment for the differentiation of primary and secondary amyloid (Romhányi, 1979).

For electron microscopy 1 cm^3 cubes of the spleen, kidney and liver from 2 mice and 2 hamsters killed at 18 weeks were fixed in a similar formaldehyde solution at 4° for 4 h. Postfixation was performed in 1% OsO_4 in Millonig's buffer solution at 4° for 1 h before embedding in Durcupan ACM (Fluka AG, Switzerland). Ultrathin sections were cut with an LKB Ultratom III and stained with uranyl acetate and lead citrate. Electron micrographs were taken on Gevaert 23D50 plates with a JEOL 100B electron microscope at the 2nd Central Laboratory for Electron Microscopy, Semmelweis Medical University, Budapest, Hungary.

RESULTS

Skin lesions

A small nodule developed within 1–2 weeks after inoculation at the site of infection both in mice and hamsters. At about 4 weeks the lesions ulcerated. Healing started around 12 weeks and was completed after 16 weeks.

Histology showed *at 5 weeks* a central large ulcer with fibrin and necrotized cells on the surface (Fig. 1). Beneath this layer there was a thick zone of large, pale, heavily-parasitized histiocytes intermingled with a few plasma cells (Fig. 1, insert). At the periphery there was a dense inflammatory infiltrate dominated by plasma cells (Fig. 2b). In the small arteries within and around the inflammation in 4 mice and 3 hamsters necrosis of the smooth muscle cells or subintimal fibrinoid change could be found (Fig. 2a). *At 9 weeks* the necrosis in the centre of

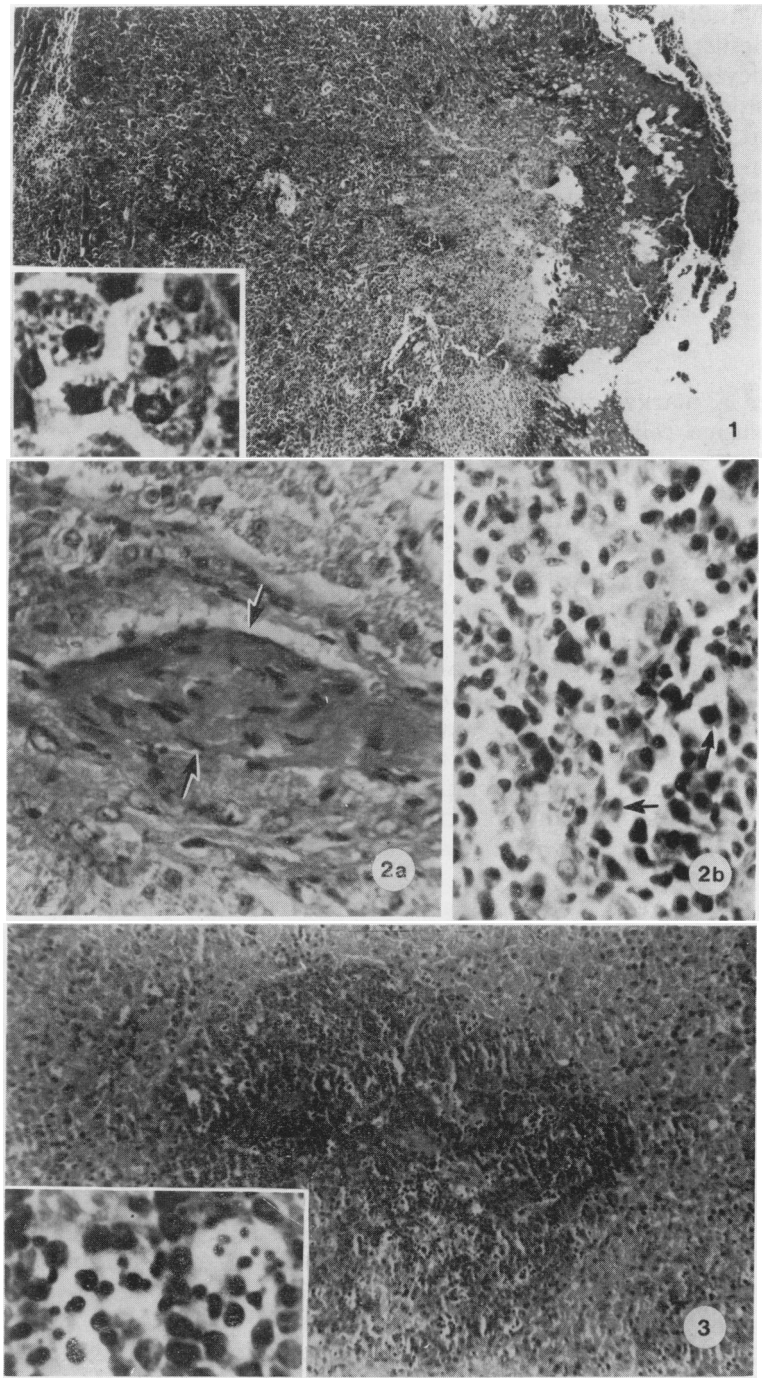

FIG. 1.—Five weeks, mouse, skin. Sharp ulcer with large superficial necrosis. Beneath the necrosis there is a thick layer of heavily parasitized histiocytes. Insert: large histiocytes containing numerous parasites. H. & E. ×30. Insert: ×420.

FIG. 2.—Five weeks, mouse, skin. A: Showing fibrinoid change in a small vessel (arrows). B: Detail of the cell-infiltrate from the periphery with plasma cells (arrows). H. & E. A: ×240. B: ×180.

FIG. 3.—Nine weeks, mouse, spleen. Enlarged Malpighian corpuscle with necrotized lymphocytes. Insert: "tingible body" macrophages containing phagocytosed cell-fragments. H. & E. ×60. Insert: ×180.

the lesion was not so marked, and in the surrounding infiltrate activated, non-parasitized histiocytes with basophilic cytoplasm, accompanied by many small lymphocytes and plasma cells, were seen. Moreover, in 6 hamsters but no mice epithelioid cell granulomata were also observed. *18 weeks* after inoculation the lesions were healed by fibrous tissue in all animals.

Lymph nodes

At 5 weeks a marked increase in the number of plasma cells in the medullary cords and the presence of pale, large histiocytes were the most characteristic changes in both mice and hamsters. *At 9 weeks* the number of small lymphocytes was also increased in the paracortical areas but they were not the dominating cell type in the lymph nodes. In 4 mice and 3 hamsters parasitized histiocytes were present in the subcapsular sinuses. *At 18 weeks* histology showed many histiocytes, some containing parasites, and a diminished number of small lymphocytes in all mice and 3 hamsters. In 4 hamsters there were no *Leishmania* parasites seen in the histiocytes but the paracortical areas were well populated with small lymphocytes. No amyloid deposits were found in the lymph nodes examined.

Spleen

Splenomegaly was detected in all mice but one, which was killed 5 weeks after inoculation, and in 3, 6 and 5 hamsters killed at 5, 9 and 18 weeks, respectively. *At 5 weeks* histology revealed enlargement of the Malpighian corpuscles caused by the accumulation of immunoblasts and plasma cells at the periphery and of small lymphocytes around the central artery. In the cords of the red pulp there were also increased numbers of plasma cells and pale, non-parasitized histiocytes. *At 9 weeks* a striking feature was the necrosis of individual lymphocytes and the presence of "tingible body" macrophages in the large Malpighian corpuscles (Fig. 3). Perifollicularly, and extending into the red pulp, a homogeneous, eosinophilic material accumulated in all the mice and in 6 of the hamsters (Fig. 4). This material showed green-birefringence after Congo-stain in polarized light, but was isotropic following performiat treatment: hence it was "secondary" amyloid. Scattered within the amyloid there were still many plasma cells and histiocytes. Numerous macrophages in all the mice and some of the 6 hamsters contained *Leishmania* parasites. Granulomata of the parasitized histiocytes were also observed in 2 mice and 3 hamsters. The histology of the spleen showed a different picture in one hamster: only scattered necrosis of the lymphocytes and a few "tingible body" macrophages were present in the enlarged Malpighian corpuscles and no amyloid was detected. *At 18 weeks* the Malpighian corpuscles were dramatically diminished in size and almost completely obliterated due to the accumulation of large masses of amyloid in all mice and in 5 hamsters (Fig. 5). No longer were "tingible body" macrophages seen in the corpuscles, which were composed of a small number of lymphocytes and plasma cells. The dominating cell-type in the amyloid of the red pulp was still the plasma cell along with some pale histiocytes, but the numbers of both were also diminished. In 2 mice and 4 hamsters granulomata composed of parasitized histiocytes were also found with plasma cells at the periphery (Fig. 6). In 2 hamsters neither parasites nor amyloid were seen, and the Malpighian corpuscles were of normal size without signs of necrosis.

Electron microscopy of the spleen revealed 8 nanometer thick filaments typical for amyloid (Fig. 4, insert). In the centre of the granulomata necrotized cells and undamaged amastigotes were observed (Fig. 7). In both mice several macrophages could also be seen containing rests of phagocytosed RBC-s or siderosomes (Fig. 8).

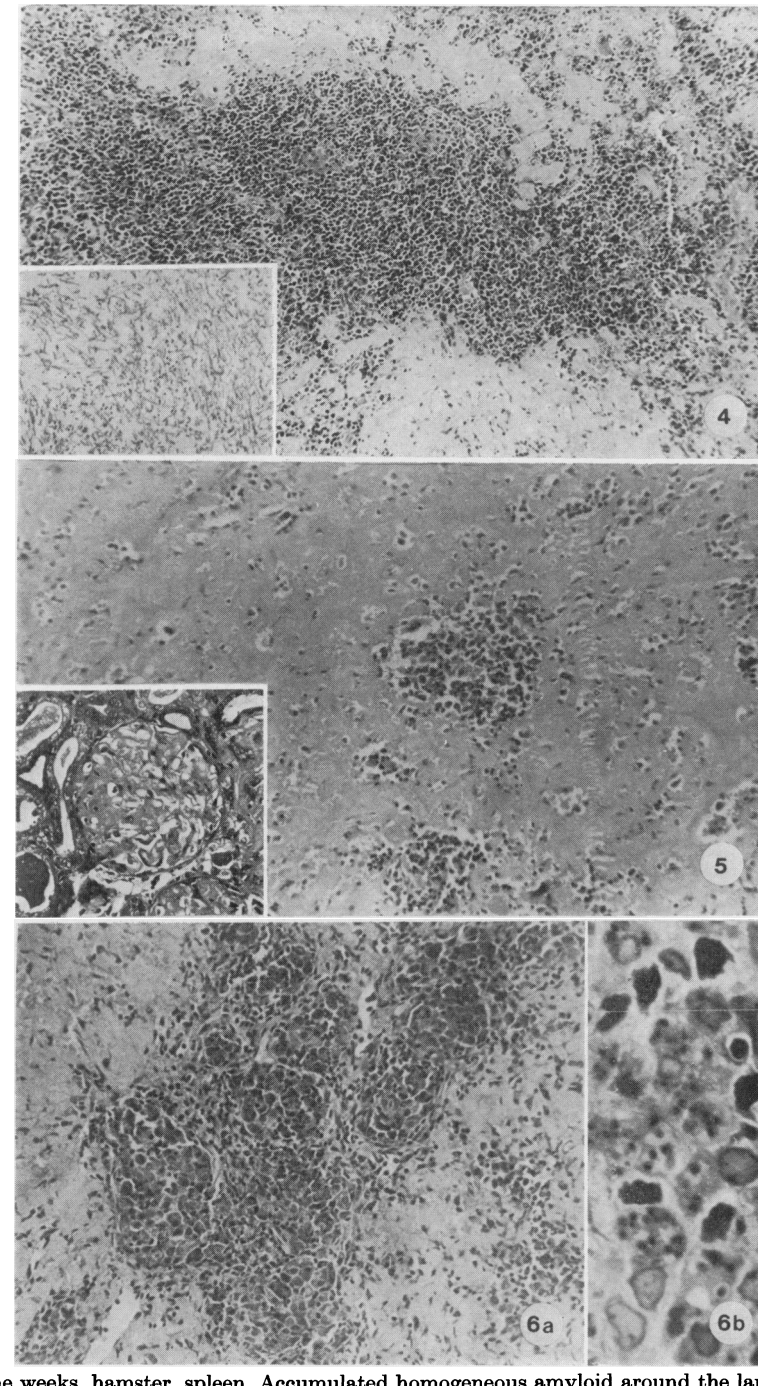

FIG. 4.—Nine weeks, hamster, spleen. Accumulated homogeneous amyloid around the large Malpighian corpuscle. Insert: amyloid filaments of 8 nm diameter. H. & E. × 60. Insert: uranyl acetate and lead citrate. × 15,000.

FIG. 5.—Eighteen weeks, mouse, spleen. Atrophic Malpighian corpuscle and large amount of amyloid with scattered plasma cells. Insert: 18 weeks, hamster, kidney. Amyloid-deposits in the glomerulus. H. & E. × 100.

FIG. 6.—Eighteen weeks, hamster, spleen. A: Showing granulomata surrounded by amyloid. The granulomata are composed of parasitized histiocytes and a few plasma cells at the periphery. H. & E. × 140. B: High power view of parasitized histiocytes from a granuloma. 1 μm section, toluidine blue. × 480.

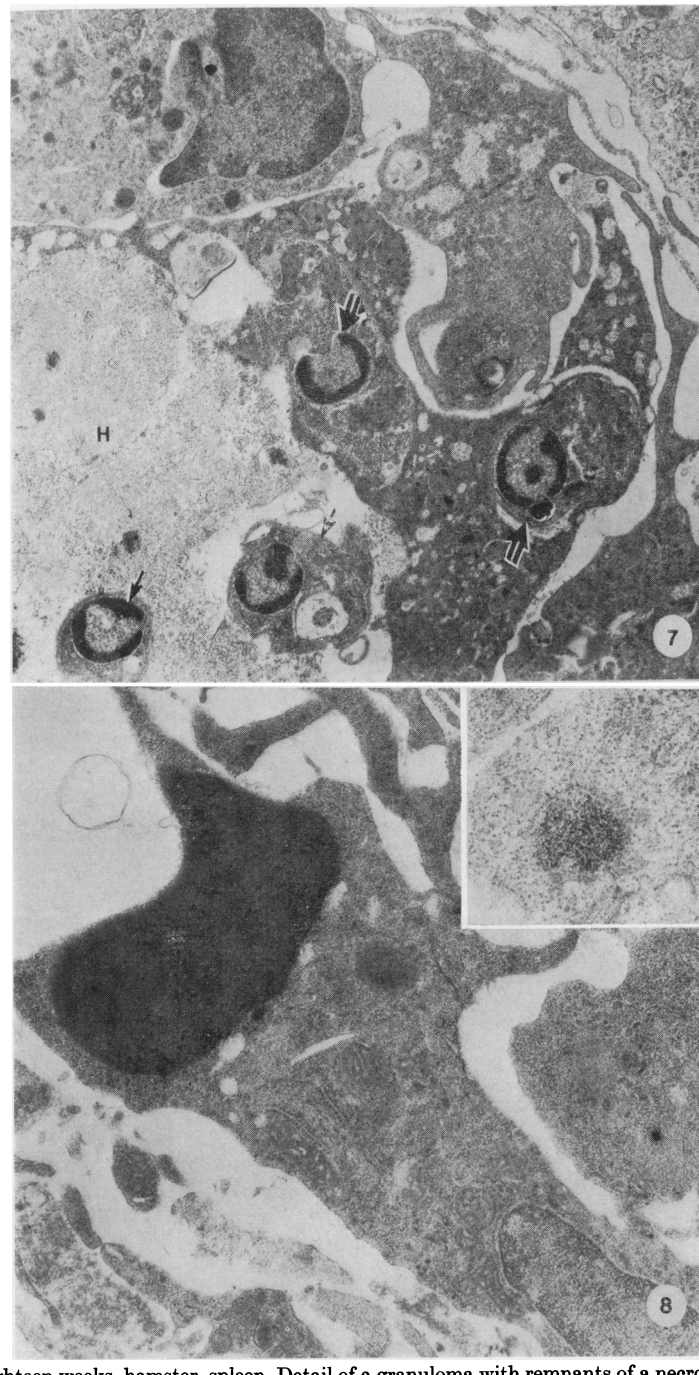

FIG. 7.—Eighteen weeks, hamster, spleen. Detail of a granuloma with remnants of a necrotized histiocyte (H) and viable, extracellular amastigotes (arrows). At the periphery there are macrophages containing amastigotes in the cytoplasm (double arrow). Uranyl acetate and lead citrate. × 9600.

FIG. 8.—Eighteen weeks, mouse, spleen. Fragment of a phagocytosed red blood cell in the cytoplasm of a macrophage. Insert: siderosome with ferritin granules. Uranyl acetate and lead citrate. × 12,000. Insert: × 32,000.

Kidney and liver

At 5 weeks there was no histological alteration seen in these organs, but at 9 and 18 weeks in all mice and in 6 and 5 hamsters, respectively, the glomeruli and some of the small arteries showed heavy deposits of amyloid (Fig. 5, insert). The liver sinuses were also obliterated in these animals by large amounts of amyloid. Furthermore, in 3 mice and 1 hamster there were granulomata found in the liver composed of parasitized histiocytes.

Control groups

Neither the skin nor the spleen, lymph nodes, kidney or liver showed any pathological changes in the control animals.

DISCUSSION

The defence against infection by *Leishmania* parasites is a complex mechanism, composed of an early, natural, genetically-regulated immunity, and a late, acquired one (Bradley, 1977). A delicate relationship should exist between the cell-types of the natural and the acquired resistances, otherwise the development of the curative cell-mediated immunity will be impaired, and the infection will become a non-healing disease. The natural resistance is under genetic influence (Howard, Hale and Liew, 1982) and the failure of this control is thought to be responsible for the development of a non-healing visceral disease in susceptible inbred mice strains after inoculation with the dermatotropic *L. tropica* (Nasseri and Modabber, 1979; Djoko-Tamnou *et al.*, 1981; Leclerc *et al.*, 1981), while successful control results in a self-healing cutaneous disease in resistant mice strains, even after *L. donovani* infection (Blackwell, Freeman and Bradley, 1980; Blackwell, 1982).

Our experiments seem to strengthen this theory by showing the failure of the acquired resistance to develop in all the mice and in the majority of hamsters. This failure led to the visceral spreading of the parasites, although they were obtained from a human cutaneous lesion. An impaired acquired immunity was demonstrated in these investigations by a "noncure" type of cellular response, a selective T-cell depletion, and the development of a reactive, systemic amyloidosis while the lack of amyloidosis, the disappearance of *Leishmania* parasites, and the normalization of splenic histology in some of the hamsters can point to the effective role of the genetic control in these animals.

The "non-cure" cellular response

Bradley and Kirkley (1977) described first the occurrence of "noncure" granulomata in outbred PO mice. These granulomata were composed of large, pale, heavily parasitized histiocytes and some plasma cells but no lymphocytes. Similar reaction was also observed in susceptible B10HTG (Blackwell *et al.*, 1980) and in BALB mice (Howard *et al.*, 1980). In our experiments the cellular response was similar with the occurrence of parasitized histiocytes, plasma cells and granulomata of the same type, as well as the scarcity of small lymphocytes and activated histiocytes in the majority of the animals. The cause of the insufficient activation of the macrophages may be either the depletion of T-cells, through the absence of lymphokines from sensitized T-cells (Howard *et al.*, 1982) or their inadequate response due to the failure of the genetic control (Blackwell, 1982).

T-cell depletion

In the present experiments an initial hyperplasia of the Malpighian corpuscles was observed. Later, however, the small lymphocytes necrotized at the centre and "tingible body" macrophages appeared. This necrosis, along with the accumulation of amyloid later on, has led to the atrophy of the Malpighian corpuscles. Parallel with this process the number of small lymphocytes has diminished in the lymph nodes. Similar depletion of T-cells occurred in cases of fatal human visceral leishmaniasis (Veress *et al.*, 1977). These findings are in agree-

ment with the observations of Djoko-Tamnou *et al.* (1981) and Leclerc *et al.* (1982) who found a significant fall in the percentage of T-cells in the spleen of *L. tropica* infected BALB/c mice. The cause of the T-cell depletion is a possible excess-load of antigen. This is known to lead to the induction of specific T-suppressor cells (Gershon and Kondo, 1971).

Amyloidosis

The third sign showing the impairment of the immune response was the development of reactive, systemic amyloidosis, a previously seldom-encountered complication of experimental leishmaniasis. The first histologically documented description of amyloid in hamsters infected with *L. donovani* was published by Gellhorn *et al.* (1946). Amyloid depositions of the spleen, kidney and liver were also observed in *L. mexicana* infected mice and hamsters (Coutinho-Abarth and Coelho, 1965). Duarte, Sesso and Brito (1978) dealt with the role of mesangial cells in amyloidosis of hamsters infected with *L. donovani* without describing the histomorphological changes in other organs. The relationship between impaired cellular immunity and amyloidosis is not as well understood as the connection between the hyperplasia of pyroninophilic cells and amyloid SAA protein-production in the first phase of amyloidosis (Glenner, 1980) It may be that an amyloidogenic factor is released from the necrotizing lymphocytes (Claesson and Hardt, 1972), or the T-cell depletion and amyloid-formation is an expression of immunologic tolerance (Cathcart, Mullarkey and Cohen, 1970). One can also assume that the T-cell depletion caused by antigen overload can lead to a "defect" of the information-exchange between T-lymphocytes and macrophages. As a consequence of this disturbance the macrophages might produce AA fibrils instead of other protein products, hence the development of amyloidosis.

A striking feature of leishmaniasis in our investigations was the discrepancy between local healing and the development of a systemic disease with impaired cellular immunity. The histomorphological alterations at the site of the parasite inoculation were similar to those described by Ridley (1979) in Old World cutaneous leishmaniasis and by Monroy *et al.* (1980) in *L. enriettii*-infected guinea-pigs. These were an initial infiltrate of pale, parasitized histiocytes, followed by necrosis of these cells and the skin, leading to the ulceration; and in the late phase the accumulation of activated histiocytes and small lymphocytes, resulting in local healing. It was Bryceson *et al.* (1970) who first suggested an immunologically induced necrosis as an important part of the local defence in experimental cutaneous leishmaniasis. Monroy *et al.* (1980) in a detailed study, showed that the majority of the parasites were extruded through the ulcer but that the healing was not a result of the activation of macrophages after the parasite load was reduced. Taking into consideration the various courses of infection locally and in the visceral organs in the present experiments we can assume that it was a local factor (namely, the ulceration) that played a role in the healing. The disturbance of the local circulation can also have been a promoting factor in the necrosis; this is supported by the local vasculitis observed in several animals.

The present investigations also showed ultrastructural evidence of erythrophagocytosis in the spleen of 2 mice. Similar observations were earlier described in cases of human kala-azar (Woodruff, 1973 Veress *et al.*, 1977). Decker-Jackson and Honigberg (1978) have presented data showing that *Leishmania* parasites have antigens cross-reacting with components of blood cells. This could be the basis of autoimmune destruction of the red blood cells leading to haemolytic anaemia, a feature of human visceral leishmaniasis (Woodruff, 1973). In the light of these data one can assume that a similar mechanism takes place in *Leishmania* infected mice.

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