DISTINCT ULTRASTRUCTURAL ASPECTS IN DIFFERENT BIOPSIES OF A SINGLE PATIENT WITH DIFFUSE CUTANEOUS LEISHMANIASIS

ACHILEA LISBOA BITTENCOURT; LUIZ ANTONIO RODRIGUES DE FREITAS*; MARGARIDA L. POMPEU**; MARIA LUCIA VIEIRA* & ALDINA BARRAL***

Departamento de Anatomia Patológica, UFBa, Hosp. Univ. Prof. Edgard Santos, Rua João das Botas, s/nº Canela, 40140 Salvador, BA, Brasil *Centro de Pesquisa Gonçalo Muniz, **Departamento de Patologia UFCe, ***Laboratório de Imunologia, Hosp. Prof. Edgard Santos, UFBa, Salvador, BA, Brasil

The authors investigated the relation between parasites and host-cells in active and regressed lesions of a patient with diffuse cutaneous leishmaniasis, evaluating the frequency of different cell types, and the location and integrity of amastigotes. No correlation was found between parasite integrity and size of parasitophorous vacuoles. They observed ultrastructural findings characterizing a cell mediated immune response: macrophages lysis, parasitic destruction inside macrophages, close contact between parasitized macrophages and lymphocytes and between parasites and lymphocytes, lymphocytic infiltration and fibrosis. They suggest that in DCL there is a limited cellular immune response, although insufficient to control infection.

Key words: diffuse cutaneous leishmaniasis -- human leishmaniasis – Leishmania – skin biopsie

Diffuse cutaneous leishmaniasis (DCL) has clinical and immunopathological aspects very different from cutaneous leishmaniasis (CL). The patients with DCL present widespread specific immunosupression of the cell-mediated-immune response and are resistent to specific treatment (Convit et al., 1972).

The literature generally states that the histological picture of DCL consists exclusively of parasite-loaded macrophages (Balzer et al., 1960; Convit & Pinardi, 1974; Medina & Romero, 1957). However, Bittencourt & Freitas (1983) demonstrated variability of the histopathological pattern showing the presence of mononuclear infiltrate, fibrosis and necrosis and differences in the intensity of parasitism among different lesions in the same patient even without treatment. Bryceson (1969) in Ethiopia observed a tuberculoid pattern in relapses of DCL after treatment. An infiltration of the epidermis by parasitized macrophages was also previously described (Bittencourt & Guimarães, 1968).

The purpose of this study is to investigate ultrastructurally the relationship of parasite and host cells in different lesions, evaluating the frequency of different cell types as well as location and integrity of amastigotes.

MATERIAL AND METHODS

The patient — A 32 year old man with disseminated large, non-ulcerated erythematous nodules, most on the face, arms, legs and abdomen which had been present for four years. Frequently there was coalescence of these nodules forming infiltrated plaques. Less frequently were observed flat lesions with a wrinkled surface. The patient never used specific therapy for leishmaniasis and stated that some lesions involuted spontaneously. There was no mucosal infiltration. Skin test and *in vitro* lymphocyte proliferation to *Leishmania* antigen were always negative. The indirect immunofluorescent anti-*Leishmania* test was positive, titer up to 1/16.000. Leishmania was isolated by culture and characterized as *Leishmania amazonensis* by monoclonal antibody reactivity pattern (serodeme), by isoenzyme profiles (zymodeme) and by KDNA restriction fragments profiles (schizodeme) (Barral, 1988). The patient was treated with pentavalent antimonium (20 mg / per kg / per day).

Biopsies — Four biopsies were done before treatment, three in large erythematous nodules and one in a lesion which is regressed, flat with a wrinkled surface. One other biopsy was done

in an erythematous lesion which had decreased after 24 days of treatment with antimonial pentavalent therapy.

Ultrastructural microscopy – The skin samples including epidermis and dermis were processed as previously reported (Barral-Netto, et al., 1987) and examined with Zeiss E-109 microscope at 50 KV5. In order to make a quantitative evaluation of the parasitized and non-parasitized inflammatory cells, a differential counting of 200 cells on average was made using one or more ultra thin sections. The parasites were also counted, taking into consideration their location and integrity. The parasitophorous vacuoles were subdivided into small (which could be filled by only one parasite), medium (by 2 to 6 parasites) and large size (filled by more than 6 parasites). The following criteria were used as indicators of degeneration of *Leishmania:* 1. Irregular forms of the parasites. 2. Dilatation of the flagelar pocket. 3. Increase in number of size of the multivesicular bodies and lipid goblets. 4. Dilatation of the endoplasmatic reticulum or mitochondria. 5. Reduction in number of ribosomes (Alexander & Vikerman, 1975; Hentzer & Kobayasi, 1977, Wery & Groodt-Lasseel, 1966). One of the following aspects was used as criteria for *Leishmania* destruction: 1. Presence of dark electron dense homogeneous parasites lacking the details of organelles. 2. Rupture of plasmatic membrane. 3. Lysis of the cytoplasm (Sandbank & Ben-David, 1979; Wery & Groodt-Lasseel, 1966). The following morphological indicators of macrophage ativation were used: the presence of microvilli and pinocytic vacuoles, cytoplasmic expansion with increased number of organells, decrease in the chromatin, increase in the number or size of lysosomes and evidence of enhanced non-parasitic phagocytosis (Adams, 1976; North, 1978).

RESULTS

A great variation in the ultrastructural pattern was observed even in sections of one single lesion.

Active lesions – A marked predominance of macrophages and a heavy macrophage parasitism was observed in all three lesions (Table I). There was a predominance of damaged parasites and the parasitism was more intense in macrophages than in the interstitium (Table II). Rarely amastigotes were seen inside neutrophils and eosinophils. The lymphocytes represented 13% of the inflammatory cells. A close contact between parasitized macrophage and lymphocytes (Fig. 1) and between lymphocytes and amastigotes (Fig. 2) was observed. Plasma cells and eosinophils were rarely observed. The size of the parasitophorous vacuoles varied from area to area but extravacuolar cytoplasmatic localization of amastigotes was not frequently observed. No correlation was found between parasitic integrity and size of vacuoles (Table III – Fig. 1). Thirty five per cent of macrophages, parasitized or not, were necrotic. In areas, focal agglomerations of necrotic cells were observed in all three biopsies. Foci of collagen deposition were seen only in one biopsy, associated with a marked predominance of dead parasites and larger areas of necrosis (Table I). Less than 1% of macrophages showed morphological signs of activation. Epithelioid cells and giant cells were rarely observed. Many well preserved parasites in the cytoplasm of keratinocytes and in macrophages between keratinocytes were seen in two biopsies (Fig. 3).

Regressed lesions – In these lesions there was a great decrease in parasitism and presence of fibrosis (Table I). The collagen deposition was more marked in the spontaneously involuted lesion (Fig. 4) in which there was a predominance of parasites in the interstitium, numerous neutrophils (Table I) and large areas of necrosis. In the regressed lesions it were also observed scarce giant and epithelioid cells and a close contact between lymphocytes and lysed and parasitized macrophage (Fig. 4).

COMMENTS

A great variation was observed in the ultrastructural aspects in both active and regressed lesions. Besides the parasite-loaded macrophages with large parasitophorous vacuoles, macrophages with small vacuoles and with few parasites, foci of necrosis, infiltration of lymphocytes and areas of collagen deposition were also found. These differences were more marked between the active and the regressed lesions. In these latter lesions there was a marked reduction in the number of macrophages and in the intensity of parasitism, and a more marked fibrosis was observed. Fibrosis is described in CL and in experimentally infected resistent inbred mice but not in the BALB/c mice

TABLE I

Frequency of inflammatory cells, tissular parasitism and fibrose in the lesions

Lesions	% of inflammatory cells					Fibrose	Ama/Cell
	Mo	Mop	Neu	L	Other cells		
Active	27	48	12	13	0	+	4:1
Spontaneously regressed	29	2	56	9	4	++++	1:1
Regressed after treatment	48	6	29	14	3	+	2:1

Mo — non parasitized macrophages; Mop — parasitized macrophages; Neu — neutrophyls; L — lymphocytes; Ama — amastigotes.

TABLE II

Parasite integrity related to its location in the lesions

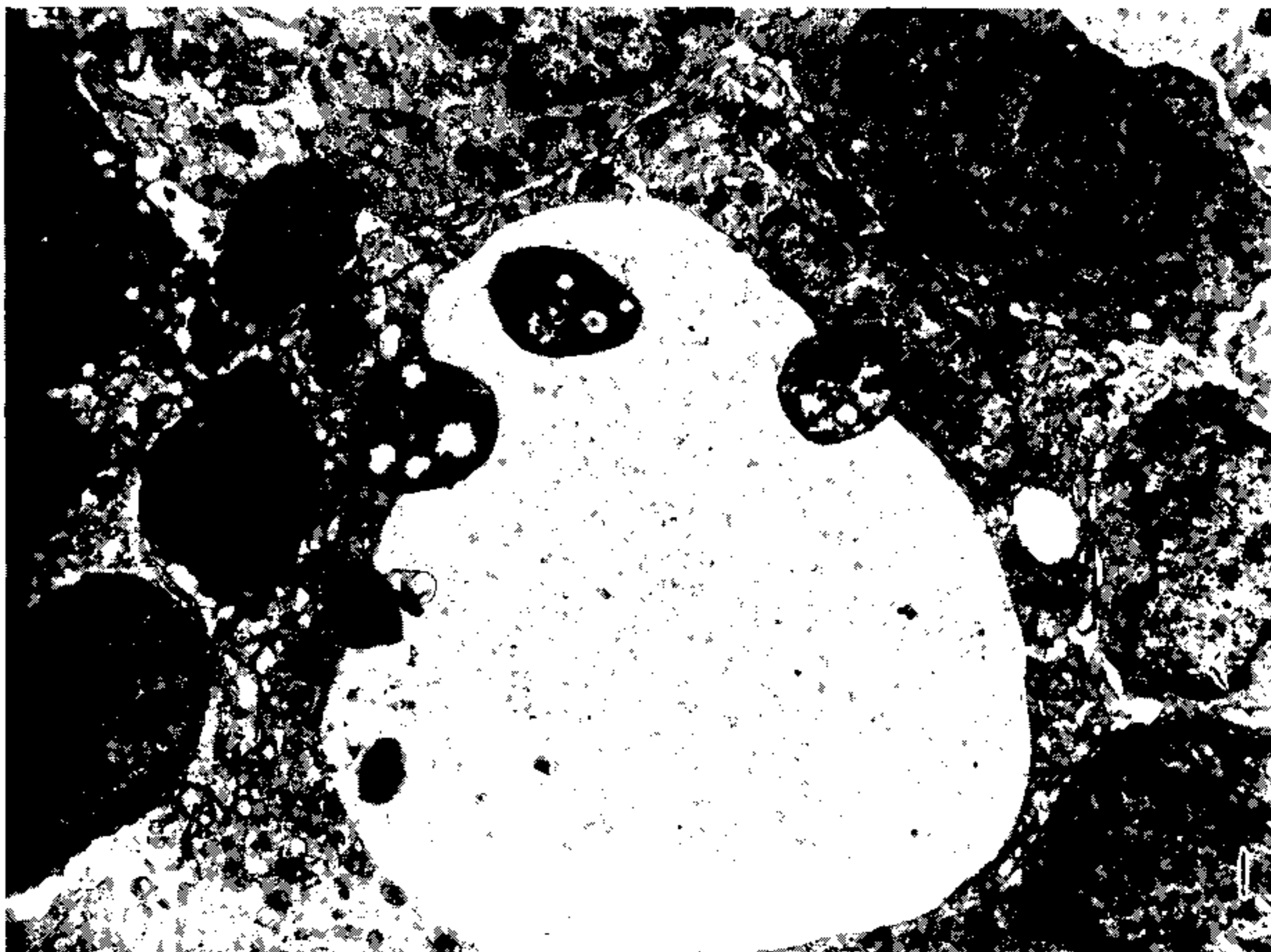| Parasite location	Lesions					
	Active		Spontaneous regressed		Regressed after treatment	
	Preserved	Damaged	Preserved	Damaged	Preserved	Damaged
Intracel.	189 (18%)	878 (82%)	0	6 (100%)	11 (34%)	21 (66%)
Extracel.	48 (11%)	406 (89%)	0	37 (100%)	5 (63%)	3 (37%)

Fig. 1: active lesion. There are lymphocytes around and in close contact with parasitized well preserved macrophage. See degenerated amastigotes within a large parasitophorous vacuole. X 3.000.
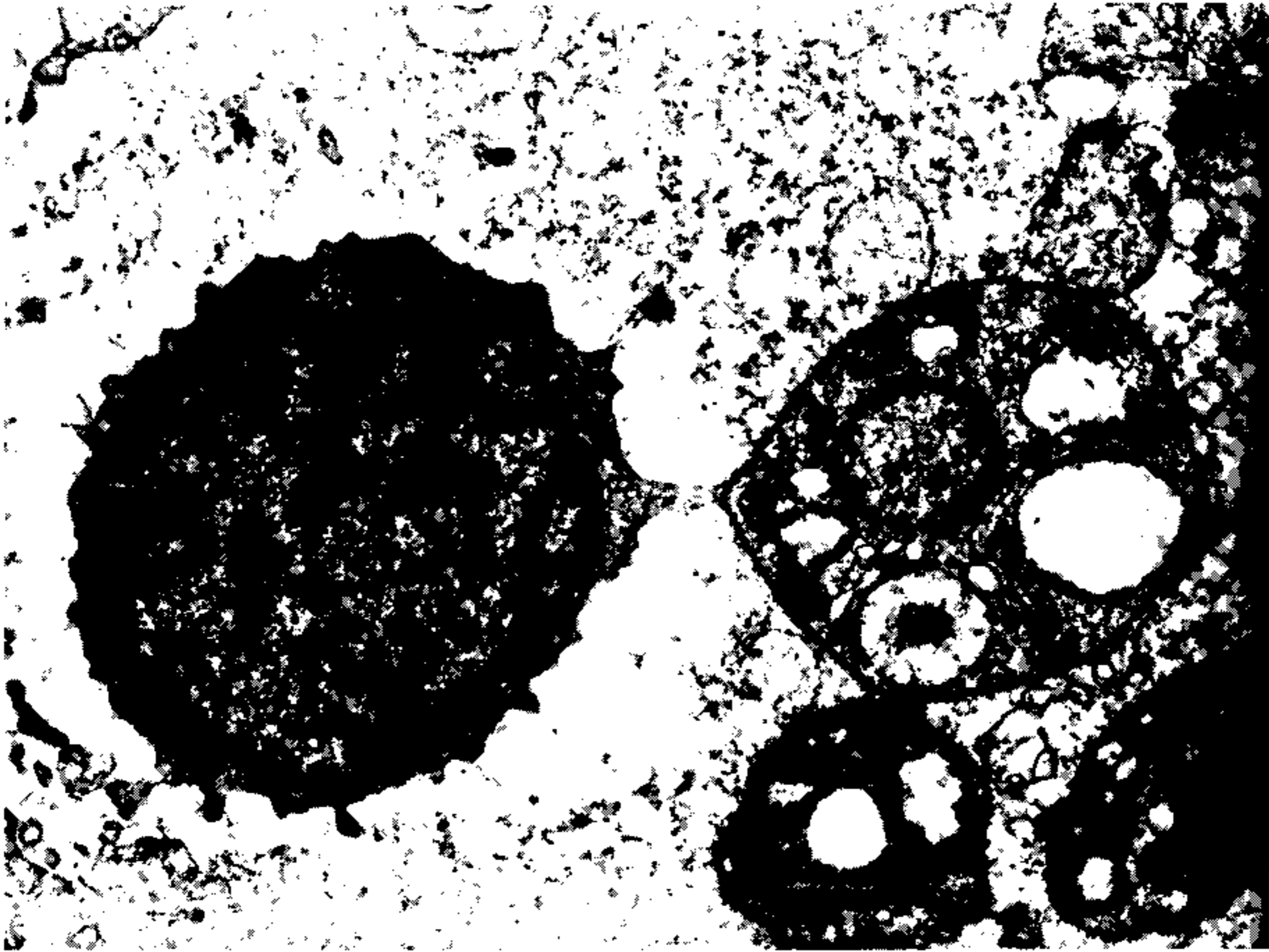
Fig. 2: active lesion. A degenerated amastigote in close contact with a lymphocyte. X 7.000.
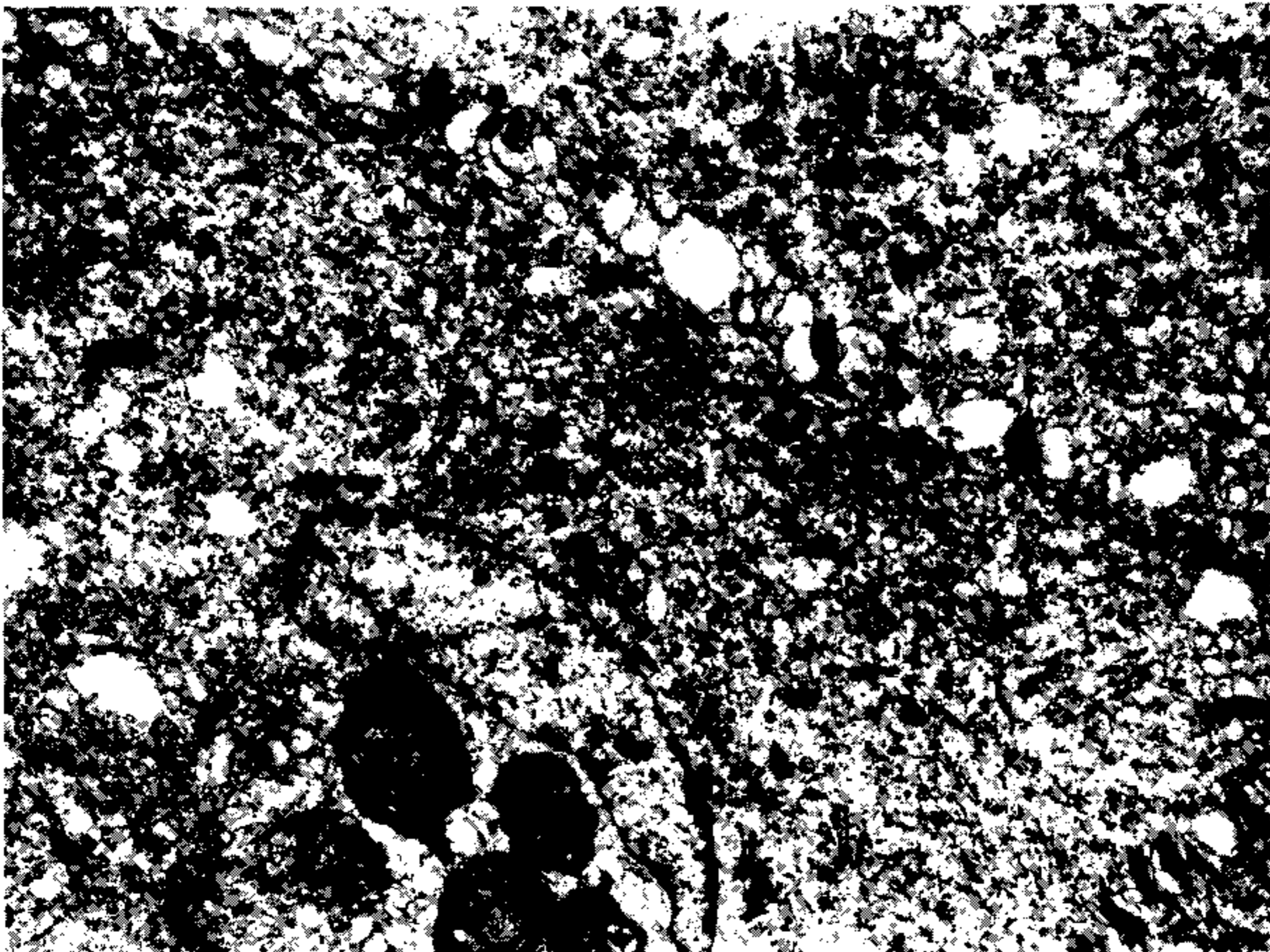

Fig. 3: epidermis of the active lesion. See well preserved amastigotes within the keratinocyte. X 3.000.
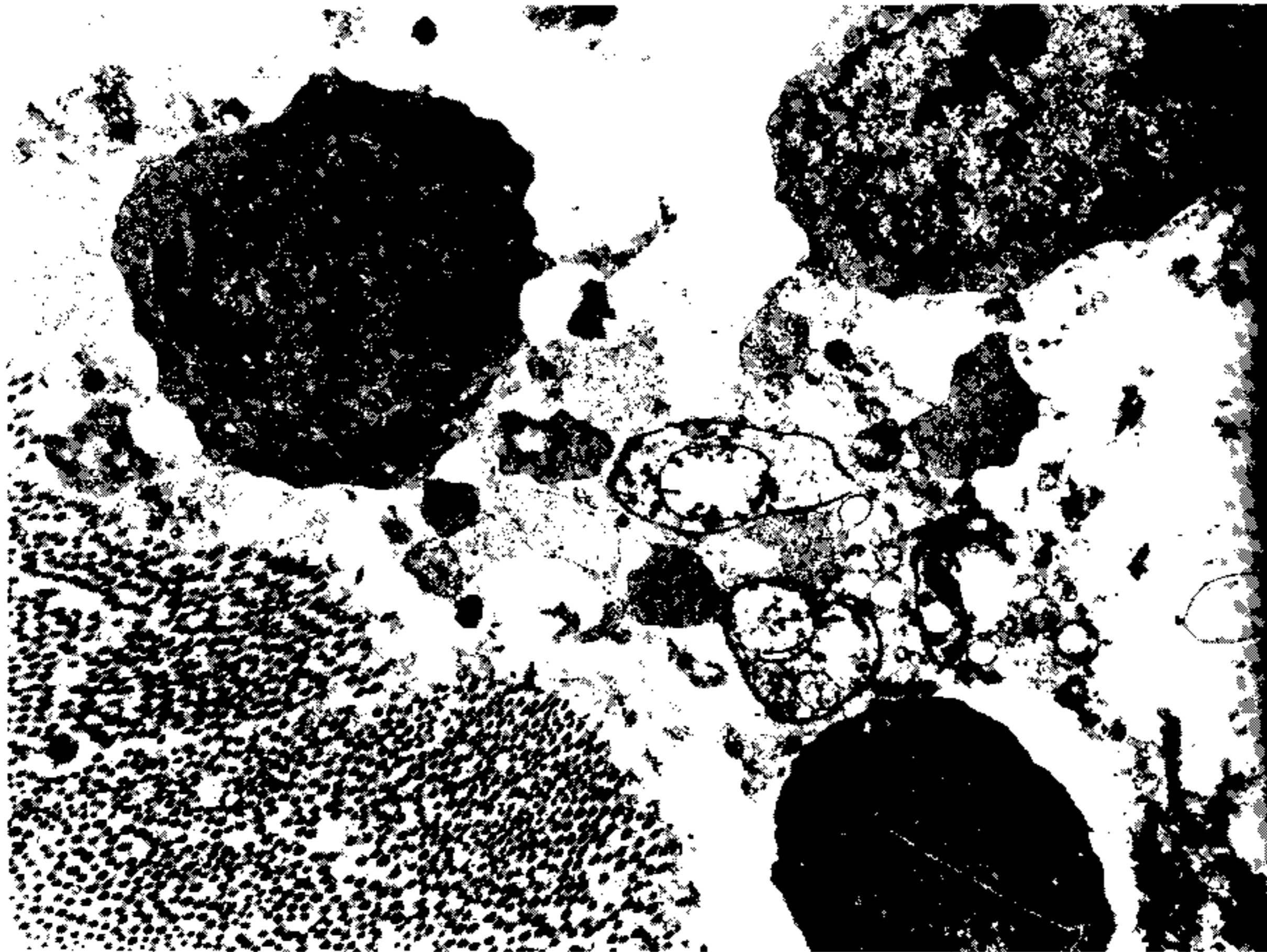
Fig. 4: spontaneously involuted lesion. A lysed macrophage with deposition of collagen. X 3.000.

(Andrade et al., 1984; Bretana et al., 1983). However it was observed in the immunized BALB/c mice (Barral-Netto et al., 1987). Fibrosis in parasitic infections is related to immune host response (Andrade et al., 1984). In the regressive lesions a marked increase of neutrophils was also observed, probably correlated with the presence of a more marked necrosis.

A marked predominance of damaged parasites was observed in the regressive lesions (Table II). In contrast to CL (Hassan et al., 1984), apoptosis was not seen but lytic parasitized macrophages were often observed. Besides, well preserved macrophages with damaged parasites were also found.

In contrast with these observations, some authors have shown ultrastructurally that amastigotes in DCL remain well preserved within the macrophages and that parasite damage occurs only after treatment (Bretaña et al., 1983; Zaar et al., 1982). This same aspect was observed experimentally in both susceptible and resistent mice but in the resistent strain parasitized macrophages were frequently necrotic and degenerated amastigotes were often found in the interstitial tissue (Andrade et al., 1984).

TABLE III

Parasite's integrity related to the size of parasitophorous vacuoles

VP size	Parasite's condition (%)		
	Well preserved	Degenerated	Necrotic
Large	21	72	07
Medium	44	41	15
Small	24	70	06

VP — Parasitophorous vacuoles.

The close contact between lymphocytes and parasitized macrophages were seen in both types of lesions. The macrophages frequently appeared lytic. Macrophage lysis is generally attributed to T cytotoxic lymphocytes. However Pham & Mauel (1987) demonstrated *in vitro* that lymphocytes with L3T4 phenotypes besides killing amastigotes can produce macrophage lysis. Otherwise the close contact between T helper lymphocytes and parasitized macrophages increase the leishmanicidal effect of those cells (Panosian et al., 1984).

According to some author, well preserved parasites were found in large parasitophorous

vacuoles while is small vacuoles the damaged parasites predominate (Alexander & Vickerman, 1975; Veress et al., 1981). In Table III we can see that no correlation was observed between the size of these vacuoles and the parasitic integrity. Although eosinophils are frequently observed in infected BALB/c and in C3H mice (Grimaldi et al., 1984; Pompeu et al., 1987) in the present study they were rarely found.

An interesting finding was the presence of well preserved amastigotes in the interior of keratinocytes in the epidermis and also in macrophage lying between these cells. This aspect was observed only when there was a heavy dermal infiltration of loaded parasitized macrophages. The presence of parasites in the epiderms was previously observed on the light microscopic level and considered an invasion of the epidermis by parasitized macrophages (Bittencourt & Guimarães, 1968). Epithelial cells are not generally considered to have phagocytic properties, but studies employing ferritin have demonstrated the capacity of phagocytosis by keratinocytes (Sagebriel, 1972).

Leishmania amazonensis was isolated in this patient and this species is implicated as the agent of diffuse leishmaniasis in Brazil (Lainson, 1981). In the susceptible BALB/c mice this species causes disease similar to DCL (Perez et al., 1978). Despite the clinical presentation and immunological aspects of this patient typical of DCL there are distinct ultrastructural findings characterizing a cell mediated immune response which include: macrophage lysis, parasitic destruction inside macrophages, close contact between parasitized macrophages and lymphocytes and between parasites and lymphocytes, lymphocytic infiltration and presence of fibrosis. Giant cells and activated macrophages were seen, although rarely. These aspects are generally seen only in CL (Hassan et al., 1984; Ridley & Wells, 1986).

The presence of active or regressive lesions in different areas of the body, as well as lesion regression in the absence of treatment, suggest that lymphocyte maldistribution is not involved in this process.

Is seems that in DCL there is a limited cellular immune response, insufficient to control the infection, but able to promote the spontaneous regression of some lesions.

REFERENCES

ADAMS, D. O., 1976. The granulomatous inflammatory response. A review. *Amer. J. Pathol., 84:* 164-191.

ALEXANDER, J. & VICKERMAN, K., 1984. Fusion of host cell secondary lysosomes with the parasitophorus vacuoles of *Leishmania mexicana* infected macrophages. *J. Protozool., 22:* 502-508.

ANDRADE, Z. A.; REED, S. G.; ROTERS, S. B. & SADIGURSKY, M., 1984. Immunopathology of experimental cutaneous leishmaniasis. *Am. J. Pathol., 114:* 137-148.

BALZER, R. J.; DESTOMBES, P.; SCHALLER, K. F. & SERIE, C., 1960. Leishmaniose cutanèe pseudolepromateuse en Ethiope. *Bull. Soc. Path. Exot., 53:* 293-298.

BARRAL, A., 1988. *Caracterização fenotípica e genotípica de cepas de* Leishmania *isoladas de diversas formas clínicas da leishmaniose humana no Estado da Bahia.* Tese, Universidade Federal da Bahia, p. 123.

BARRAL-NETTO, M; FREITAS, L. A. R. & ANDRADE, Z. A., 1987. Histopathologic changes induced by vaccination in experimental cutaneous leishmaniasis of BALB/c mice. *Am. J. Pathol., 127:* 271-278.

BITTENCOURT, A. L. & FREITAS, L. A. R., 1983. Leishmaniose tegumentar difusa. Aspectos anátomo-patológicos. *Medicina Cutânea ILA,* (Espanha), *11:* 265-270.

BITTENCOURT, A. L. & GUIMARÃES, N. A., 1968. Imunopatologia da leishmaniose tegumentar difusa. *Medicina Cutânea ILA,* (Espanha), *2:* 395-402.

BRETAÑA, A. L.; AVILA, J. L.; LIZARDO, G.; CONVIT, J. & RONDON, A. J., 1983. Leishmania species. Comparative ultrastructure of experimental nodules and diffuse human cutaneous lesions in American leishmaniasis. *Exp. Parasitol., 55:* 377-385.

BRYCESON, A. D. M., 1969. Diffuse cutaneous leishmaniasis in Ethiopia. I The clinical and histological features of the disease. *Trans. R. Soc. Trop. Med. Hyg., 63:* 708-737.

CONVIT, J. & PINARDI, M. E., 1974. Cutaneous leishmaniasis. The clinical and immunopathological spectrum in South America p. 160-169. In *Trypanosomiasis and leishmaniasis with special reference to Chagas' Disease.* Ciba Foundation Simposium. Associated Scientific Publishers, Amsterdam.

CONVIT, J.; PINARDI, M. E. & RONDON, A. J., 1972. Diffuse cutaneous leishmaniasis: a disease due to an immunological defect of the host. *Trans. R. Soc. Trop. Med. Hyg., 66:* 603-610.

GRIMALDI, G.; SOARES, M. J. & MORIEARTY, P., 1984. Tissue eosinophilia and *Leishmania m. mexicana* eosinophil interations in murine cutaneous leishmaniasis. *Parasite Immunol., 6:* 397-408.

HASSAN, A. M.; VERESS, B. & KUTTY, M. K., 1984. The ultrastructural morphology of human cutaneous leishmaniasis of low parasite load. *Acta Dermatol Venereol., 64:* 501-505.

HENTZER, B. & KOBAYASI, T., 1977. The ultrastructural changes of *Leishmania tropica* after treatment with pentamidine. *Ann. Trop. Med. Parasitol., 71:* 157-166.

LAINSON, R., 1981. Epidemiologia e ecologia da leishmaniose tegumentar na Amazônia. *Hilèia Médica,* Belém, *3:* 35-40.

Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. *85* (1), jan./mar. 1990

59

MEDINA, R. & ROMERO, J., 1957. Estudio sobre la leishmaniasis tegumentaria en Venezuela. *Dermatol. Venezol., 1:* 30-86.

NORTH, R. J., 1978. The concept of the activated macrophage. *J. Immunol., 121:* 806-816.

PANOSIAN, C. B.; SYPEK, J. P. & WYLER, D. J., 1984. Cell contact-mediated macrophage activation for anti-leishmanial defense. I Lymphocyte effector mechanism that is contact-dependent and non cytotoxic. *J. Immunol., 133:* 3358-3365.

PEREZ, H.; ARREDONDADO, B. & GONZALEZ, M., 1978. Comparative study of American cutaneous leishmaniasis and diffuse cutaneous leishmaniasis in two strains of inbred mice. *Inf. Immunity, 22:* 301-307.

PHAM, T. V. & MAUEL, J., 1987. Studies on intracellular killing of *Leishmania major* and lysis of hosts macrophages by immune lymphoid cells *in vitro. Paras. Immunol., 9:* 721-36.

POMPEU, M. L.; VIEIRA DOS SANTOS, M. L.; BARRAL-NETTO, M. & FREITAS, L. A. R., 1987. Ultrastructural analysis of cutaneous leishmaniasis in BALB/c mice immunized against *Leishmania m. amazonensis. Mem. Inst. Oswaldo Cruz, 82:* IM-72, Supp. I.

RIDLEY, M. J. & WELLS, C. W., 1986. Macrophage parasite interaction in the lesions of cutaneous leishmaniasis. An ultrastructural study. *Am. J. Pathol., 123:* 79-85.

SAGEBIEL, R. W., 1972. *In vivo* and *in vitro* uptake of ferritin by Langerhans cells of the epidermis. *J. Invest. Dermatol., 58:* 47-54.

SANDBANK, M. & BEN-DAVID, E., 1979. The role of lysosomes in the healing process of cutaneous leishmaniasis. *Int. J. Derm., 18:* 50-54.

VERESS, B.; ABDALLA, R. E. & HASSAN, A. M., 1981. Electron microscope investigation on leishmaniasis in the Sudan: II ultrastructural morphology of macrophage-parasite interaction in human and hamster macrophages *in vivo. Ann. Trop. Med. Parasitol., 75:* 607-613.

WERY, M. & GROODT-LASSEEL, M. G., 1966. Ultrastructural de *Trypanosoma cruzi* en culture sur milieu semi-synthetique. *Ann. Soc. Belge. Med. Trop., 46:* 337-348.

ZAAR, K.; WUNDERLICH, F. & BELEHU, A., 1982. Electron microscopical studies on cutaneous leishmaniasis in Ethiopia. I The diffuse form and its treatment with pentamidine. *Ann. Trop. Med. Parasitol., 76:* 595-605.