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## Electron microscopical studies on cutaneous leishmaniasis in Ethiopia

### II. Parasite and host cell differences between the localized and the diffuse form\*

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#### Summary

The ultrastructure of *Leishmania aethiopica* parasites and their host cells was investigated in lesions of 7 patients suffering from diffuse cutaneous leishmaniasis (DCL) and in lesions of 4 patients with localized cutaneous leishmaniasis (LCL). The appearance of host cells and parasites varied considerably in both disease forms. Host cell variations occurred especially in the number of cytoplasmic vesicles, the size of the parasitophorous vacuoles, and the number of amastigotes per parasitophorous vacuole. Differences concerned the occurrence of a special macrophage-type in DCL-lesions which was characterized by an electron-translucent cytoplasm and a low degree of parasitization, larger parasitophorous vacuoles with higher numbers of amastigotes per vacuole in infected macrophages from DCL-patients, and the number of electron dense granules in host cell vacuoles of DCL-patients. The parasites inducing DCL and LCL significantly differed in size and membrane structure: Amastigotes had a length of  $2.27 \pm 0.48 \mu\text{m}$  and a width of  $1.77 \pm 0.40 \mu\text{m}$  in DCL-lesions, and  $1.92 \pm 0.40 \mu\text{m}$  and  $1.48 \pm 0.32 \mu\text{m}$  in LCL-lesions. Promastigotes obtained from DCL-patients revealed  $2078 \pm 308$  integral membrane particles (IMP)/ $\mu\text{m}^2$  and  $892 \pm 246$  IMP/ $\mu\text{m}^2$  on the P- and E-fracture faces of plasma membranes, while the corresponding values of LCL-derived promastigotes amounted to  $1690 \pm 376$  IMP/ $\mu\text{m}^2$  and  $652 \pm 274$  IMP/ $\mu\text{m}^2$ , respectively.

**Key words:** cutaneous leishmaniasis; self-healing and non-selfhealing forms; *Leishmania aethiopica*; thin-sectioning and freeze-etching electron microscopy.

\* This paper is dedicated to the late Prof. Ayele Beleh, who initiated the work.

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## Introduction

Cutaneous leishmaniasis is a widespread tropical disease with a major focus in Ethiopia, where almost 0.5% of the total population is affected by this disease (Belehu, 1980). Causative agent is the trypanosomatid flagellate *Leishmania aethiopica* (Bray et al., 1973). *L. aethiopica* induces two forms of cutaneous leishmaniasis (CL) in Ethiopia: the self-healing localized cutaneous leishmaniasis (LCL) and the non-selfhealing diffuse cutaneous leishmaniasis (DCL). In the self-healing form the parasites remain confined to a single skin lesion, whereas in DCL a single localized nodule insidiously expands over a period of several years and finally involves large areas of the skin at multiple sites of the body (Bryceson, 1969). In the lesion the parasites, like other leishmanias, reside and multiply in the hosts' mononuclear phagocytes – cells which play an important role in the vertebrate immune system.

Of primary importance for healing in LCL and for non-healing in DCL are presumably the interactions of macrophages with their intracellular amastigotes. At this level, healing and non-healing could be explained by at least 3 possibilities. First, healing could be dependent upon the presence of resistant macrophages, which in contrast to susceptible macrophages, can develop mechanisms to control the growth of their intracellular parasites. Indeed, resistance or susceptibility of different mouse inbred strains towards cutaneous infections with the same *Leishmania*-isolate is genetically fixed to a non H-2 linked macrophage trait (Howard et al., 1982). Secondly, parasites could differ in their ability to survive in the highly detrimental environment provided by macrophages. In line with this possibility is the recent finding that different strains of *Leishmania* display striking differences in their sensitivity towards the leishmanicidal activity of the same macrophage population (Scott and Sher, 1986). Thirdly, some macrophages could be susceptible for only certain parasite strains. Therefore, only this combination of parasite and host cell could result in a non-healing DCL-condition.

It is conceivable that the proposed different interactions between host cells and parasites in the self-healing versus the non-selfhealing disease manifest themselves in altered ultrastructural organizations. This prompted us to investigate the structural features of parasites and host cells in lesions of LCL- and DCL-patients.

## Material and Methods

### *Patients*

The leishmaniasis patients were Ethiopian citizens who had been admitted to the 'All African Leprosy and Rehabilitation Training Centre' (ALERT). They varied in age, sex and duration of the lesions.

In the 4 LCL-patients the duration of the lesion varied from 2 to 24 months. None of these patients had received any kind of treatment before the biopsies were taken. The 7 DCL-patients were classified according to their multiple and spread out lesions, and their lack of skin sensitivity towards

leishmania. None of the DCL-patients had lesions for less than 2 years and for more than 12 years. Four of the DCL-patients were old cases who had received treatment before and now reported because of relapses.

In all cases punch biopsies were taken under local anaesthesia and split for histological and electron microscopical examinations. Parasites were routinely isolated from all lesions and confirmed to be *L. aethiopica* by isoenzyme analysis.

### *Histology*

The biopsy material was preserved in 10% formalin and paraffin embedded. Semi-thin sections were cut and stained with eosin-haematoxylin. In some biopsies semi-thin sections of epon embedded specimens were stained with eosin-haematoxylin following the procedure of Munger (1961).

### *Thin-sectioning electron microscopy*

The biopsy material was fixed with 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) at 25°C for 1 h. The specimens were rinsed with cacodylate buffer and post-fixed with 2% (w/v) OsO<sub>4</sub> for 4 h in an icebath. The specimens were then dehydrated in graded solutions of ethanol/propylene oxide and embedded in epon. Sections were cut on a Reichert-ultramicrotome, double-stained with lead citrate and uranyl acetate and viewed in a Zeiss EM 9 or in a Siemens Elmiskop Ia. At least 50 host cells and parasites were evaluated for each biopsy. The results were summed for the group of the LCL- and DCL-patients, respectively.

### *Freeze-etch electron microscopy*

Promastigotes were grown in RPMI-1640/20% fetal bovine serum (FBS, Flow-Laboratories) harvested by centrifugation at 2000×g, 10 min at 25°C and washed twice in phosphate buffered saline. Then, the promastigotes were fixed with 2% glutaraldehyde at growth temperature. After 30 min at 25°C the glutaraldehyde was washed out and the promastigotes were stepwise glycerinated up to a level of 25% during a 3-h period. Promastigotes were frozen in Freon 22 cooled by liquid nitrogen, fractured and replicated at -100°C in a Balzers freeze-etch device. The integral membrane particle (IMP)-frequency was evaluated on plane fracture faces of an area of 1 µm<sup>2</sup>. In each isolate at least 20 E- and 20 P-faces were evaluated. Mean IMP-densities were calculated for the LCL- and DCL-group.

### *Statistical analysis*

Statistical analysis was performed by using the two-tailed Student t-test for independent means. Statistical significance was judged according to standard tables (Documenta-Geigy).

## **Results**

### *Histological survey*

Light microscopical examination of most biopsies showed no injury or pathological changes in the epidermis and only in 2 LCL-patients signs of ulcerations were detected. Further, the epidermis was not infested by parasites, which were exclusively situated in the dermis. A scattered distribution of amastigotes was typical for LCL-derived specimens (Fig. 1), whereas a high density of parasites was associated with DCL-tissue (Fig. 2). The dermis revealed massive lympho-histiocytic infiltration with occasional polymorphonuclear leucocytes and plasma cells. Usually more cells of the histiocyte-macrophage lineage were found in DCL-infiltrates compared with sections from LCL-lesions (Figs. 1 and 2).



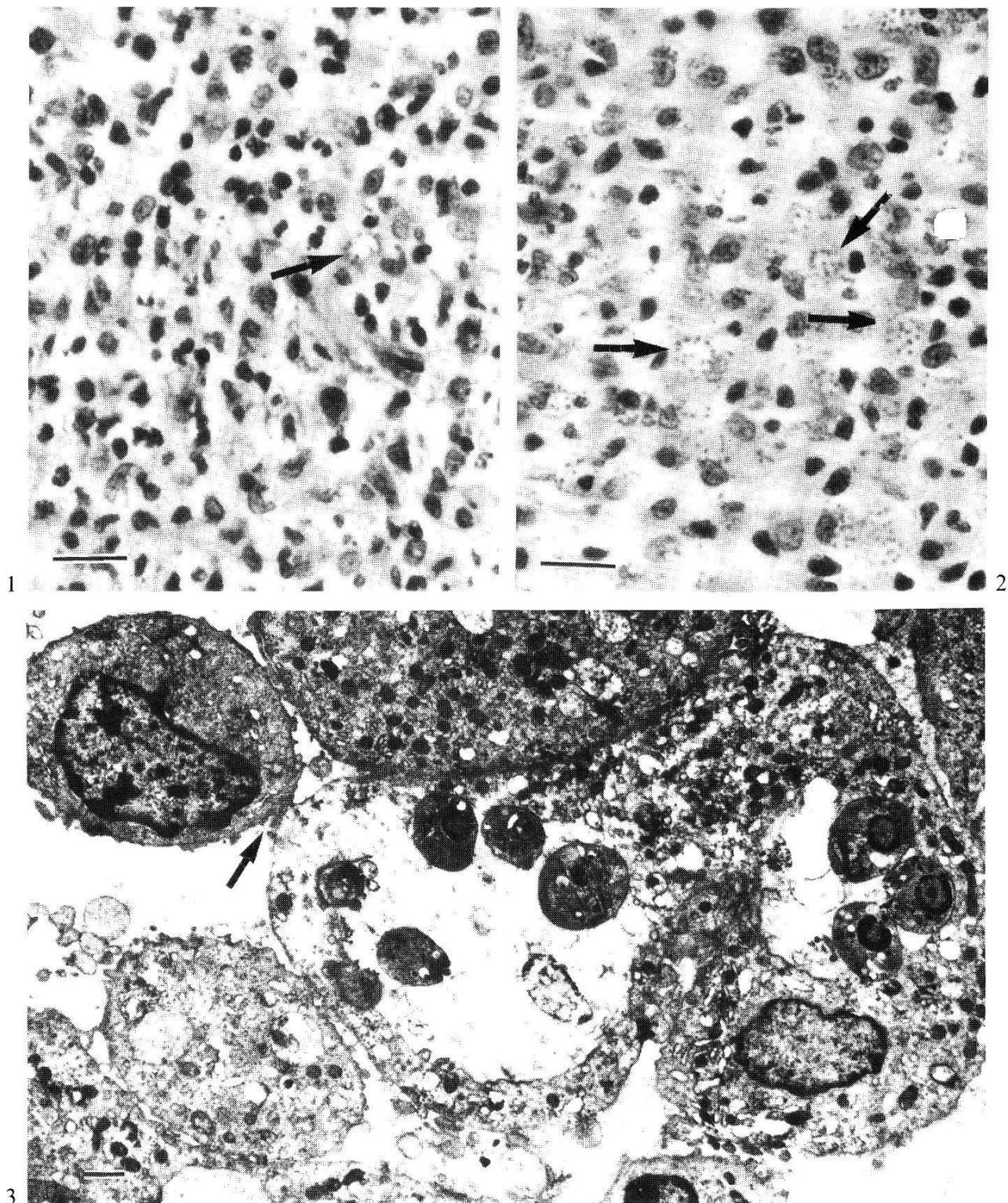


Fig. 1. Light micrograph of a semi-thin section showing the lympho-histiocytic infiltration in the dermis of a LCL-patient. The infiltration is mainly composed of lymphocytes and occasional parasitized histiocytes (arrow) are visible (bar = 30  $\mu$ m).

Fig. 2. Histological survey from a lesion of a DCL-patient. The cellular infiltrate is predominated by histiocytes with numerous parasites (arrows) in huge vacuoles (bar = 30  $\mu$ m).

Fig. 3. A lymphocyte in close contact (arrow) with a disintegrating parasitized macrophage in the lesion of a DCL-patient. The disintegration process appears to start at the contact-site. The ultrastructure of amastigotes is still intact (bar = 1  $\mu$ m).

Thin-sectioning electron microscopy (TSEM) confirmed that in LCL-cases a significant smaller amount of cells was infected with parasites as compared to DCL-patients. In addition, only a small number of lymphoid cells was observed in DCL-infiltrates, whereas numerous lymphocytes were generally found in LCL-lesions. It is remarkable that lymphocytes were frequently detected in close association with infected host cells. In DCL-patients lymphocytes were occasionally observed to be tightly associated with disintegrating parasitized host cells (Fig. 3).

#### *Ultrastructure of parasitized host cells*

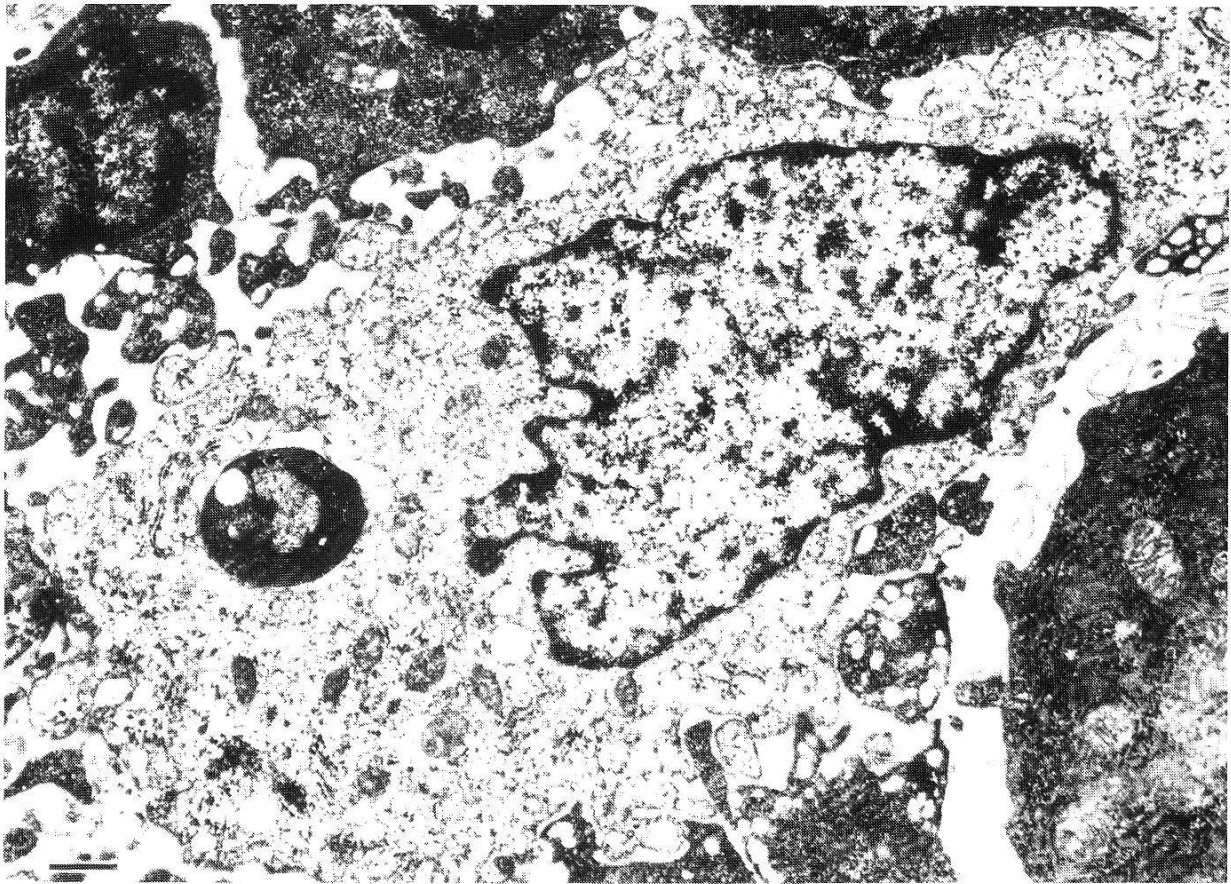
The micromorphology of the infected host cells, especially the appearance of the cytoplasm, varied considerably in DCL- and LCL-lesions, even in the same biopsy. Characteristic for most of the infected cells in both LCL- and DCL-biopsies were numerous small unilamellar vesicles in the cytoplasm. Some cells, however, contained only few vesicles embedded in an electron-translucent cytoplasmic ground substance (Fig. 4, Table 1). The most conspicuous variation occurred in the distribution and the number of amastigotes in the infected host cells. Some macrophages contained only one parasite either in a large or small parasitophorous vacuole (for definition see footnote to Table 1). A representative example for a small parasitized vacuole is shown in Fig. 3, where the parasite occupies more than half of the total vacuolar area. Often the small vacuoles did not show any free vacuolar area since the parasitophorous vacuole membrane surrounded closely the entire parasite surface. However, most of the host cells were parasitized by more than one amastigote. In these macrophages the parasites could be found either scattered over several vacuoles containing different numbers of parasites or in one huge vacuole with up to 30 amastigotes. Beside parasites, some parasitophorous vacuoles also contained electron dense granules with a mean diameter of approximately 200 nm, which were preferably located at the vacuolar periphery (Fig. 5). Incidentally, such electron dense granules could also be found in small vesicles located in the host cell cytoplasm, which were occasional in close contact with parasitophorous vacuoles.

Table 1 summarizes some semi-quantified characteristics of parasitized host cells from LCL- and DCL-patients. This evaluation suggests that the difference of parasitized host cells in LCL- and DCL-lesions is a quantitative rather than a definite feature, i.e. differently structured subpopulations of macrophages can be found in both forms of CL but to a different extent (Table 1). The only exception were the electron-translucent host cells, which were present only in DCL-patients (Table 1).

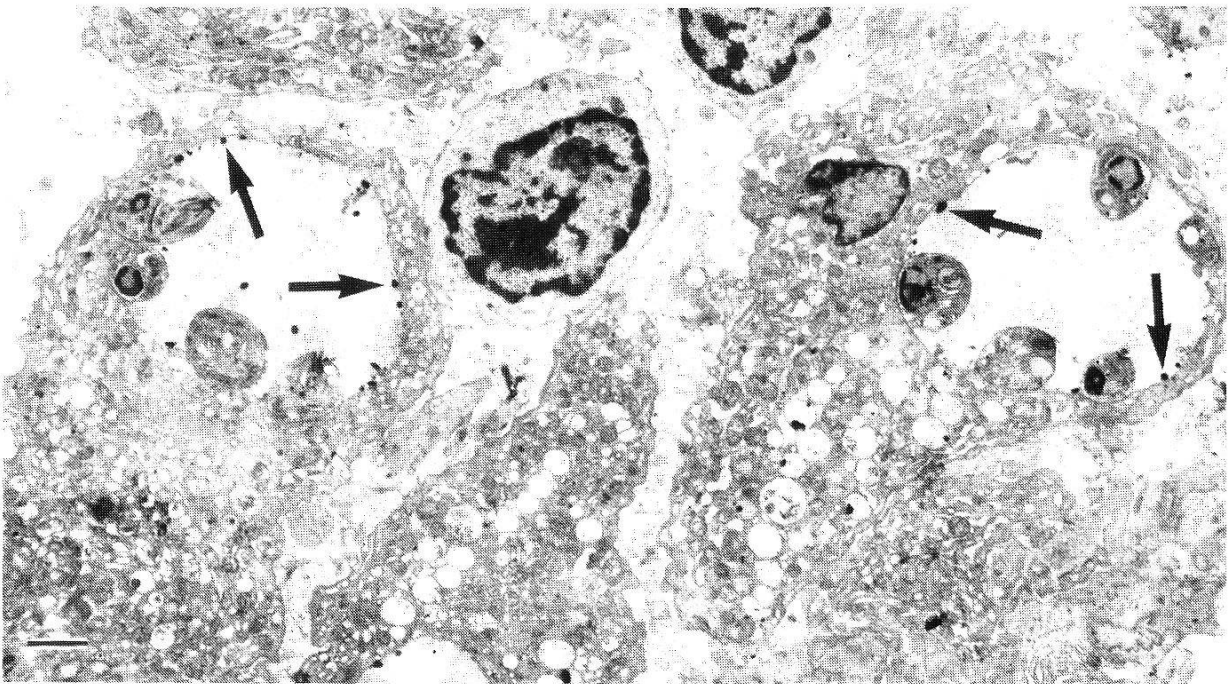
#### *Morphology of amastigotes*

Amastigotes in the parasitophorous vacuoles of host cells revealed a spherical to ovoid shape and occasional elongated forms were detected in both forms





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Fig. 4. A DCL-macrophage with electron-translucent cytoplasm. This macrophage-type contained only few vesicles and single parasites in small parasitophorous vacuoles (bar = 1  $\mu$ m).

Fig. 5. Large parasitophorous vacuoles of DCL-host cells containing amastigotes and numerous electron dense granules (arrows) (bar = 1  $\mu$ m).

Table 1. Morphological characteristics of *L. aethiopica*-infected macrophages in patients with localized and diffuse cutaneous leishmaniasis

Characteristics	Cutaneous leishmaniasis		P <sup>c</sup>
	diffuse form <sup>a</sup>	localized form <sup>b</sup>	
Low cytoplasmic stain . . . . .	occurrence in 3 out of 7 cases with a frequency of 23%	not found	n.d. <sup>d</sup>
Number of parasites per parasitophorous vacuole . . . . .	2.62 ± 0.84	1.68 ± 0.98	>0.10
Frequency of parasitophorous vacuoles [%]:			
large <sup>e</sup> . . . . .	47.5 ± 19.9	25.5 ± 17.4	>0.10
small <sup>f</sup> . . . . .	52.5 ± 19.9	74.5 ± 17.4	>0.10
Parasitophorous vacuoles with electron dense granules	occurrence in 3 out of 7 cases with a frequency of 37.0 ± 6.4%	occurrence in 1 out of 4 cases with a frequency of 17.4%	n.d.

<sup>a</sup> Means from seven patients

<sup>b</sup> Means from four patients

<sup>c</sup> P was calculated according to the two-tailed Student t-test for two independent means

<sup>d</sup> n.d.: not done

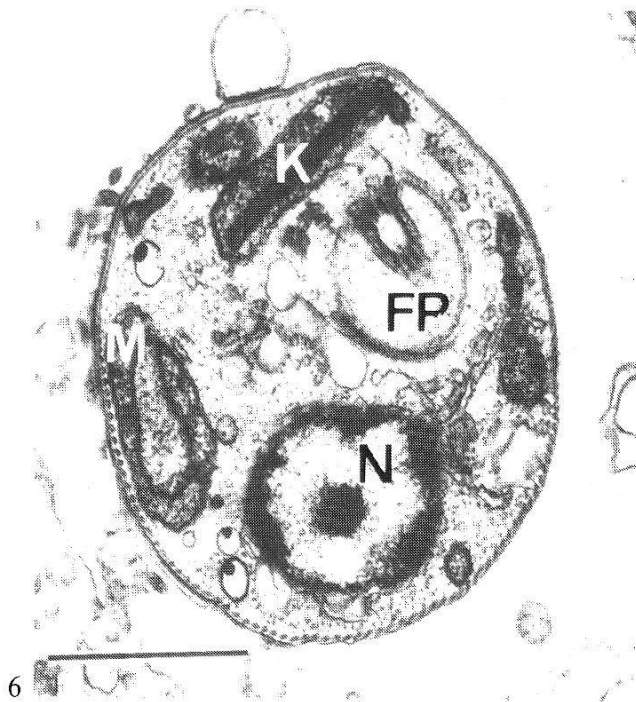
<sup>e</sup> Parasitophorous vacuoles with more than one parasite and with one parasite (if the free vacuolar area > area of the parasite)

<sup>f</sup> Parasitophorous vacuoles with one parasite (if the area of the parasite exceeds the free vacuolar space)

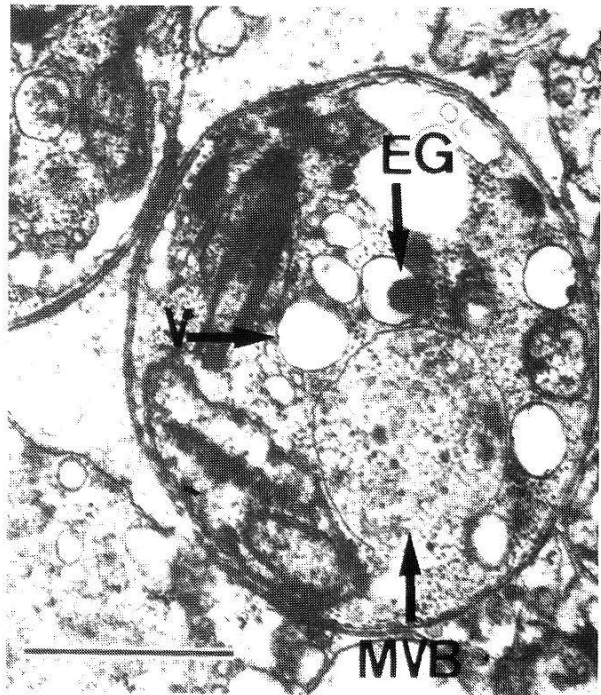
of the disease. The DCL-parasites were larger than the LCL-parasites (Table 2). The ultrastructure of amastigotes varied considerably. Figs. 6–9 show representative parasites selected from DCL- and LCL-patients. The parasites had a spherical nucleus with nuclear envelope adjacent heterochromatin. The kinetoplast with K-DNA was situated near the flagellar pocket, which accommodated a rudimentary flagellum. The cell periphery revealed the typical cytoskeleton consisting of plasma membrane-associated microtubules. In addition, vesicles, “lipid” droplets, electron dense granules surrounded by a membrane and a large multivesicular body were embedded in the parasites’ cytoplasm (Figs. 6–9). Quantitative evaluation of these subcellular parameters did not show a difference between LCL- and DCL-parasites (Table 2).

### *Membrane characteristics of promastigotes*

The difference in size could reflect either an inherent feature of the parasites or a host cell-induced effect on parasites. In order to obtain a first indication about possible parasite-inherent differences, we investigated LCL- and DCL-parasites in the promastigote culture-form by freeze-etch electron micro-



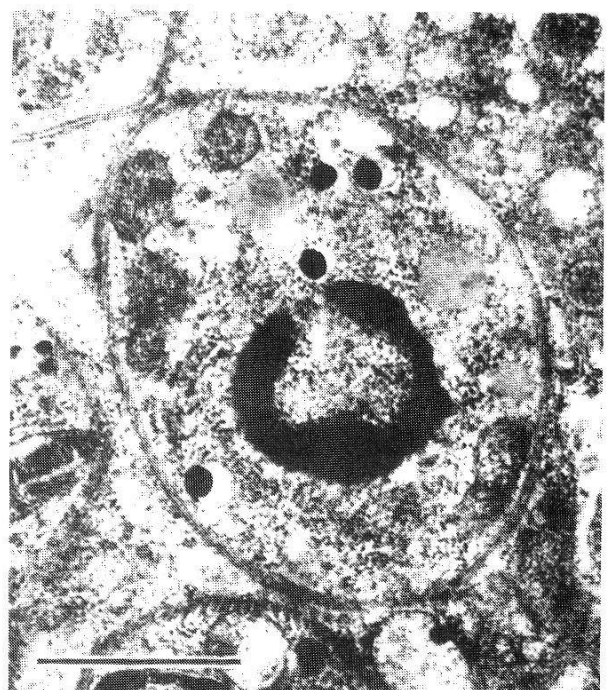
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Fig. 6. Amastigote of *L. aethiopica* in the parasitophorous vacuole of a macrophage in the lesion of a DCL-patient. N = nucleus, M = mitochondria, K = kinetoplast, FP = flagellar pocket (bar = 1  $\mu$ m).

Fig. 7. *L. aethiopica* amastigote from a DCL-patient. MVB = multivesicular body, V = cytoplasmic vesicles, EG = vesicle with an electron dense granule (bar = 1  $\mu$ m).

Fig. 8. *L. aethiopica* parasite with numerous lipid droplets in a LCL-macrophage (bar = 1  $\mu$ m).

Fig. 9. *L. aethiopica* amastigote in a macrophage in the lesion of a LCL-patient with membrane-enclosed electron dense granules and lipid droplets in the cytoplasm (bar = 1  $\mu$ m).



Table 2. Morphological characteristics of *L. aethiopica* amastigotes from patients with localized and diffuse cutaneous leishmaniasis

Characteristics	Cutaneous leishmaniasis		P <sup>c</sup>
	diffuse form <sup>a</sup>	localized form <sup>b</sup>	
Length [ $\mu\text{m}$ ] .....	$2.27 \pm 0.48$	$1.92 \pm 0.40$	$>0.001$
Width [ $\mu\text{m}$ ] .....	$1.77 \pm 0.40$	$1.48 \pm 0.32$	$>0.001$
Frequency of amastigotes with lipid droplets [%] ..	$61.7 \pm 33.7$	$42.4 \pm 13.7$	$>0.10$
Number of lipid droplets per amastigote .....	$1.4 \pm 0.99$	$2.2 \pm 0.14$	$>0.10$
Frequency of amastigotes with electron dense granules [%] .....	$50.5 \pm 16.5$	$64.0 \pm 15.9$	$>0.10$
Number of electron dense granules per amastigote	$1.58 \pm 0.24$	$1.66 \pm 0.19$	$>0.10$
Frequency of amastigotes with multivesicular body [%] .....	$28.9 \pm 7.5$	$27.3 \pm 9.5$	$>0.10$

<sup>a</sup> Means from seven patients

<sup>b</sup> Means from four patients

<sup>c</sup> P was calculated by the two-tailed t-test for two independent means

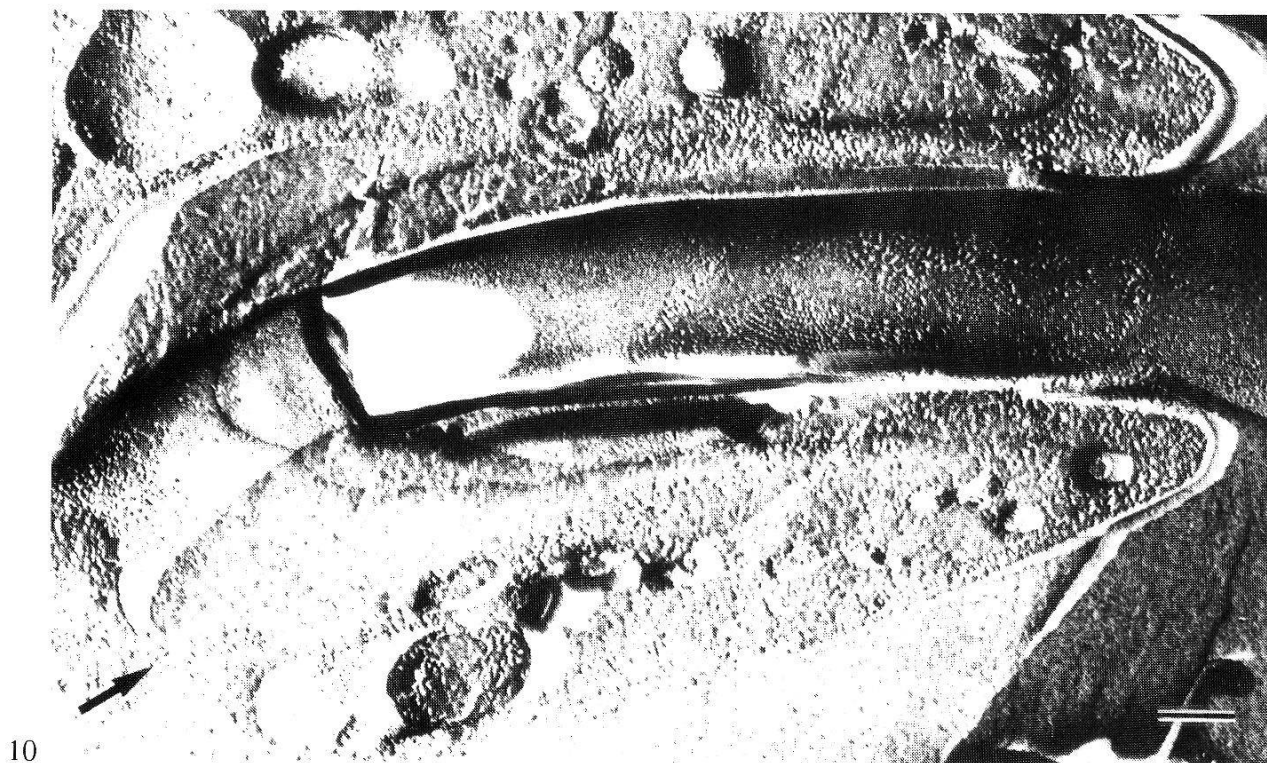
scopy. Promastigotes revealed a special arrangement of integral membrane particles (IMP) at the base of the flagellum the so-called “flagellar necklace” (Wunderlich and Speth, 1972). Moreover, quasi-crystalline arrays of IMP on P-faces and small pits on corresponding E-faces (Fig. 10) could be regularly observed on flagellar membranes within the flagellar pocket, especially at those sites, where the flagellar membrane was in close contact with the plasma membrane.

Figs. 11 and 12 show typical P- and E-fracture faces of plasma membranes from LCL-promastigotes. The P-face is occupied with numerous uniformly distributed IMP, whereas the E-face exhibits a significant lower IMP-frequency. A highly significant difference between LCL- and DCL-promastigotes could be detected in the particle density of the parasites' plasma membrane E- and P-faces (Table 3).

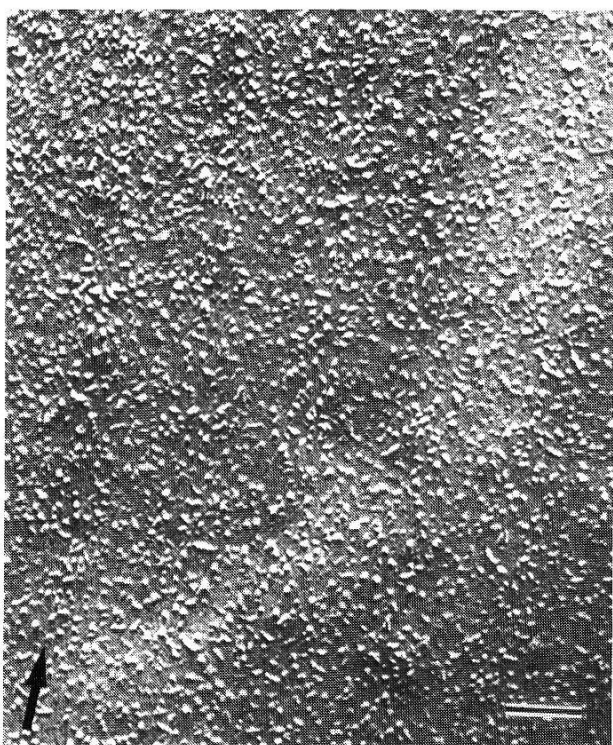
## Discussion

The present study has compared structural features of *L. aethiopica* amastigotes and corresponding host cells directly in lesions of patients suffering from the self-healing localized and the non-selfhealing diffuse cutaneous leishmaniasis in Ethiopia. Our data show that less parasites are present in LCL-lesions compared to DCL-lesions. Furthermore, LCL-parasites are usually more scattered in smaller vacuoles than DCL-parasites, which are frequently found densely packed in large vacuoles. Obviously, parasite multiplication is under





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Fig. 10. Flagellar pocket of a promastigote isolated from a LCL-patient as monitored by freeze etch electron microscopy. At the base of the flagellum a "flagellar necklace" is visible and on the flagellum membrane plaques of IMP are conspicuous (bar = 0.1  $\mu$ m; arrow indicates direction of shadowing).

Fig. 11. Freeze-etch electron micrograph of a P-face from a LCL-promastigotes. Arrow indicates shadowing direction (bar = 0.1  $\mu$ m).

Fig. 12. E-face of a LCL-promastigote. The IMP-frequency is significantly lower than that on P-faces. Arrow indicates shadowing direction (bar = 0.1  $\mu$ m).

Table 3. Frequency of integral-membrane-particles (IMP) in plasma membranes of *Leishmania aethiopica* promastigotes isolated from localized cutaneous leishmaniasis (LCL) and diffuse cutaneous leishmaniasis patients (DCL)

Plasma membrane	Cutaneous leishmaniasis		P <sup>c</sup>
	diffuse form <sup>a</sup>	localized form <sup>b</sup>	
P-face .....	2078 ± 308 IMP/μm <sup>2</sup>	1690 ± 376 IMP/μm <sup>2</sup>	>0.001
E-face .....	892 ± 246 IMP/μm <sup>2</sup>	652 ± 274 IMP/μm <sup>2</sup>	>0.02

<sup>a</sup> Means from seven isolates

<sup>b</sup> Means from four isolates

<sup>c</sup> P was calculated with the two-tailed Student t-test

more stringent control in LCL- than in DCL-patients. This is in accordance with previous results on the Ethiopian cutaneous leishmaniasis and similar findings on South American cutaneous leishmaniasis (Bryceson, 1969; Convit et al., 1972).

The parasitized host cells varied considerably in their ultrastructural appearance in both LCL- and DCL-lesions. The major variation concerned the range of size and parasite number of the parasitophorous vacuoles and the number of cytoplasmic vesicles. This could reflect different activation stages of macrophages. Activation is probably accompanied by more intense membrane flow (Cohn, 1978) entailing increased vesiculation of the macrophage cytoplasm. In bacterial granulomatous infections, for example, distinct stages of macrophage maturation have been defined at the ultrastructural level with an increasingly developed endomembrane system towards the activated pole (Adams, 1974, 1976).

Despite the high variation, distinct differences could be revealed between host cells in DCL- and those in LCL-lesions, though they are not present in all biopsies of one group. One difference concerned the electron dense granules in the vacuoles of parasitized host cells. This type of granules is found at the same frequency among DCL- and LCL-biopsies, though, they are much more abundant in DCL-patients. The function and significance of these electron dense granules is unknown. Concerning their genesis 2 speculations are conceivable: (i) Since electron dense granules occur in both parasitophorous vacuoles and non-parasitized smaller vesicles, they could be derived from the macrophage, (ii) the macrophage granules could be derived from parasites since electron dense granules were also found within amastigotes. Indeed, it is known that amastigotes secrete so-called "excretory factors" (El-On et al., 1979) via membrane flow into the parasitophorous vacuole (Hernandez, 1983). Another difference occurred in the staining of the host cell cytoplasm. In accordance with

our previous studies (Zaar et al., 1982) we observed a unique macrophage-type with electron translucent cytoplasm and poorly developed endomembranes in 3 out of 7 DCL-biopsies. This macrophage-type was usually parasitized by singly scattered parasites in small parasitophorous vacuoles with a tightly apposed parasitophorous vacuole membrane. This situation is indicative of a non-activated macrophage with limited phagocytic capacity. A third difference was the close contact of lymphocyte in 2 DCL-biopsies with disintegrating host cells. These macrophage associated lymphocytes could possibly represent cytotoxic T-cells which recognize, attack and destroy parasitized host cells. In this way amastigotes could become liberated which subsequently could infect other parts of the skin via the blood stream and thus contribute to the dissemination of the DCL-infection.

Amastigotes also displayed a variable ultrastructure in DCL- and LCL-lesions but no difference was detected between the 2 disease forms. Nevertheless, DCL-parasites are larger than LCL-parasites. The size of amastigotes was already reported by Gardener et al. (1977) to be a species specific parameter. For example, *Leishmania major* and *Leishmania donovani* have long diameters of 3.22  $\mu\text{m}$  and 2.08  $\mu\text{m}$ , respectively. Thus, both LCL- and DCL-parasites of *L. aethiopica* are clearly different from *L. major*. Moreover, a difference in the IMP-frequency between LCL- and DCL-parasites could be revealed in the plasma membranes by freeze etch electron microscopy. It is interesting to note that the IMP-frequency in both types of *L. aethiopica* promastigotes was similar to those of *Leishmania mexicana* exhibiting 2040 IMP/ $\mu\text{m}^2$  and 890 IMP/ $\mu\text{m}^2$ , respectively (Benchimol and DeSouza, 1980).

The difference in size and plasma membrane structure between DCL- and LCL-parasites suggested that the non-healing course of DCL could depend not only on host factors but also on separate strains of *L. aethiopica*. This is in line with recent findings showing different immunogenicity of LCL- and DCL-parasites as revealed by their lymphokine generating capacity (Akuffo, 1986).

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