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Cell populations in the lesion of human cutaneous leishmaniasis: a light microscopical, immunohistochemical and ultrastructural study

P. Esterre, J.P. Dedet, C. Frenay, M. Chevallier, and J.A. Grimaud

Laboratoire de Parasitologie, Institut Pasteur de Guyane, Cayenne, French Guiana and Unité de Physiopathologie des Fibroses Tissulaires, CNRS URA 1459, Institut Pasteur de Lyon, France

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Summary. To characterize the in situ cellular immune response in localized cutaneous leishmaniasis (LCL), the authors studied frozen skin biopsies from 50 patients with LCL due to *Leishmania braziliensis guyanensis*. A panel of 31 monoclonal antibodies was used, which defined the number and distribution of inflammatory cell subsets. Skin inflammatory infiltrates were composed of T cells (with a local CD4/CD8 ratio of 1.05 ± 0.7 vs 1.48 ± 0.3 in peripheral blood), macrophages and a smaller number of B cells, natural killer cells and granulocytes. Most of the T cells expressed activation markers (interleukin-2 and transferrin receptors, HLA-DR$^+$) and an increase in T-cell-receptor $\gamma\delta$ expression was noted. Analysis of the CD4$^+$ subpopulations with newly available reagents showed that helper T cells (CD4$^+$CD45RO$^+$) exceeded the suppressor/inducer subset (CD4$^+$CD45RA$^+$) by 1.4:1. There were no differences between local immune variables from patients with primary infection (45 patients) and those with recurrence (5). In 7 patients, biopsies were analysed before and 1 month after specific treatment, and did not show significant differences except for a small increase of dermal CD1a$^+$ (Langerhans) cells/mm^2. The observed pattern of cellular skin infiltration suggests an immune-mediated tissue injury including T-cell-mediated cytotoxicity and delayed hypersensitivity reactions in addition to direct parasitic action.

Key words: Human cutaneous leishmaniasis – Skin – Immunohistochemistry – Electron microscopy

Introduction

Leishmania species are intracellular parasites pathogenic for mammals, including man. Their interactions with the host immune system are complex. In cutaneous leishmaniasis, depending upon the immune response and perhaps the parasite species, the disease manifests itself in different clinical and histopathological forms: localized cutaneous leishmaniasis (LCL), a muco-cutaneous form (MCL) and, more rarely, diffuse cutaneous leishmaniasis (DCL). In French Guiana only LCL is found; it is due to *L. braziliensis guyanensis* in about 97% of human cases (Dedet 1990; Desjeux and Dedet 1989) and accompanied by lymphangitis in 18% of cases (Dedet 1990; Dedet and Desjeux 1989). Cellular immune mechanisms, involving interactions between T lymphocytes and macrophages, are considered to play a major role in the local and general immunoregulation of cutaneous leishmaniasis. The different forms of the disease can be explained by the extent and the nature of the immune response, which appears to be specific and well developed in LCL, inadequate in MCL and absent in DCL (Castes et al. 1983). Therefore, the LCL form represents a suitable system for studying local control of the disease.

Immunohistochemical staining techniques, using monoclonal antibodies (mAb) against surface antigens of inflammatory cells, permit investigation of the in situ cellular immune response in skin lesions and lymphatic nodules in the LCL form. Particular attention has been paid to the influence of the treatment in this early phase of the disease.

Materials and methods

Fifty patients with leishmaniasis confirmed by the laboratories of the Pasteur Institute of Cayenne, French Guiana [positive lesion smears (Fig. 1) and/or cultures in enriched RPMI medium] were studied. In 16 cases, the disease was related to *L. braziliensis guyanensis*, by isoenzyme electrophoresis of culture isolates. The group of patients was homogeneous and consistent with the clinical and epidemiological characteristics of LCL encountered in French Guiana, with a recent infection (mean duration of illness: 2.2 ± 2.7 months) affecting young adults (mean age: 29.1 ± 9.3 years) with a variable number of lesions (mean number: 3.9 ± 3.4). Forty-five

Correspondence to: P. Esterre, Physiopathologie des Fibroses Tissulaires, CNRS URA 1459, Institut Pasteur de Lyon, Avenue Tony Garnier, F-69365 Lyon Cedex 07, France

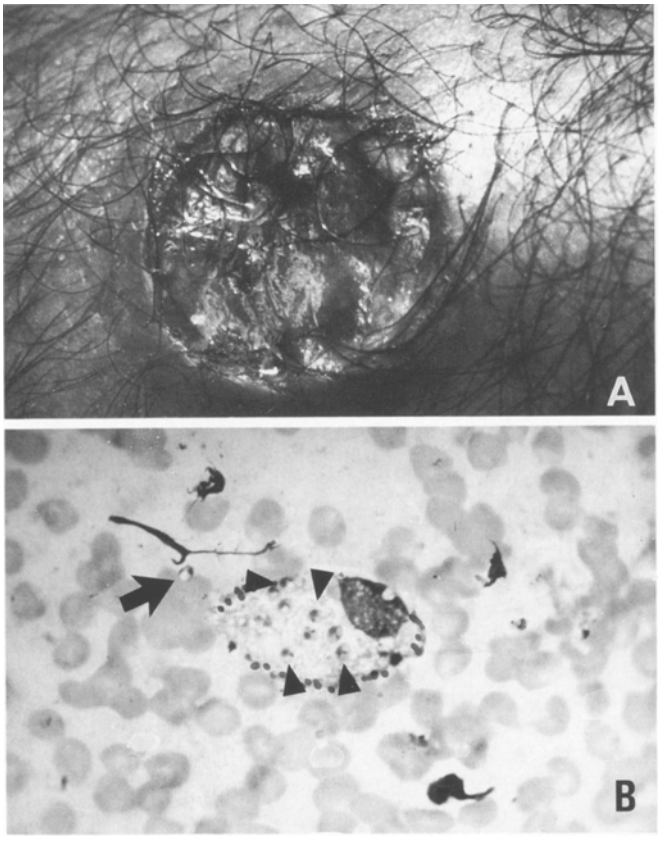

Fig. 1. A classic ulcerative wet lesion, with inflamed and raised edges (**A**). The cutaneous smears (**B**) showed intra-macrophagic (*arrowheads*) or, more rarely, extra-cellular amastigotes (*arrow*). Giemsa, ×1000

cases were primary infections; the 5 other cases presented with recurrences. None of the patients had mucocutaneous involvement. A subcutaneous lymphangitic extension was frequently observed in the draining territory of the cutaneous lesion (Dedet 1990; Dedet and Desjeux 1989).

In 7 patients of the same group consent was given for a second skin biopsy 1 month after the end of treatment with pentamidine (administered every 2 days by intramuscular injections, total dose of about 760 mg). In 3 patients, a lymphatic nodule of the arm was sampled in addition to the skin biopsy. The control group was composed of four biopsies from individuals undergoing general surgery. Cutaneous biopsies were performed at the peripheral part of the lesion, within the inflammatory active outline, using a 3-mm punch biopsy (Stiefel, FRG) and after local anaesthesia with lidocaine (Roger Bellon, France). The lymphatic nodules were excised in total. Each biopsy was embedded in Tissue Tek OCT medium (Miles, Naplesville, Ill., USA) immediately after punch excision, and immersed in liquid nitrogen. Frozen serial sections were cut in a Leitz cryostat and processed as described below.

A panel of 31 mAbs, described in Table 1 with their specificity and origin, was used for immunoperoxidase staining. All were used at a concentration predetermined by checkerboard titration and vials were conserved at −20° C before immunostaining. Whenever possible, a second mAb of a similar specificity but from different commercial or experimental sources was used in a cross-checking strategy. Biotinylated reagents (anti-mouse IgG or IgM, horseradish peroxidase, alkaline phosphatase) were purchased from Vector (Burlingame, Calif., USA). The chromogens, diaminobenzidine tetrahydrochloride or 3-amino-9-ethylcarbazole, naphtol AS-MX phosphate and Mayer's haematoxylin were obtained from Sigma (St. Louis, Mo., USA).

Cryostat sections (5 μm) were air-dried before fixing in cold acetone and staining. We used a standardized technique (Hsu et al. 1981) with an avidin-biotin-peroxidase complex (ABC), using commercial kits (Vector; Immunotech, Luminy, France). In some cases we used a double staining procedure (Chen et al. 1987), the second primary mAb being revealed with an alkaline phosphate substrate (ABC-AP kit, Vector) or, more easily interpreted, using an alkaline phosphatase-antialkaline phosphatase complex (APAAP kits, Immunotech and Dakopatts, Santa Barbara, Calif., USA). Negative controls without the primary antibody were included in each run, and blocking of avidin binding (Wood and Warnke 1982) and endogenous peroxidase (Kelly et al. 1987) were performed prior to deposition of the first mAb. In addition, for each patient a section was stained with haematoxylin and eosin to evaluate the extent of the infiltrate, and the number of amastigotes per section was evaluated on skin smears stained with Giemsa.

The number of positive cells within the infiltrate was evaluated blind, 10 microscopic fields (magnification ×250) being counted by two independent observers for each commercial mAb. To normalize the results, the number of positive cells was calculated per square millimetre of inflammmatory infiltrate (Ridel et al. 1988; Sobel et al. 1984) and the results expressed as a mean percentage ± standard error. We preferred to use this more precise means of data analysis instead of the semi-quantitative method of scoring recommended by others (Ridley and Ridley 1984). It must be noted that the granuloma in LCL is too poorly delineated to calculate its total area precisely. To assess the uniformity of cell subset distribution within the lesion, we examined several sections of the same biopsy. For Langerhans and related cells, the density was calculated using an ocular grid of known surface area both in the dermis and in the adjacent epidermis, on 14 microscopic fields at ×25 magnification. To compare the data collected on the same patient during two periods of time, we used a Wilcoxon test included in a statistical software package (Statist, Medical Data Processing Lab., Medical Faculty, Nancy, France), considering there to be a significant difference if the P value was <0.05. The histopathological analysis of each biopsy was performed with haematoxylin and eosin stains, and cellular density indexed as described by Ridley and Ridley (1983). The parasite index was evaluated by two observers in most of the patients, according to the logarithmic scale proposed by the same authors (Ridley and Ridley 1983).

Analysis of the circulating mononuclear cell subsets was performed on 6 patients, by indirect immunofluorescence using standard procedures (Nakane and Kawaoi 1974) after Ficoll-Hypaque gradient isolation, sampled before and after treatment. This phenotypic analysis was performed with a limited panel (CD2, CD3, CD4, CD8, CD14 and CD37) of mAbs and compared with that of 4 healthy controls using Student's t-test.

The ultrastructural study was carried out on a second group (20 patients) whose clinical, histological and laboratory results, particularly the parasite species (*L. braziliensis guyanensis*) involved, were identical to the first. Modifications of the extracellular matrix of the skin lesion have been described in detail elsewhere (Esterre et al. 1991). We compare here images obtained by electron microscopy on cellular infiltrates with light microscopic data.

Results

Except in a few cases, the observations on smears indicated a consistent presence of parasites (Fig. 1 A), with a mean density of 4.3 ± 5.4 amastigotes per field at ×100 magnification even though they were not readily seen by histopathological staining. This may have been due to a deleterious effect of histopathological fixatives and to the limits of conventional staining, as proved by the superiority of the immunoperoxidase technique using species-specific mAbs. Unfortunately, the lymphatic

Table 1. Characteristics and specificity of the monoclonal antibodies used in this study

Antibody	Cluster of differentiation[a]	Isotype	Principal reactivity and distribution	Source
IOT11	CD2	IgG_1	>95% E-rosette$^+$ cells	Imm
O275	CD2	IgG_1		AB
IOT3	CD3	IgG_{2a}	95% mature peripheral	Imm
Anti Leu4	CD3	IgG_1	T cells; thymocytes	BD
IOT4	CD4	IgG_{2a}	Mature peripheral hel-	Imm
O516	CD4	IgG_1	per T cells; 75% thymocytes	AB
K203	CD29	IgG_{2a}	Broad; CD4$^+$ T cells subset	AB
UCHL1	CD45RO	IgG_{2a}	Granulocytes; monocytes; CD4$^+$ T-cell subset	Dk
ALB11	CD45RA	IgG_1	Granulocytes; monocytes	Imm
Anti Leu18	CD45RA	IgG_1	CD4$^+$ T cells subset	BD
IOT8	CD8	IgG_1	Mature peripheral supp.	Imm
OX8	CD8	IgG_{2a}	/cytotoxic T cells	AB
IOT9	CD71	IgG_1	Transferrin receptor	Imm
IOT14	CD25	IgG_{2a}	IL-2 receptor	Imm
Anti TCR		IgG_1	$\alpha\beta$ T-cell antigen receptor	BD
Anti TiγA		IgG_{2a}	$\gamma\delta$ T-cell antigen receptor	TH
IOT2a		IgG_{2b}	Class II major histo-	Imm
G157		IgM	compatibility molecu- les (HLA-DR)	AB
Anti Leu10		IgG_1	(HLA-DQ).	BD
IOM2	CD14	IgG_1	Monocytes (>90%);	Imm
Anti LeuM3	CD14	IgG_{2b}	macrophages	BD
IOB1	CD37	IgG_1	B lymphocytes	Imm
IOB1a	CD21	IgG_1	Non-circulating B	Imm
Anti Leu7	CD57	IgM	Resting NK cells; some CD8$^+$ T cells and tissues.	BD
Anti Leu11b	CD16	IgM	Resting NK; some granulocytes	BD
Anti Leu 19	CD56	IgG_1	Resting and activated NK	BD
NKH1a	CD56	IgM	Non MHC-restricted cyto- toxic CD8$^+$ T lymphocytes	TH
ION3	CDw65	IgG_1	Granulocytes	Imm
IOT6a	CD1a	IgG_1	Epidermal Langerhans and	Imm
L404	CD1a	IgG_1	thymocytes	AB
IOT6b	CD1b	IgG_{2a}	Dermal dendritic cells	Imm

[a] CD clusters established at the 4th Workshop of Human Leucocyte Differentiation Antigens, Vienna, Austria, 1989
AB, Gift from Alain Bernard, Institute Gustave Roussy, Villejuif, France;
TH, gift from Thierry Hercend, Institut Gustave Roussy, Villejuif, France;
BD, Becton-Dickinson, Mountain View, Calif., USA;
Dk, Dako Corp., Santa Barbara, Calif., USA;
Imm, Immunotech, Marseille-Luminy, France

nodules sections were of too poor a quality for detailed histological and cytological observations, perhaps in part because of the prominent necrosis in this tissue.

In skin sections routinely stained with haematoxylin and eosin, infiltrates were composed of lymphocytes and macrophages, and arranged diffusely without showing a well-organized granuloma (Fig. 2). Plasma cells, in contrast to giant Langerhans cells and neutrophils, were common. This predominantly mononuclear infiltrate extended from the upper to the lower dermis, sometimes surrounding a central zone of necrosis. The epidermis overlying the dermal infiltrate showed moderate ulceration with hyperplasia. No significant difference between the biopsies taken before and after treatment was noted.

The immunophenotypic analysis of cell subsets is given in Table 2. In spite of wide variations from one patient to another, as shown by some large standard error values, these data showed several striking features. All the samples showed a rich T-cell component, scattered among macrophages and other cells. Most of these T lymphocytes presented an activated phenotype, expressing interleukin-2 (IL-2; CD25$^+$, Fig. 4) or transferrin (CD71$^+$) receptors, or major histocompatibility complex (MHC) class II molecules on their surface. Helper/inducer (CD4$^+$) and suppressor/cytotoxic (CD8$^+$) lymphocytes were equally represented, with a local CD4/CD8 ratio of 1.05 ± 0.7. This ratio, calculated on a sample of 30 biopsies processed by the two mAbs was in

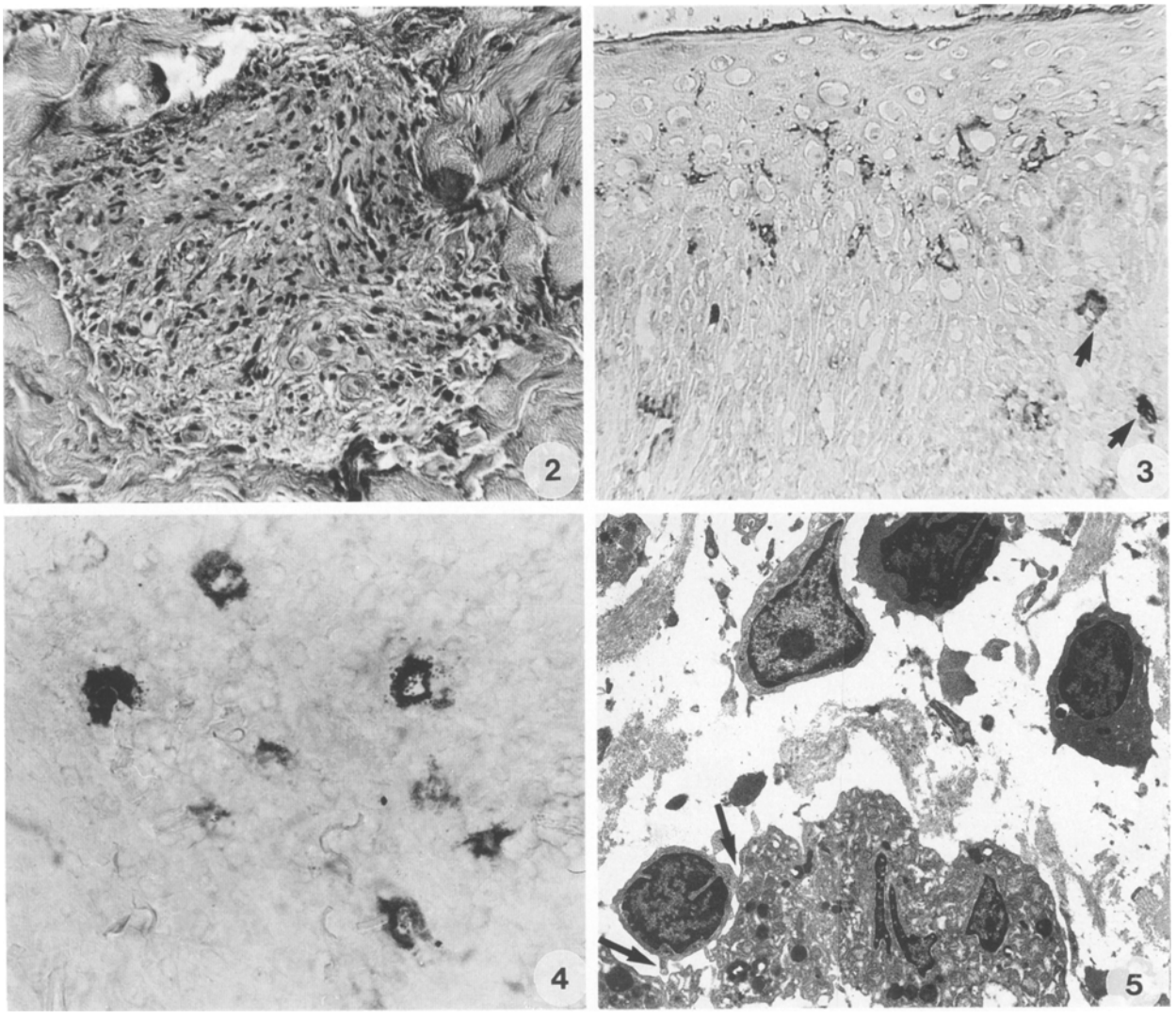

Fig. 2. An inflammatory infiltrate, mainly composed of lymphocytes, plasmocytes and histiocytes, surrounded by a dense connective matrix. Lower dermis; H&E, ×250

Fig. 3. CD1a$^+$ cells observed in the epidermis (Langerhans cells, with a typical dendritic pattern) and in the upper dermis (*arrows*) at the periphery of an inflammatory reaction. Immunohistochemistry, ×250

Fig. 4. Activated T lymphocytes bearing the interleukin-2 receptor (CD25$^+$ cells) within a granuloma. Immunohistochemistry, ×400

Fig. 5. Ultrastructural observation of the inflammatory cells within an acute granulomatous reaction. Many lymphocytes are easily identified, sometimes in close cell-cell contacts (*arrows*) with activated macrophages. Electron microscopy, ×6300

contrast to the ratio observed in the peripheral blood of 6 patients at the same disease stage (1.48 ± 0.3). The surface markers studied on circulating mononuclear cells were not significantly different between patients and healthy controls (data not shown).

Analysis of CD4 subpopulations in situ indicated a significant predominance of helper ($CD4^+CD29^+$)/memory ($CD4^+CD54RO^+$) phenotypes, outnumbering inflammatory/naive ($CD4^+CD45RA^+$) cells in a ratio of 1.4:1. In contrast to normal control skin biopsies, where the majority of T cells bear the TCR $\alpha\beta$ complex, we found that 21.7% of the T ($CD2^+$) cells in the lesions (on a sample of 10 biopsies) showed the TCR$\gamma\delta$ complex. On the other hand, there was no evidence of non-MHC restricted cytotoxic ($CD3^+$ $CD56^+$) lymphocytes in our samples.

Macrophage-related antigens (Leu M3 and IOM2) were frequently observed at both membrane and cytoplasmic levels and the corresponding cells represented a main population in the lesions. With double-labelling techniques (IOM2 plus *Leishmania*-specific mAbs) used on a sample of 20 frozen biopsies, we observed a mean percentage of infected macrophages of 6.14 ± 6.7 with a mean number of parasites per cell rising to 0.95 ± 1.0. These results must be compared with the number of extracellular amastigotes, 0.78 ± 2.2, calculated at ×100 magnification on the same sample. Some macrophages, scattered within the infiltrate, were positive for IL-1 β and tumour necrosis factor-α (TNF-α) (data not shown) but, unexpectedly, endogenous interferon-γ (IFN-γ) remained undetectable with the same immunoenzymatic techniques.

Table 2. Skin infiltrates in localized cutaneous leishmaniasis

Antibodies	CD	Percentage of positive cells[a]	Number counted
IOT11	CD2	47.8±36.2	48
IOT3	CD3	44.3±20.0	20
IOT4	CD4	27.3±20.2	40
IOT8	CD8	22.6±11.0	40
IOT14	(IL-2r)	34.5±26.6	43
Leu M3	CD14	37.3±23.9	38
IOM2	CD14	34.7±14.6	20
IOB1	CD37	8.6± 9.6	30
Leu 7	CD57	30.9±16.9	30
Leu 11b	CD16	18.1±10.0	30
Leu 19	CD56	6.7± 3.9	30
NKH1a	CD56	7.5± 6.0	20
IOT3/IOT2a	CD3/(DR+)	15.2± 5.0	10
IOT11/IOT9	CD2/(transfer+)	34.7±22.7	23
IOT3/Leu 19	CD3/(CTL subset)	0.0	20
IOT11/TCR	CD2/($\alpha\beta$+)	8.5± 6.9	10
IOT11/TiγA	CD2/($\gamma\delta$+)	4.1± 2.2	10
IOT4/K203	CD4/CD29	14.3± 5.6	10
IOT4/UCHL1	CD4/CD45RO	16.4± 5.9	10
IOT4/Leu 18	CD4/CD45RA	11.9± 8.9	10
IOT4/ALB11	CD4/CD45RA	12.9±10.6	10
ION3	(Granulocytes)	2.2± 2.7	15

[a] Results expressed as mean percentage ± standard error

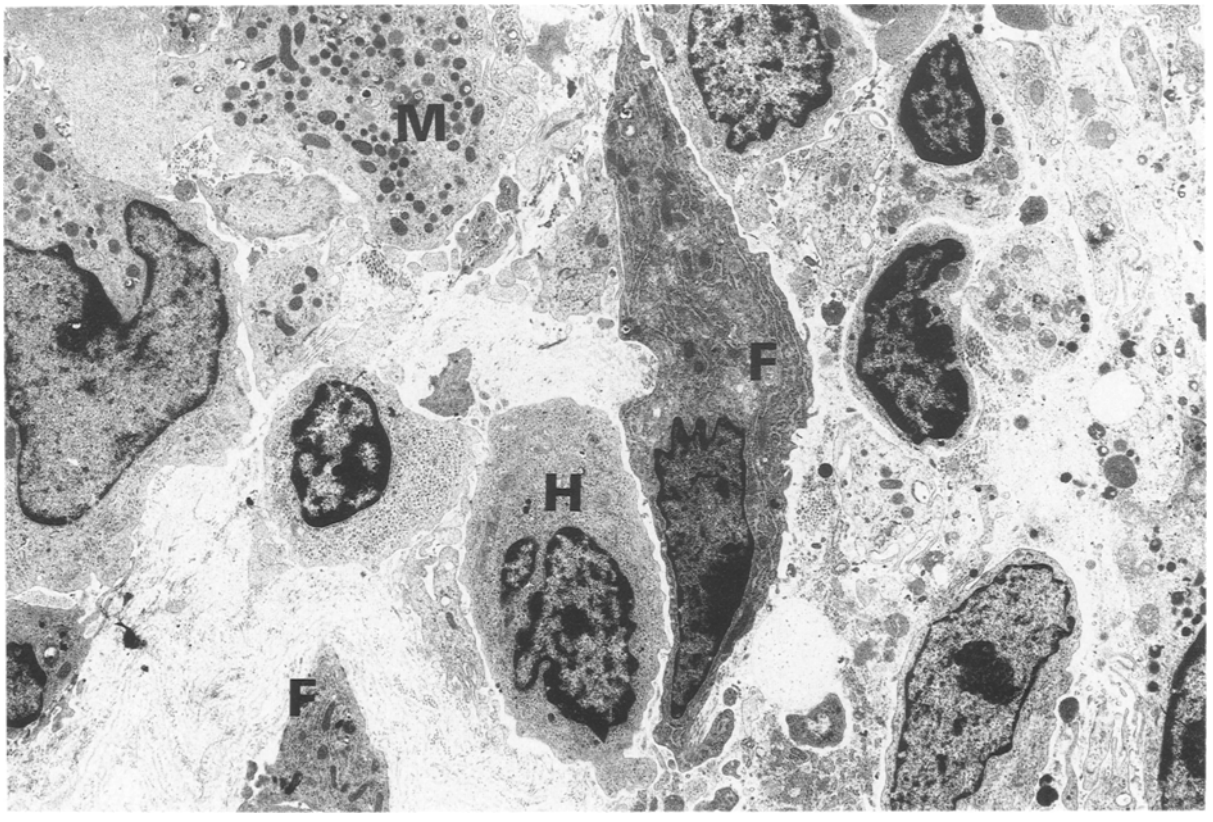

Fig. 6. The dense connective matrix, observed at the periphery of the lesion, is synthesized by fibroblasts (*F*) which are present from the beginning of the inflammatory reaction. Histiocytes (*H*), which are poorly distinguished with fibroblasts by standard histological techniques, represent a quiescent subset of macrophages easily parasitized by *Leishmania*. Activated macrophages (*M*) possess numerous cytoplasmic organelles and are the effector cells responsible for the defence mechanisms in the leishmaniasis lesion. Electron microscopy, ×2700

Table 3. Dendritic cells in the infiltrates of cutaneous leishmaniasis

Antibody	CD	Positive cells(cells/mm^2)[a]	
		in epidermis	in dermis
IOT6a	CD1a	887.5±250.6	87.7±43.6
L404	CD1a	598.2±208.7	82.6±54.8
IOT6b	CD1b	0.0	38.8±23.2

[a] Results are expressed as mean count±standard error. The sample was composed of 21 biopsies

Table 4. Comparison of the skin infiltrates before and after specific treatment for cutaneous leishmaniasis

Antibody	CD	Percentage of positive cells[a]	
		Before treatment	After treatment
IOT11	CD2	38.8±27.4	39.5±15.6
IOT4	CD4	29.3±20.8	21.2±16.4
IOT8	CD8	14.4± 6.3 (n=3)	19.2± 9.5
IOT14	(IL-2r)	37.6±20.5	36.3±17.7 (n=6)
Leu M3	CD14	50.4±37.8	44.9±33.6
Leu 11b	CD16	8.1± 2.0	9.8± 2.8 (n=3)

[a] Results are expressed as mean percentage±standard error. The number of tested biopsies is 7 except when indicated in parentheses

About 6% of the infiltrating cells apparently possessed a NK phenotype. Histological data on neutrophils in these early inflammatory lesions were consistent with the results obtained with a granulocyte marker on frozen sections: a small percentage of ION3$^+$ cells was observed but some biopsies were negative. However, in contrast to haematoxylin and eosin observations, few B cells were revealed by a specific mAb, confirming our previous study (Ridel et al. 1988), which had also indicated that these B lymphocytes were mature.

The study of Langerhans and related cells was performed on 21 lesions (Table 3, Fig. 3) compared with 4 control biopsies. The results obtained in the epidermis with the different mAbs were in line with classical estimates and our controls (617.0±188.1 CD1a$^+$ cells/mm^2). More interesting is the finding of dendritic CD1b$^+$ cells in the dermis, probably belonging to the Langerhans set but double-marker techniques will have be used to test this hypothesis.

The immunophenotypic analysis of cells in situ, in lesions biopsied just before and 1 month after a specific treatment, are indicated in Table 4. There was no statistically significant difference between the two matched samples, largely perhaps because the number of biopsies was too small and/or the time period between the biopsies was too short to detect slight changes in these cell populations. We found similar results with Langerhans (CD1a$^+$) cell density in the epidermis (468.2±147.2 cells/mm^2 before treatment versus 505.8±153.2 cells/

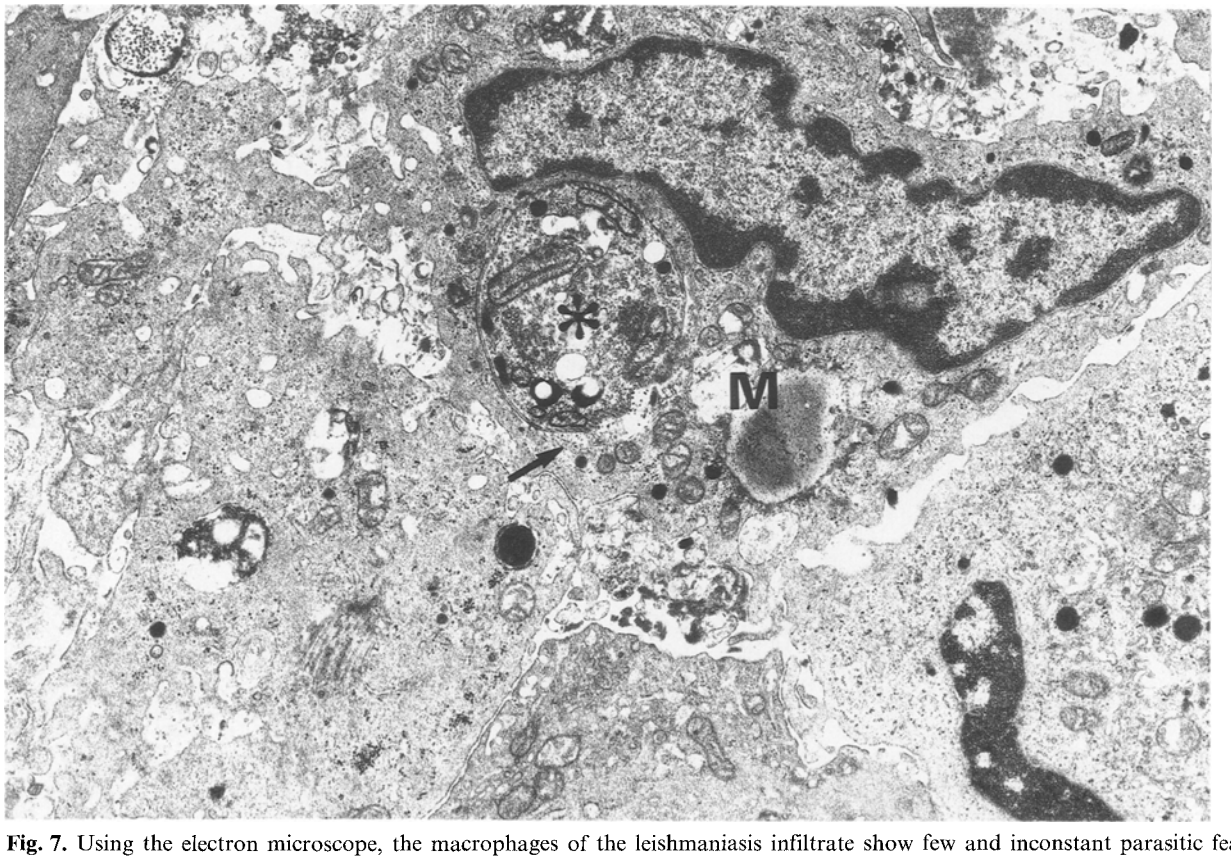

Fig. 7. Using the electron microscope, the macrophages of the leishmaniasis infiltrate show few and inconstant parasitic features. Note the phagolysosome membrane observed (*arrow*) around the amastigote form (*star*). Electron microscopy, ×6300

mm^2 after treatment), and a slight but not significant increase of dendritic ($CD1b^+$) cells in the dermis (56.5 ± 40.9 cells/mm^2 before versus 82.1 ± 34.8 cells/mm^2 after).

We have studied the cellular interactions occurring in the inflammatory infiltrate ultrastructurally (Fig. 5), concurrently with the associated tissue remodelling process. These observations confirmed the extension of the infiltrate from the upper to the lower dermis, as seen with histopathological techniques, and the scarcity of plasmacytes. We believe that the majority of the cells identified as "histiocytes" on haematoxylin and eosin staining are in fact fibroblasts (Fig. 6). We observed amastigote forms in only 5 biopsies (Fig. 7). Some of the parasites were dispersed in the extracellular space; some were found in phagolysosomes and exhibited structural alterations. It must be pointed out that macrophages were always slightly parasitized. In many sections a close contact between macrophages, obviously in an "activation" state, and lymphocytes, some with a "large granular" morphology, was observed (Fig. 5). In those areas where connective tissue damage was evident and was associated with a lytic activity due to "fibroclasts", we were able to see some macrophages in a state of lysis but we never observed the direct destruction of parasitized macrophages mediated by large granular lymphocytes (probably NK) as described elsewhere (Ridley and Wells 1986).

Ultrastructurally the few dendritic cells observed in the dermis lacked Birbeck granules, a fact which favours the identification of $CD1b^+$ dermal cells as dendritic and not Langerhans cells.

Discussion

While the characteristics of cellular immunity in cutaneous leishmaniasis are comparatively well defined in peripheral blood level (Barbier et al. 1985; Carvalho et al. 1985; Castes et al. 1983, 1984; Rada et al. 1987), there are fewer studies on cellular immunity in situ (Modlin et al. 1985; McInnes and Rennick 1988; Nilsen and Mshana 1987; Ridel et al. 1988). In this study, we have analysed the immunocompetent cells present in the lesion of LCL, using a comprehensive panel of mAbs. This immunohistochemical approach has been made concurrently with histological and ultrastructural investigations. It is particularly interesting to compare these results with what is known on the immunoregulation of experimental cutaneous leishmaniasis (Liew 1983, 1986; Louis and Milon 1987; Scott 1988, 1989; Titus et al. 1989).

We have been able to confirm previous reports that the bulk of inflammatory cells are T lymphocytes (Huszar et al. 1987; Ridley and Ridley 1983, 1984), with approximately the same numbers of $CD4^+$ and $CD8^+$ cells (Carvalho et al. 1985; Huszar et al. 1987; Modlin et al. 1985; Nilsen et al. 1987), most of them with an activated phenotype ($CD25^+$, $CD71^+$ or $HLA\text{-}DR^+$) as confirmed by electron microscopy. Newly available mAbs have allowed us to analyse $CD4^+$ subsets, showing a predominance of "helper" ($CD4^+$ $CD45RO^+$) T lymphocytes. Another interesting result is the high percentage of TCR-$\gamma\delta^+$ lymphocytes from a sample of 10 biopsies, confirming other data (20%, in a sample of 7 patients) recently published on leishmaniasis and with granulomatous reactions in leprosy (Modlin et al. 1989), as compared with normal skin (Groh et al. 1989; Modlin et al. 1989). These "$\gamma\delta$T cells" may play a direct role in granuloma formation, as recently suggested (Bloom 1989), together with some monokines (IL-1 and TNF-α) and a lymphokine (IL-4) possibly secreted by activated T lymphocytes (Kindler et al. 1989; McInnes and Rennick 1988; Shikama et al. 1989).

While macrophages are very frequently encountered in the inflammatory infiltrate, it must be emphasized that they are rarely parasitized, as seen at the ultrastructural level. This last result is in contrast with the high parasite density observed on 15 cutaneous smears, corresponding to frozen biopsies, showing 4.5 ± 5.4 amastigotes at the same magnification (Esterre et al. 1991). This forces us to consider the possibility that the "histiocytes" classically described with the histological technique are probably fibroblasts as observed with the electron microscope. The same tool also permits observation of the cell-cell contacts between T lymphocytes and macrophages (Fig. 5) that may promote delivery of activation signals from lymphocytes to the resting macrophages (lymphokine-mediated activation). However, on that occasion, we failed to confirm Wyler's theory of a non-lymphokine-mediated anti-leishmanial defence (Panosian et al. 1984; Sypek and Wyler 1990; Wyler et al. 1987) that is to say, we observed no direct contact between infected macrophages and T lymphocytes (contact-mediated activation).

Perhaps because of the small sample and technical difficulties, no significant difference was seen between biopsies analysed before and after treatment. We observed a slight increase in Langerhans cells ($CD1a^+$) in the dermis, where dendritic $CD1b^+$ cells of unknown origin and function were also seen. We must add that, in contrast to results obtained with immunofluorescence techniques (Groh et al. 1989), our immunoenzymatic techniques revealed no staining of Langerhans cells with anti-CD3 or anti-TCR$\gamma\delta$ mAbs. This problem may be resolved rapidly by using in situ hybridization together with immunohistology (V. Groh, personal communication).

The immunoenzymatic study suggests the participation of few B lymphocytes and NK cells, and this result is supported by the scarcity of plasmacytes and large granular lymphocytes observed with the electron microscope. It is worth noting that the discrepancy between the present NK scoring and a previous one (Ridel et al. 1988) may be accounted for by differences in specificity with respect to both the mAbs and the immunostaining kits used. In this respect, new techniques of tissue processing (Bourges et al. 1989; Sato et al. 1986, 1990) may allow us to study soft tissues like lymph nodes.

This area of intense research actually suggests that the critical event producing such an immunological granuloma may be a (lymphokine-mediated) down-regulation of IFN-γ (Liew 1983) or TNF-α (Titus et al. 1989)-

mediated macrophage activation (Scott 1989). Taken together, these data and others will help us towards a better understanding of the precise mechanisms responsible for the local modulation – in terms of cellular and matricial heterogenity – of the granuloma formation and the evolution of leishmaniasis from LCL to DCL or MCL forms.

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References

Barbier D, Goyot P, Dedet JP (1985) *Leishmania braziliensis guyanensis* dermal leishmaniasis: cell-mediated immunity related to clinical features. Trans R Soc Trop Med Hyg 79:47–50

Bloom BR (1989) Immunology of bacteria and parasites. Immunol Today 10:397–399

Bourges M, Esterre P, Guerret S, Grimaud JA (1989) A simplified method of tissue processing for immunostaining with good preservation of antigens and morphology. Cell Mol Biol 35:231–237

Carvalho EM, Johnson WD, Barreto E, Marsden PD, Costa JLM, Reed S, Rocha H (1985) Cell mediated immunity in American cutaneous and mucosal leishmaniasis. J Immunol 135:4144–4148

Castes M, Agnelli A, Verde O, Rondon AJ (1983) Characterization of the cellular immune response in American cutaneous leishmaniasis. Clin Immunol Immunopathol 27:176–186

Castes M, Agnelli A, Jordon AJ (1984) Mechanisms associated with immunoregulation in human American cutaneous leishmaniasis. Clin Exp Immunol 57:279–286

Chen K, Demetris AJ, Van Thiez DH, Whiteside TC (1987) Methods in laboratory investigation. Double immunoenzyme staining method for analysis of tissues and blood lymphocytes subsets with monoclonal antibodies. Lab Invest 56:114–120

Dedet JP (1990) Cutaneous leishmaniasis in French Guiana: a review. Am J Trop Med Hyg 43:25–28

Dedet JP, Desjeux P (1989) Epidemiological aspects of human cutaneous leishmaniasis in French Guiana. Trans R Soc Trop Med Hyg 83:616–620

Desjeux D, Dedet JP (1989) Isoenzyme characterization of 112 *Leishmania* isolates from French Guiana. Trans R Soc Trop Med Hyg 83:610–612

Esterre P, Dedet JP, Guerret S, Chevallier M, Frenay C, Grimaud JA (1991) Matrix remodelling and fibroblast phenotype in early lesions of human cutaneous leishmaniasis. Pathol Res Pract 187:924–930

Groh V, Porcelli S, Fabbi M, Lanier LL, Picker LJ, Anderson T, Warnke RA, Bhan AK, Strominger JL, Brenner MB (1989) Human lymphocytes bearing T cell receptor γ/δ are phenotypically diverse and evenly distributed throughout the lymphoid system. J Exp Med 169:1277–1294

Hsu A, Raine L, Fanger H (1981) Use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 29:577–580

Huszar M, Shor R, Trau H, Gazit E, Passwell JH (1987) The T cell phenotypes in the lesion of patients with cutaneous leishmaniasis. Clin Exp Dermatol 12:103–107

Kelly J, Whelan CA, Weir DG, Feighery C (1987) Removal of endogenous peroxidase activity from cryostat sections for immunoperoxidase visualisation of monoclonal antibodies. J Immunol Methods 96:127–132

Kindler V, Sappino AP, Grau GE, Piguet PF, Vassalli P (1989) The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. Cell 56:731–740

Liew FY (1983) Functional heterogeneity of $CD4^+$ T cells in leishmaniasis. Immunol Today 10:40–45

Liew FY (1986) Cell-mediated immunity in experimental cutaneous leishmaniasis. Parasitol Today 2:264–270

Louis J, Milon G (1987) 20th Forum in Immunology. Immunobiology of experimental leishmaniasis. Ann Inst Pasteur/Immunol 138:737–795

McInnes A, Rennick DM (1988) Interleukin 4 induces cultured monocytes/macrophages to form giant multinucleated cells. J Exp Med 167:598–611

Modlin RL, Tapia FJ, Bloom BR, Gallinoto ME, Castes M, Rondon AJ, Rea TH, Convit J (1985) In situ characterization of the cellular immune response in American cutaneous leishmaniasis. Clin Exp Immunol 60:241–248

Modlin RL, Pirmez C, Hofman FM, Torigian V, Uyemura K, Rea TH, Bloom BR, Brenner MB (1989) Lymphocytes bearing specific $\gamma\delta$ T-cell receptors accumulate in human infectious disease lesions. Nature 339:544–547

Nakane PK, Kawaoi A (1974) Peroxidase-labeled antibody. A new method of conjugation. J Histochem Cytochem 22:1084–1091

Nilsen R, Mshana RN (1987) In situ characterization of the cutaneous immune response in Ethiopian cutaneous leishmaniasis. Scand J Immunol 26:503–512

Panosian CB, Sypek JP, Wyler DJ (1984) Cell contact-mediated macrophage activation for antileishmanial defense. Lymphocyte effector mechanism that is contact dependent and noncytotoxic. J Immunol 133:3358–3365

Rada E, Trujillo D, Castellanos PL, Convit J (1987) Gamma interferon production induced by antigens in patients with leprosy and American cutaneous leishmaniasis. Am J Trop Med Hyg 37:520–524

Ridel PR, Esterre P, Dedet JP, Pradinaud R, Santoro F, Capron A (1988) Killer cells in human cutaneous leishmaniasis. Trans R Soc Trop Med Hyg 82:223–226

Ridley DS, Ridley MJ (1983) The evolution of the lesion in cutaneous leishmaniasis. J Pathol 141:83–96

Ridley MJ, Ridley DS (1984) Cutaneous leishmaniasis: immune complex formation and necrosis in the acute phase. Br J Exp Pathol 65:327–336

Ridley MJ, Wells CW (1986) Macrophage-parasite interaction in the lesion of cutaneous leishmaniasis. An ultrastructural study. Am J Pathol 123:79–85

Sato Y, Mukai K, Watanabe S, Goto M, Shimosato Y (1986) The AMeX method. A simplified technique of tissue processing and paraffin embedding with improved preservation of antigens for immunostaining. Am J Pathol 125:431–435

Sato Y, Mukai K, Matsuno Y, Furuya S, Kagami Y, Miwa M, Shimosato Y (1990) The AMeX method. A multipurpose tissue-processing and paraffin-embedding method. II. Extraction of spooled DNA and its application to Southern blot hybridization analysis. Am J Pathol 136:267–271

Scott P (1988) Immunoregulation of cutaneous leishmaniasis. T cell lines that transfer protective immunity or exacerbation belon to different T helper subsets and respond to distinct parasite antigens. J Exp Med 168:1675–1684

Scott P (1989) The role of TH1 and TH2 cells in experimental cutaneous leishmaniasis. Exp Parasitol 68:369–372

Shikama Y, Kobayashi K, Kasahara K, Kaga S, Hashimoto M, Yoneya I, Hosoda S, Soejima K, Ide H, Takahashi T (1989)

Granuloma formation by artificial particles in vitro. Macrophages and monokines play a critical role in granuloma formation. Am J Pathol 134:1189–1199

Sobel RA, Blanchette BW, Bhan AK, Colvin RB (1984) The immunopathology of experimental allergic encephalomyelitis. Quantitative analysis of inflammatory cells in situ. J Immunol 132:2393–2401

Sypek JP, Wyler DJ (1990) T-cell hybridomas reveal two distinct mechanisms of anti-lesihmanial defense. Infect Immun 58:1146–1152

Titus RG, Milon G, Marchal G, Vassalli P, Cerottini JC, Louis J (1987) Involvement of specific Lyt-2^+ T-cells in the immunological control of experimentally induced murine cutaneous leishmaniasis. Eur J Immunol 17:1429–1433

Titus RG, Sherry B, Cerami A (1989) Tumor necrosis factor plays a protective role in experimental murine cutaneous leishmaniasis. J Exp Med 170:2097–2104

Wood GS, Warnke R (1982) Suppresion of endogenous avidin binding activity in tissues and its relevance to biotin-avidin detection systems. J Histochem Cytochem 29:1196–1204

Wyler DJ, Bellerr DI, Sypek JP (1987) Macrophage activation for antileishmanial defense by an apparently novel mechanism. J Immunol 138:1246–1249