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Clinical and immunopathological aspects of disseminated cutaneous leishmaniasis

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The clinico-pathological and immunological findings in eight patients from Bahia, Brazil with disseminated cutaneous leishmaniasis are described. This condition differs from anergic diffuse cutaneous leishmaniasis (DCL) and from classical American cutaneous leishmaniasis (ACL). The number of lesions in these patients ranged from 75 to 800 and were characterized by papules and an acneiform type of lesion with a few ulcers rather than nodules that are the main characteristic of DCL. On the other hand the high incidence of mucosal disease (38%) in patients with disseminated cutaneous leishmaniasis make the prevalence of mucosal involvement in this condition higher than that observed in ACL. *L. amazonensis* (five cases) and *L. braziliensis* (two cases) were the causal agents in the patients where the infecting agent was characterized. Antibody titers in disseminated cutaneous leishmaniasis were higher than those observed in ACL and patients with the highest antibody titers had mucosal envolement. Abnormalities in cellular immunity that are not observed in ACL such as decrease in CD_4+ cells and absence of T cell response to leishmania antigen were observed in several patients with disseminated cutaneous leishmaniasis but restoration of these abnormalities occurred after treatment. In spite of the great number of lesions, the therapeutic response was good in six patients with disappearance of the lesions in a period shorter than that observed in ACL. In the two patients that presented therapeutic failure the causal agent was *L. amazonensis*. In such patients there was a predominance of ulcerated lesions, and a high titer of antibody was detected.

Introduction

Disseminated cutaneous leishmaniasis is clinically characterized by the presence of multiple papular and ulcerated lesions. The differential diagnosis of this condition is made with the anergic type of disease called diffuse cutaneous leishmaniasis (Convit et al., 1972; Bittencourt and Guimarães, 1968; Bryceson, 1970). Patients with diffuse cutaneous leishmaniasis (DCL) have nodular disseminated lesions with heavily parasitized macrophages and relatively poor lymphocyte infiltration (Bryceson, 1970; Convit et al., 1972). Additionally patients with DCL lack a T cell

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proliferative response and intradermal skin test reaction to leishmania antigens (Bryceson, 1970; Petersen, et al., 1982). Multiple cutaneous lesions due to leishmania infection have also been documented in post-Kala-azar dermal leishmaniasis (Haldar et al., 1983), and in a rare clinical presentation of cutaneous leishmaniasis called leishmanids (Berlin and Brenner, 1982). In the New World, patients with disseminated leishmania infection have been described (Costa et al., 1986), but the clinical picture, the pathology of this condition and host immune response during this infection has not been studied. Here we describe clinical, histopathological, and immunological findings in eight patients with disseminated cutaneous leishmaniasis, and indicate clear differences between this form of leishmania infection, DCL and classical American cutaneous leishmaniasis (ACL).

Material and methods

Subjects

The patients were seen at the Hospital Universitário Professor Edgard Santos in the state of Bahia, Brazil in the period from 1986 to 1992. Data of the clinical history and of a complete physical examination were obtained, and parasite culture, skin test and immunological studies were performed.

Parasite isolation, cultivation and characterization

The isolation was made by culture in tubes of NNN blood agar overlaid with a modified liver infusion tryptose from samples obtained by aspiration of the lesions. The culture from the biphasic medium were expanded for growth in modified LIT medium and kept at $25 \geqslant$ C. Procedures for both serodeme analysis with monoclonal antibodies and enzyme electrophoresis have been reported previously (Grimaldi et al., 1987).

Histopathological studies

Skin biopsies were performed with a 4 mm Baker's biopsy punch (Baker/Cummins Pharmaceuticol Inc, Miami, Fl), after local anesthesia (1% lidocaine), and fixed in buffered formol. Sections of paraffin embedded tissue were stained with hematoxylin-eosin.

Immunological studies

Immunological studies included evaluation of T cell surface markers, 'in vitro' test of lymphocyte proliferation, and determination of IFN-γ in supernatants of cultures of peripheral blood mononuclear cells (PBMC) stimulated with leishmania lysate. PBMC were separated from heparinised venous blood by gradient centrifugation using Ficol Hypaque. For proliferation assays (Carvalho et al., 1985), PBMC were adjusted to 10^6/ml in RPMI (Gibco, New Island, NY) containing penicillin and streptomycin and supplemented with 10% AB normal human serum. Aliquots of 2×10^5 cells in 0.2 ml were cultured in triplicate in flat-bottomed microtiter plates

(Linbro Chemical, New Haven, CT) and stimulated with soluble *L. amazonensis* lysate (5 µg/ml), cell proliferation was measured by [^{3}H]thymidine uptake after 5 days of incubation (Carvalho et al., 1985). The number of T cells, CD_4^+ cells and CD_8^+ cells were determined by reaction with monoclonal antibodies anti OKT_{11}, OKT_4, OKT_8 (Ortho Diagnostic, Paritan, NJ) followed by addition of a fluorescent rabbit anti-mouse immunoglobulin (Carvalho, et al., 1985). Determination of IFN-γ production was evaluated in supernatants of lymphocyte cultures (3×10^6 cells/ml stimulated with 20 µg of crude Leishmania antigen). IFN-γ levels were determined by a sandwich ELISA (Kemp et al., 1991).

Antigen and serological test

Leishmania promastigote lysate was prepared from a cloned isolate of *L. amazonensis* (MHOM BR86-BA125) from a patient with cutaneous leishmaniasis. Antigen used for skin test and T cell studies were prepared as previously described (Carvalho et al., 1985; Reed et al., 1986). Serological tests were performed by indirect immunofluorescence using promastigotes of *L. amazonensis* (Badaró et al., 1983).

Results

The clinical picture of the cases presented includes the presence of papules and acneiform lesions ranging in number from 75 to more than 800 and a few ulcers (Fig. 1). These lesions were disseminated and present on the face, chest, abdomen, and limbs. The clinical profile, antibody titer to leishmania antigen and therapeutic response in these patients are shown in Table 1. All patients were adult males with an age range of 23 to 62 years. Fever, chills and malaise were complaints of six patients in the initial phase of illness (before or in the first week after the appearance of the dissemination). In four individuals reference was made to an initial lesion in one leg followed by dissemination to the entire body after a period as short as two days. In only one case the initial lesion had a long duration (six months) before dissemination. Mucosal lesions were detected in two patients in the initial examination (patients no. 5, no. 7). A third patient (no. 4) developed mucosal disease during follow-up. Leishmania was isolated in culture in seven patients and *L. amazonensis* was the causal agent in five of them. Anti-leishmania antibody was documented in all patients with titers ranging from 1:64 to 1:1024. In three patients who had mucosal involvement, antibody titers were higher than 1:512 and two of them failed to respond to antimonial therapy. The response to antimonial therapy was prompt in the other six patients. Five of this patients were completely free of active lesions in a period of two months. In such cases a scar with minimal skin retraction was documented after cure. In one of these six patients who respond to antimonial therapy mucosal involvement was documented at initial evaluation, but the patient was free of cutaneous and mucosal involvement, 30 and 90 days respectively after initiation of therapy.

In five of the six patients who were cured, two series of antimonial therapy were given (20 mg Sb/kg/day for 20 days), with an interval of 10 days between the series, and in one patient only one course of antimonial therapy was used with disappearance of the lesions in thirty days. In two patients (no. 4, no. 5) there was only

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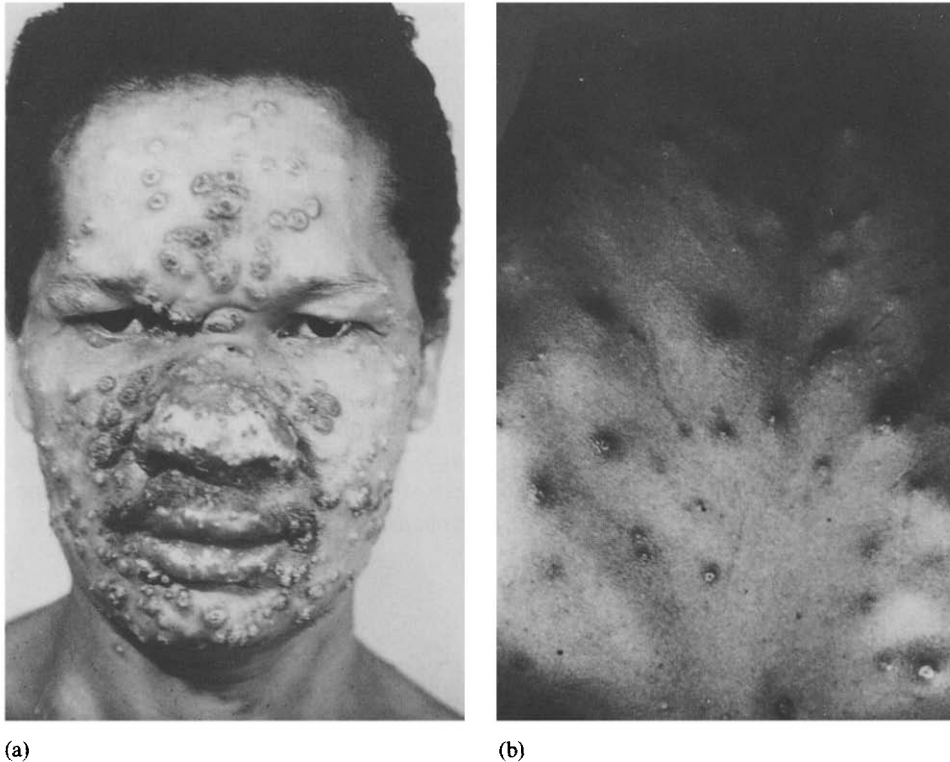(a)   (b)

Fig. 1. (a) Numerous acneiform lesions in the face. In the nose a diffuse infiltration with small ulcer is observed. (b) See pustules of different sizes surrounded by an erythematous halo (acneiform lesions) on the chest.

partial response with healing of the majority of the lesions but with persistence of a few ulcers and reactivation of the disease in healed areas upon cessation of therapy. One of these two patients had mucosal disease in the initial evaluation. In the other patient in addition to skin lesions a mucosal disease was detected 6 months after the initiation of the treatment.

The histopathological studies (Table 2) revealed a mononuclear infiltrate with predominance of plasma cells. Granulomatous reaction and parasitism were observed in two patients. Only one case presented with pseudocarcinomatous hyperplasia. Biopsies of the acneiform lesions were performed in three patients and all showed a follicular pattern. A heavy inflammatory reaction was observed around the follicles. The involvement was predominant in the follicular infundibulum (Fig. 2a) although all the follicule may be compromised (Fig. 2b). A partial disruption of the follicular epithelial sheath with marked spongiosis and exocytosis was observed associated with intrafollicular plugging (Fig. 2a). In one acneiform lesion of very recent onset (three days of appearance) a marked infiltration of polymorphonuclear cells with predominance eosinophils was observed around one follicule (case 4).

Data of the skin test, leishmania antigen specific lymphocyte proliferative response and circulating T cells subsets are also shown in Table 2. Delayed type hypersensitivity to leishmania antigen was absent in four patients (nos. 2,3,5,6). Absence of lymphocyte proliferation to leishmania lysate was documented in three of the four

TABLE 1

Clinical and parasitological findings of disseminated cutaneous leishmaniasis

Patients	Age/sex	Illness duration (months)	Constitutional symptoms[a]	Number of lesions	Mucosal involvement	Parasite identification	Antibody titer	Therapeutic response to antimonials
1	23/M	3	Yes	360	No	L. braziliensis	1:64	Cure
2	24/M	1	No	300	No	L. amazonensis	1:128	Cure
3	35/M	2	Yes	75	No	L. amazonensis	1:512	Cure
4	37/M	2	Yes	800	Yes	L. amazonensis	1:1024	Failure[c]
5	42/M	6	No	130	Yes	L. amazonensis	1:1024	Failure[c]
6	45/M	3	Yes	100	No	L. amazonensis	1:128	Cure
7	46/M	2	Yes	330	Yes	ND[b]	1:512	Cure
8	62/M	3	Yes	250	No	L. braziliensis	1:512	Cure

Fever, chills, malaise, aches. [b]Not done. [c]Both patients were successfully treated with amphotericin B.

TABLE 2

Histopathological findings and T cell responses in disseminated cutaneous leishmaniasis

Patients	Age/sex	Histopathological findings				Skin test (mm)	Immunological findings blastogenesis		T cell markers (mm^3)		
		Parasitism	Infiltration	Folicular pattern	Granuloma formation		Control media	Leishmania Ag	CD4	CD8	CD4/CD8
1	23/M	−	Diffuse	+	−	8	653±77	7378±1011	ND	ND	ND
2	24/M	−	Focal	−	−	Negative	717±35	706±137	962	405	2.3
3	35/M	−	Diffuse	−	−	Negative	2418±173	20673±656	477	841	0.56
4	37/M	+	Focal	+	−	15	2565±245	26191±1351	578	1026	0.56
5	42/M	+	Diffuse	−	−	Negative	722±301	2526±952	561	432	1.3
6	45/M	−	Diffuse	−	−	Negative	1081±271	2449±261	1398	714	1.9
7	46/M	−	Focal	+	+	12	696±99	14771±107	663	933	0.71
8	62/M	−	Diffuse	−	+	10	225±13	86778±3824	973	546	1.7

+ Present; ND — Not done.

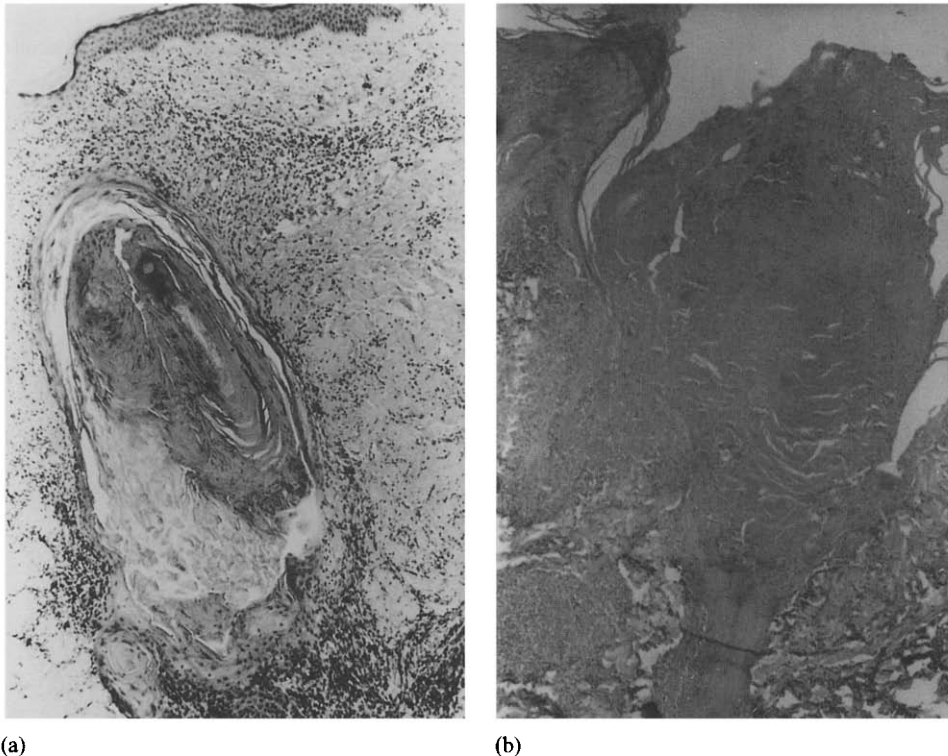(a)   (b)

Fig. 2. (a) A longitudinal section of a hair follicle shows a marked dilatation with plugging of the infundibulum. A partial disruption of the follicular epithelial sheath and a chronic inflamatory infiltrate can also be observed. HE, ×64. (b) An oblique section showing dilatation and plugging of the whole hair follicle with atrophy of the follicular epithelial sheath. A chronic inflammatory infiltrate is seen around the follicle. HE, ×64.

patients with negative skin test (nos. 2,5,6). In all cases lacking T cell response to leishmania antigen (absence of lymphocyte proliferation and/or negative skin test) the infecting agent was *L. amazonensis*. No correlation between the degree of T cell proliferation to leishmania antigens and the therapeutic response could be documented. Of the three patients whose lymphocytes did not proliferate to leishmania lysate only one did not respond to antimonial therapy. In the three patients that had negative lymphocyte proliferative responses during active disease, restoration of this response was observed in subsequent evaluations after cure in two patients and in a third patient after resolution of the majority of the lesions. Low counts of CD_4+ cells (less than mean minus one standard of controls $\neq 694$ cells mm^3) was observed in four patients (nos. 3,4,5,7). In three of them *L. amazonensis* was the causal agent. The two patients who failed to antimonial therapy had low CD4 counts. CD_4+ cells observed in these patients was however much higher than one observes in AIDS patients, and HIV antibodies were absent in all cases with disseminated cutaneous leishmaniasis. In two patients (nos. 4,7) IFN-γ levels were determined in supernatant of lymphocyte culture stimulated with PHA or leishmanial antigen. High levels of IFN-γ were detected (456 pg/ml and 394 pg/ml respectively) in

TABLE 3

Clinical and immunopathological findings in disseminated cutaneous leishmaniasis, diffuse cutaneous leishmaniasis and classical American cutaneous leishmaniasis

Clinical and immuno-pathological findings	Clinical forms of leishmaniasis		
	Disseminated	DCL	ACL
Clinical presentation	papules and acneiform lesions	nodules	ulcers
Number of lesions	many	many	one or few
Destructive mucosal involvement	frequent	absent	infrequent
Parasite identification	*L. amazonensis* *L. braziliensis*	*L. amazonensis*	*L. amazonensis* *L. braziliensis*
Parasites in tissue	rare or absent	abundant	rare or absent
Follicular pattern	present	absent	absent
Granulomatous pattern	present or absent	absent	present or absent
Intradermal skin test	positive or negative	negative	positive
Antibody titers	mainly high	high	low
Lymphocyte proliferative response	present or absent	absent	present

cultures stimulated with PHA and (366 pg/ml and 420 pg/ml) in supernatants from cultures stimulated by leishmanial antigen.

A comparison between the clinical, histopathological and immunological findings of the disseminated cutaneous leishmaniasis with the DCL and the classical ACL is in Table 3. In disseminated cutaneous leishmaniasis multiple acneiform lesions are the main clinical findings. The causal agent is predominantly *L. amazonensis* and a follicular pattern is found in the histopathological studies. The absence of lymphocyte proliferative response to leishmania antigens and absence of DTH seen in disseminated cutaneous leishmaniasis is transitory and such patients can produce IFN-γ.

Discussion

ACL in Brazil is predominantly caused by *L. braziliensis* and the most frequent clinical form of presentation is a single ulcer in the leg. (Llanos-Cuentas et al., 1984). Immunological studies in ACL have documented a preserved T cell response to leishmania antigen and the ability of macrophages to kill intracellular amastigotes (Carvalho et al., 1985; Conceição-Silva et al., 1990). At the other pole of the disease is the diffuse cutaneous leishmaniasis characterized by multiple nodules and absence of T cell response to leishmania antigen (Bittencourt and Guimarães, 1968; Petersen et al., 1982). In Brasil all cases of DCL have been associated to *L. amazonensis* infection (Lainson, 1983; Barral et al., 1991). The cases described here of disseminated cutaneous leishmaniasis have clinical immunological and therapeutic aspects that clearly differentiate this condition from the DCL and from the classical ACL. In disseminated cutaneous leishmaniasis the main findings are the predominance of papules and acneiform type of lesions associated less frequently with ulcers. Papules are rarely observed and acneiform type of lesion are not seen in classical ACL. Otherwise, in DCL the lesion are predominantly nodular. Additionally in the present series a high frequency of mucosal disease was observed while the prevalence of

mucosal involvement in the endemic areas of cutaneous leishmaniasis is less than 5% (Jones et al., 1987). Clinically disseminated cutaneous leishmaniasis resemble the rare form of leishmaniasis reported in the old world called leishmanid that is defined as an eruption of papules and pustules that appears in a different site from the primary focus of leishmaniasis (Berlin and Brenner, 1982). The word leishmanid was used to indicate a hypersensitivity reaction since delayed hypersensitivity was present in the few cases reported (Berlin, 1953) and histopathologically a tuberculoid reaction was observed. The present study show that immunological responses in disseminated cutaneous leishmaniasis are quite variable and both a strong T cell response or absent cell mediated immunity may be present.

The histopathological studies in all of these patients revealed the same histological pattern observed in New World cutaneous leishmaniasis represented by a mono-nuclear cell infiltration with predominance of plasma cells associated with granulo-matous reaction (Bittencourt and Barral, 1991). Different from the classical ACL was the low frequency of epidermal hyperplasia and the occurrence of a follicular pattern in the acneiform lesion. Considering that acneiform lesions were observed in all cases except one, it is probably that circulating parasites present a preference for the follicular area. It is possible that a follicular pattern was not observed in all the cases due to a complete obliteration of the follicular structure by the inflammatory reaction in older lesions. As observed in classical ACL (Bittencourt and Andrade, 1967) a tendence to destroy the cutaneous appendages was observed. Occurrence of a more pronounced eosinophil infiltrate, as observed in a recent acneiform lesion, is also referred in the initial lesions in experimental leishmaniasis (Pompeu et al., 1992).

The immunological studies in patients with disseminated cutaneous leishmaniasis are different from those observed in ACL and in DCL. For instance depression of the cellular immunity was observed in several patients, but restoration of T cell response to parasite antigen could be documented after the cure or improvement of the disease. Patients with disseminated cutaneous leishmaniasis may also exibit preserved T cell reponses to leishmania antigen. The antibody titers to leishmania antigen in this form of leishmaniasis were higher than that observed in classical ACL.

Although the mechanism of dissemination of the disease is not clear, the rapid spread of the infection, absence of lymph node enlargment and the occurrence of general symptoms such as fever, chills and malaise suggest direct heamatogenous dissemination. For such dissemination, factors related to the nature of the parasite and to the host may be involved. Different from the classical ACL in Brazil where the causal agent is predominantly L. braziliensis (Grimaldi et al., 1987) and a preserved T cell response to leishmania antigen is observed (Carvalho et al., 1985) most of the patients with disseminated leishmaniasis were infected by L. amazonensis and variable degree of abnormalitites in T cell response could be documented. Therefore an association of factors related to the parasite with alteration of the immune response of the host may be involved in dissemination of the infection.

The therapeutic response to pentavalent antimonials in disseminated cutaneous leishmaniasis was quite variable. Several patients with hundreds of lesions could be free of symptoms and without scars as soon as after two months of initiation of therapy, and no evidence of cutaneous or mucosal disease was observed after a follow-up from 4 to 7 years in five of such patients. In two cases therapeutic failure was documented. The failure of response to antimonial therapy was associated with

L. amazonensis infection, high antibody titer, mucosal involvement and a low CD4 count.

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