Clinical and Immunopathological Spectrum of American Cutaneous Leishmaniasis with Special Reference to the Disease in Amazonian Brazil - A Review

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The wide variety of Leishmania *species responsible for human American cutaneous leishmaniasis combined with the immune mechanisms of the host results in a large spectrum of clinical, histopathological, and immunopathological manifestations. At the middle of this spectrum are the most frequent cases of localized cutaneous leishmaniasis (LCL) caused by members of the subgenera* Leishmania *and* Viannia, *which respond well to conventional therapy. The two pathogenicity extremes of the spectrum generally recognized are represented at the hypersensitivity pole by mucocutaneous leishmaniasis (MCL) and at the hyposensitivity pole by anergic diffuse cutaneous leishmaniasis (ADCL). Following the present study on the clinical, histopathological and immunopathological features of cutaneous leishmaniasis in Amazonian Brazil, we propose the use of the term "borderline disseminated cutaneous leishmaniasis" for the disseminated form of the disease, due to parasites of the subgenera* Leishmania *and* Viannia, *which might be regarded as intermediate between LCL and the extreme pathogenicity poles MCL and ADCL.*

Key words: cutaneous leishmaniasis - clinical classification - Amazonian Brazil

American cutaneous leishmaniasis (ACL) is a parasitic protozoal disease, widely spread in most countries of Latin America, and caused by different species of the genus *Leishmania*. There are at present fourteen recognized species of *Leishmania* within the subgenera *Leishmania* and *Viannia*, which may produce a variety of cutaneous and mucocutaneous lesions in man (Lainson & Shaw 1972, 1987, 1998). In Amazonian Brazil, ACL is regarded as a zoonotic infection of silvatic mammals, among which the parasites are transmitted by the bite of naturally infected species of phlebotomine sand flies (Diptera: Psychodidae) (Lainson & Shaw 1992, Lainson et al. 1994). In this region, the disease may be the result of infection due to seven recognized species of *Leishmania*, six of them within the subgenus *Viannia* and one within the subgenus *Leishmania* (Silveira et al. 2002) (Table). Following infection of man by these parasites, there will be some naturally resistent individuals (asymptomatic) and others with different degrees of susceptibility to infection (symptomatic). Depending on the species of the infecting *Leishmania* and the infected person's cell-mediated immune response, there develops a spectrum of clinical forms of disease, conventionally known as localized cutaneous leishmaniasis (LCL), mucocutaneous leishmaniasis (MCL), and anergic diffuse cutaneous leishmaniasis (ADCL) (Castes et al. 1983, Carvalho et al. 1985, Silveira et al. 1997a, Lainson & Shaw 1998). Recently, a new clinical feature was added to the spectrum during an attempt to characterize some cases presenting with disseminated lesions designated as borderline disseminated cutaneous leishmaniasis (BDCL) (Silveira et al. 1997b). There is some evidence indicating that the genetic background of the human host may have a major influence in determining the outcome of the disease (Blackwell 1985, 1999, Petzl-Erler et al. 1991, Lara et al. 1991). In considering this complex situation regarding the etiology of ACL, and the accompanying dificulties in interpreting this spectrum of clinical and immunopathological features, we present a brief review of these together with our own observations on cutaneous leishmaniasis in Amazonian Brazil, where *Leishmania (Leishmania) amazonensis* and *Leishmania (Viannia) braziliensis* are of principal medical interest.

LOCALIZED CUTANEOUS LEISHMANIASIS

At the middle of the clinical spectrum, LCL with one or multiple ulcerated skin lesions represents the most frequent form of the disease, having as the etiologic agent any member of the neotropical subgenera *Viannia* and *Leishmania*. *L. (V.) braziliensis*, however, is regarded as the most important parasite associated with this form of disease in the Americas (Lainson 1983, Llanos-Cuentas et al. 1984, Lainson & Shaw 1987). In patients with lesions due to *L. (L.) amazonensis* the nature of the lesion gives a clue as to the causative parasite, namely the large infiltration at the edge of lesion, and its histopathology. In these lesions there is a dense infiltrate of vacuolated macrophages in the dermis which are full of amastigotes and give the infiltrate the appearance of a macrophagic granuloma (Moraes & Silveira 1994). This differs from the histopathology in cases of LCL caused by *L. (V.) braziliensis*

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TABLE

The neotropical *Leishmania* species, etiological agents of American cutaneous leishmaniasis

Subgenus *Viannia* Lainson & Shaw, 1987		Subgenus *Leishmania* Ross, 1903	
L. (V.) braziliensis [a]	Vianna, 1911	*L. (L.) mexicana*	Biagi, 1953
L. (V.) peruviana	Velez, 1913	*L. (L.) pifanoi*	Medina & Romero, 1959
L. (V.) guyanensis [a]	Floch, 1954	*L.(L.) amazonensis* [a]	Lainson & Shaw, 1972
L.(V.) panamensis	Lainson & Shaw, 1972	*L. (L.) garnhami*	Scorza et al. 1979
L. (V.) lainsoni [a]	Silveira et al. 1987	*L.(L.) venezuelensis*	Bonfante-Garrido, 1980
L. (V.) naiffi [a]	Lainson & Shaw, 1989		
L. (V.) shawi [a]	Lainson et al. 1989		
L.(V.) colombiensis	Kreutzer, 1991		
L.(V.) lindenbergi [a]	Silveira et al. 2002		

a: recorded in Amazonian Brazil

and other species of the subgenus *Viannia*, where there is a more modest infiltration in the skin bordering the ulcerated lesion, and in which macrophages and parasites are generally scanty: in contrast, lymphocytes and plasma cells are more frequent in the infiltrate, which has the characteristics of an epithelioid granuloma (Magalhães et al. 1986) (Fig. 1 a-d). In a smaller number of patients other types of cutaneous manifestations may appear in addition to the ulcerated lesion. They may appear as verrucose vegetative lesions, papules, nodules, and infiltrations in the skin which, together, have led to the consideration of LCL as a polymorphic skin disease (Silveira et al. 1997a).

With regards to the immunology of LCL, investigations have been principally on the profile of CD4+ and CD8+ T cell subsets and the cytokines produced by these cells in the lesions of patients, with special interest in interferon-gamma (INF-γ) and interleukin (IL)-4. It is believed that these play a crucial role in determining resistence (CD4+ Th1) or susceptibility (CD4+ Th2), respectively, to leishmanial infections (Ribeiro-de-Jesus et al. 1998, Barral-Neto et al. 1998). As a result, and depending on the frequency of memory T cells CD4+ and CD8+ in the cellular infiltrate of the lesion, and the balance of CD4+ Th1/Th2 immune responses, the lesions of some patients may heal spontaneously. In most cases, however, some kind of treatment is required to end the disease. In general, it has been considered that LCL patients present an adequate cell-mediated immunity with a predominance of CD4+ Th1 immune response (Cáceres-Dittmar et al. 1993, Pirmez et al. 1993, Carvalho et al. 1995). There is not yet, however, a consensus of opinion as to which type of memory T cell (either CD4+ or CD8+ subsets) is the more prevalent in the infiltrate of lesions (Modlin et al. 1985, Barral et al. 1987, Pirmez et al. 1990, Martinez-Arends et al. 1991, Esterre et al. 1992, Isaza et al. 1996, Vieira et al. 2002). In view of the complex etiology of ACL in Amazonian Brazil, the cell-mediated immunity of the disease needs to be studied not only in terms of the clinical features of the disease but also with regards to the specific leishmanial parasite involved. Thus, although an immunocytochemistry analysis (Silveira et al. unpublished data) has shown a higher prevalence of CD8+ than CD4+ T cells in LCL in patients infected with *L. (V.) braziliensis* or *L. (L.) amazonensis* (Fig. 2), the CD4+ Th1 immune response was found to be more intense in patients with LCL due to species of the subgenus *Viannia* [e.g. *L. (V.) braziliensis*] than in those infected by species of the subgenus *Leishmania* [e.g. *L. (L.) amazonensis*]. Using a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), Silveira et al. (unpublished data) demonstrated an increased mRNA expression of IFN-γ in biopsies of cutaneous lesions of patients infected with *L. (V.) braziliensis*, whereas no expression was observed for mRNA to IL-4 in the same samples. On the other hand, patients infected with *L.* (*L.) amazonensis* showed an increased mRNA expression of IL-4 in their lesions (Fig. 3). The levels of this cytokine were, however, strikingly lower than those of IFN-γ and corresponded to only about a tenth part of the levels of IFN-γ. This suggests that even low levels of IL-4 may be able to decrease the CD4+ Th1 immune response in these patients. Moreover, it must be emphasized that the cytokines IFN-γ and IL-4 were also demonstrated in *L. (V.) braziliensis*-stimulated peripheral blood mononuclear cells (PBMC) from LCL patients before and after treatment of infections due to *L. (V.) braziliensis* and *L. (L.) amazonensis* (data not shown). Interestingly, no significant differences were recorded in the levels of IFN-γ expression in the *L. (V.) braziliensis* stimulated PBMC among LCL patients, before or after treatment, in cases infected by *L. (V.) braziliensis* or *L. (L.) amazonensis.* The levels of IFN-γ were, however, significantly higher (≥ 4 times) than those of IL-4 in the same samples (data not shown), again indicating that there were very low levels of IL-4 in the peripheral blood of LCL patients. These data, together with the clinical and cellular immune evaluations explain why patients infected with *L. (V.) braziliensis* present a higher prevalence of positive reactions to the delayed hypersensitivity skin-test reaction (DTH) to *Leishmania* antigen and to the lymphocyte proliferation assay, respectively, than those infected with *L. (L.) amazonensis* (Silveira et al. 1991, 1998).

With regards to the IgG antibody response of LCL patients, measured by the indirect fluorescent antibody test (IFAT) and the enzyme linked immune assay (ELISA), low to moderate levels (mean titre 160) of specific anti-*Leishmania* IgG antibodies may be found in cases of infection due to *L. (V.) braziliensis* (Guimarães et al. 1983, Valli et al. 1999, Corrêa et al. 2003). However, in cases of

LCL patients infected with *L. (L.) amazonensis* from Amazonian Brazil it has been shown that there is a significant increase in the level of these antibodies (mean titre 640) as measured by IFAT (Chagas et al. 1999, 2001). This supports the idea that this parasite has a greater ability to stimulate the CD4+ Th2 imune response than has *L. (V.) braziliensis*.

From the middle of the spectrum, infections not totally controlled by cell-mediated immune mechanisms may evolve to one of two polar forms of the disease; either to the cellular hypersensitivity pole, represented by mucocutaneous leishmaniasis (MCL), or to the cellular hyposensitivity pole in cases of anergic diffuse cutaneous leishmaniasis (ADCL). This deviation in the course of infection to one or other of these diseases is also influenced by the type of antigen that is stimulating the immune system of the host: in other words, by the species of parasite concerned. Thus, although *L. (V.) braziliensis* and *L. (L.) amazonensis* may be isolated from the mucous membrane tissue of patients suffering from MCL and ADCL, respectively, the immunopathological features of these two kinds of tegumentary leishmaniasis are totally different. MCL patients present a vigorous T cell immune response against *L. (V.) braziliensis* and the parasite may be isolated from the mucosal tissue as early as one year after a cutaneous infection. On the other hand, ADCL patients have a deficient T cell immune response against *L. (L.) amazonensis*: only in a few cases, and only after a very prolonged evolution of this disease (10 years, or more) has the parasite been isolated from mucosal tissue. This was the situation we observed in 2 of 12 ADCL patients from the state of Pará, in the Amazon region of Brazil. In addition, *L. (V.) braziliensis* may be found in mucosal tissue from MCL patients with no apparent cutaneous lesion, whereas *L. (L.) amazonensis* in the mucosae is always associated with active and older cutaneous lesions on the face of ADCL patients. This suggests that unlike *L. (V.) braziliensis*, which disseminates via the blood, *L. (L.) amazonensis* utilizes a contiguous mechanism for dissemination from the skin to the mucosal tissue.

MUCOCUTANEOUS LEISHMANIASIS

Although some patients may simultaneously exhibit skin and mucosal lesions, it has been observed that in the majority of cases (59%) from Amazonian Brazil the mucosal disease has resulted from an old, prolonged, and untreated (self-healing) cutaneous infection with *L. (V.) braziliensis* (Silveira et al. 1999). This parasite is recognized as the most important etiologic agent of MCL in the New World (Lainson 1983, Grimaldi et al. 1987, 1989, Lainson & Shaw 1998).

In this form of the disease (Fig. 1e), necrosis of the nasopharyngeal mucous tissue is associated with a strong T cell immune response, as evidenced by the exacerbated DTH to *Leishmania* antigens and the lymphocyte proliferation assay (Silveira et al. 1998). In this respect, it should be stressed that DTH reactions elicited by homologous antigen – *L. (V.) braziliensis* – are significantly higher (≥ 3 times) than those elicited by a heterologous antigen – *L. (L.) amazonensis* – (Silveira, personal observation). This indicates, conclusively, the antigen-specific host immune response against *L. (V.) braziliensis*. The marked cellular hypersensitivity response seen in skin-tested MCL patients may also be demonstrated in histological sections of the mucosal tissue where, in some patients, there may be a tuberculoid granulomatous reaction: this might be regarded as the extreme expression of the cellular hypersensitivity pole seen in this form of disease. Sections also show the presence of an abundant infiltrate of lymphocytes and plasma cells with few histiocytes and scanty parasites. Necrosis of the cartilaginous structures is the main sequel (Magalhães et al. 1986) (Fig. 1f).

Immunology of MCL patients has been mainly studied by immunocytochemical analysis, which has produced some evidence suggesting that CD4+ T cells are the major subsets of memory T cells infiltrating the mucosal lesions in these patients (Barral et al. 1987, Pirmez et al. 1990, Martinez-Arends et al. 1991, Esterre et al. 1994). A similar immunocytochemical assay among ACL cases from Amazonian Brazil has demonstrated that only in the group of MCL patients have the CD4+ T cells predominated over the corresponding CD8+ T cells. It would seem a great coincidence that only in MCL, associated with an extreme cellular hypersensitivity response do the CD4+ T cells have the highest concentration among all other forms of ACL studied (LCL, ADCL and BDCL) (Fig. 2). This suggests that in MCL patients we should expect to find high levels of those cytokines – INF-γ, IL-2 and tumour necrosis factor alpha (TNF-α) – that are usually linked to the CD4+ Th1 immune response. However, a mixture of CD4+ Th1 and Th2 immune responses has been observed in patients with MCL from Venezuela (Cáceres-Dittmar et al. 1993, Castes et al. 1993) and Southeast Brazil (Pirmez et al. 1993). These findings differ from our results in the Amazon region of Brazil, where a characteristic CD4+ Th1 immune response has been demonstrated in MCL patients, in the biopsies of whom there has been shown to be a high expression of mRNA to IFN-γ in the mucous membrane lesions. This contrasts with the negative results for mRNA to IL-4 in the same samples (Fig. 3). Moreover, similar to the situation in LCL patients, both of the cytokines (IFN-γ and IL-4) were demonstrated in the *L. (V.) braziliensis* stimulated PBMC from MCL patients, although we noted that the levels of IFN-γ expression were much higher (≥ 6 times) than those of IL-4 in the same samples (data not shown). This indicates that in both LCL and MCL cases infected with *L. (V.) braziliensis* there are low levels of mRNA expression of IL-4 in the *L. (V.) braziliensis* stimulated PBMC as well as a complete lack of this cytokine in the cutaneous and mucosal lesions of these patients, respectively. In this respect, and also making a correlation with the cytokine expression in the corresponding spectrum of leprosy, it is of interest to emphazise the similar findings of Stefani et al. (2003). These authors were unable to detect mRNA expression of IL-4 in biopsy samples of cutaneous lesions from a range of leprosy patients within those histopathological categories (TT, BT, and I) that are considered as type 1 immunity (with high levels of mRNA expression of IFN-γ) as is the case in our LCL and MCL patients infected with *L. (V.) braziliensis*. We feel that our results may be explained as

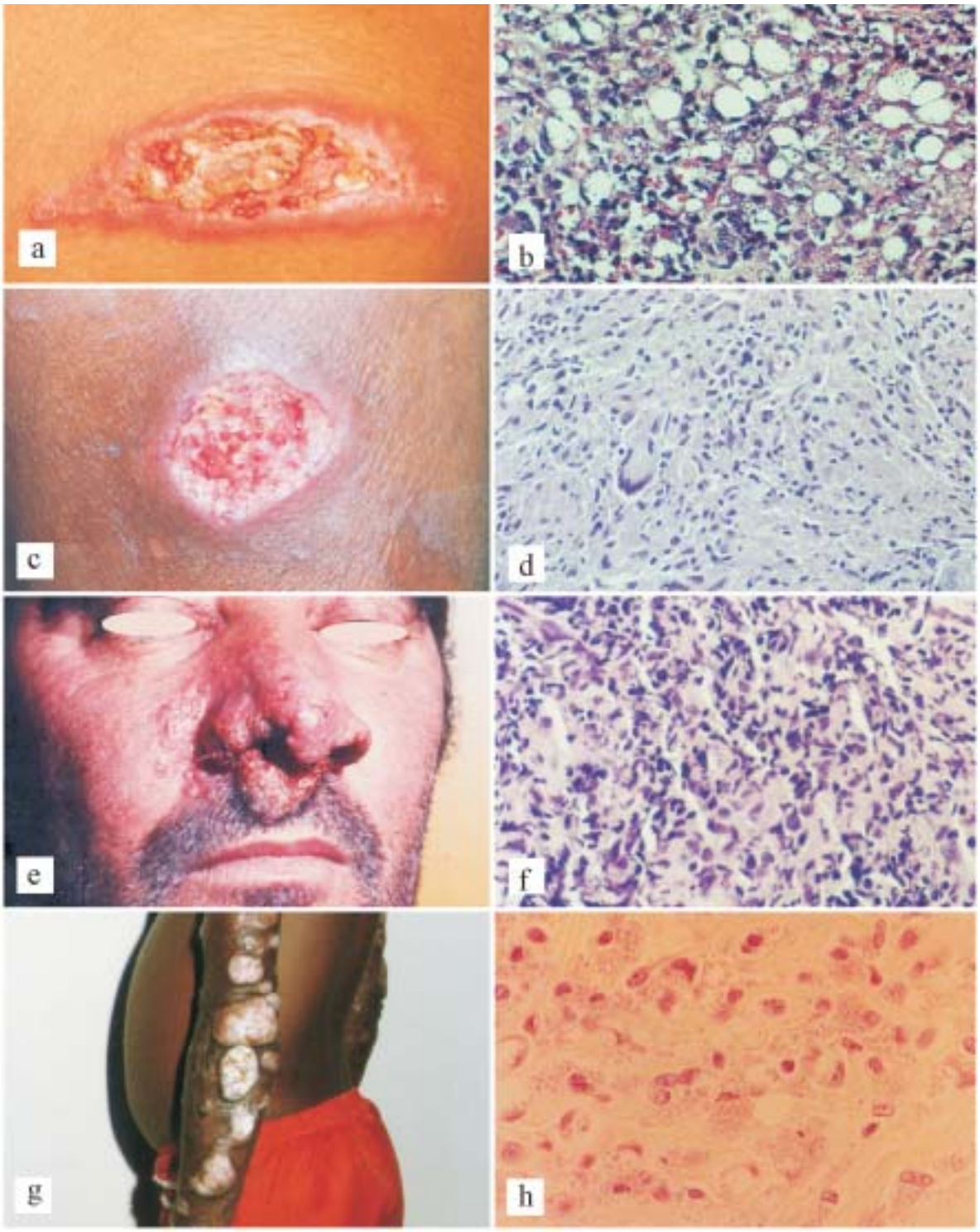

Fig. 1a: a typical ulcerated lesion in a case of localized cutaneous leishmaniasis (LCL) due to *Leishmania (Leishmania) amazonensis*, showing a large infiltration at the ulcer edge; b: a histological section of the same lesion: note the diffuse dermal infiltration of vacuolated macrophages containing abundant amastigotes and surrounding lymphocytes and plasma cells (x 400); c: a typical ulcerated lesion in a case of LCL due to *Leishmania (Viannia) braziliensis*, with a weak infiltration in the skin bordering the lesion; d: a section of the same lesion, showing a diffuse infiltration of lymphocytes and plasma cells in the dermis with scanty macrophages and parasites (x 400); e: a mucosal lesion of the nose and adjacent tissues in a case of mucocutaneous leishmaniasis due to *L. (V.) braziliensis*; f: a section of the mucosal lesion of the same patient, showing a dense and diffuse infiltration of lymphocytes and plasma cells in the corion of mucosal tissues (x 400); g: typical nodular cutaneous lesions on the arm of a patient with anergic diffuse cutaneous leishmaniasis, due to *L. (L.) amazonensis*; h: a section of a lesion from the same man: note the extensive infiltration of highly parasitized macrophages in the dermis, with rare lymphocytes and plasma cells (x 400).

follows: In the cases of LCL and MCL due to *L. (V.) braziliensis* we found a very high antigen-specific CD4+ Th1 immune response activation at the lymph nodes. It is suggested that, in consequence, the CD4+ T cells recruited from the peripheral blood to the inflamatory infiltrate of cutaneous and mucosal lesions are preferentially primed to operate as cytokine Th1 – producing cells (IFN-γ in cutaneous lesions, and IFN-γ and TNF-α in mucosal lesions). In this way, it is possible that the cellular hypersensitivity immune response recorded in these patients was largely the result of a prolonged antigen-specific CD4+ Th1 activation by *L. (V.) braziliensis*. This culminated in a high production of IFN-γ in the mucous membrane lesions and, consequently, the TNF-α. It is TNF-α that is considered to be the major cytokine responsible for damage to the mucosal tissue (Castes et al. 1993, Da-Cruz et al. 1996, Ribeiro-de-Jesus et al. 1998, Blackwell 1999). In this context, it is noteworthy that in a retrospective evaluation of 85 cases of MCL examined in Amazonian Brazil, it was concluded that the mean time between the beginning of mucosal symptoms and the inicial cutaneous lesion(s) was nearly five years (Silveira et al. 1999).

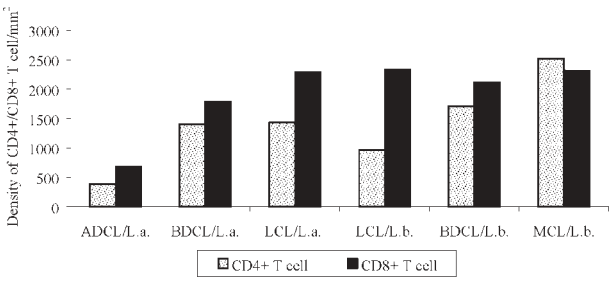

Fig. 2: immunocytochemistry profiles of CD4+ and CD8+ T cells in cutaneous and mucosal lesions of American cutaneous leishmaniasis (ACL) from Amazonian Brazil. ADCL: anergic diffuse cutaneous leishmaniasis; BDCL: borderline disseminated cutaneous leishmaniasis; LCL: localized cutaneous leishmaniasis; MCL: mucosal leishmaniasis; each clinical group contained 5 to 8 patients: ADCL had the lowest number of cases (5); L.a.: *Leishmania (L.) amazonensis*; L.b.: *Leishmania (V.) braziliensis*; CD4+ T cell: lymphocyte T CD4+; CD8+ T cell: lymphocyte T CD8+. For recognition of these two types of T cells, the following monoclonal antibodies were used: Anti-Human T cell CD45RO (clone OPD4) and Anti-Human T cell CD8 Supressor/Cytotoxic (clone DK25) (DAKO-Denmark), respectively. The technical procedures were as used by Corbett et al. (2001).

With regards IgG antibody response in MCL patients, in Amazonian Brazil there have been found, in general, moderate levels of anti-*Leishmania* IgG antibodies (mean titre 640) as measured by IFAT using *L. (L.) amazonensis* and *L. (V.) shawi* as antigens for this assay (Silveira et al. 1999, Corrêa et al. 2003). In the Southern region of Brazil, similar results have been obtained by other workers using IFAT and ELISA assays to investigate the antibody response of MCL patients (Guimarães et al. 1974, Valli et al. 1999). These results suggest the presence of a weak CD4+ Th2 immune response in the peripheral blood of MCL patients, functioning together with a very highly activated CD4+ Th1 immune response, also in the peripheral blood and particularly in the mucosal lesions of these patients. As a result, the therapy of MCL patients gives

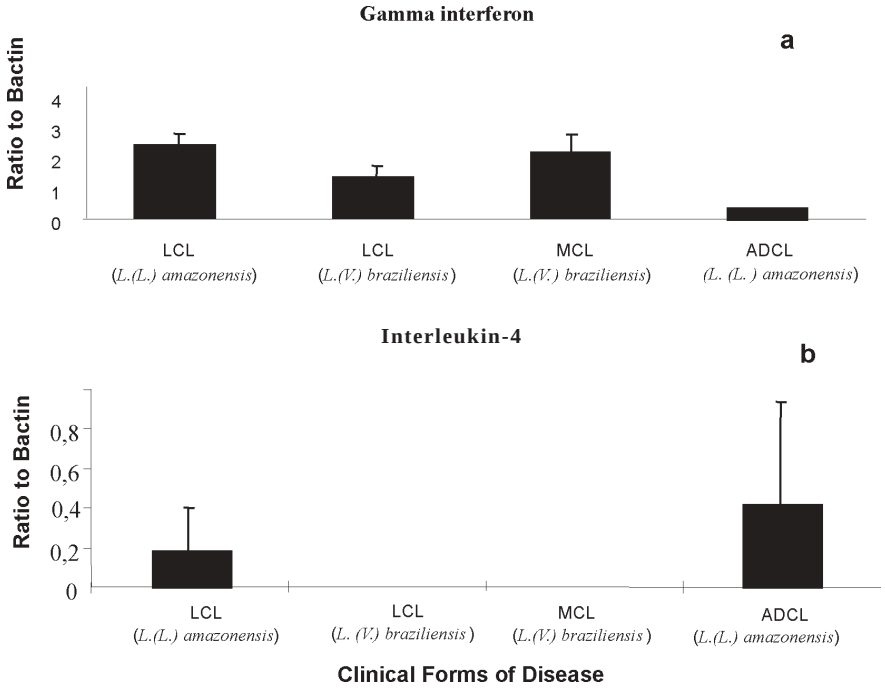

Fig. 3: semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis for cytokines in American cutaneous leishmaniasis. a: gamma interferon mRNA and b: interleikin-4 mRNA in biopsies of cutaneous and mucosal lesions from ACL patients. LCL: localized cutaneous leishmaniasis; MCL: mucocutaneous leishmaniasis; ADCL: anergic diffuse cutaneous leishmaniasis. *Patients and samples*: each clinical group contained 5 to 8 patients (ADCL had the lowest number: 5). Skin biopsy specimens (4-mm punch) were put into cryoembedding medium, flash frozen, and stored at –70°C for RT-PCR. The sequence of technical procedures of *RNA isolation*, *Reverse Transcription (RT)*, *cDNA synthesis (PCR)*, and *Hybridization of PCR product* followed that used by Moraes et al. (1999). For each cytokine, the mean concentration ± SD is shown for the four clinical groups of patients.

satisfactory results mainly in cases of short evolution, in which there is no large area of ulceration (necrosis) of the mucous tissue (Marsden et al. 1984, Marsden 1986).

ANERGIC DIFFUSE CUTANEOUS LEISHMANIASIS

ADCL is the most common form of the disease seen at the cellular hyposensitivity pole. It is a relatively rare form of New World cutaneous leishmaniasis caused by leishmanial parasites of the subgenus *Leishmania*. ADCL was first described in Venezuela (Convit & Lapenta 1946) as a bizarre form of ACL having the following characteristics: nodular lesions disseminated all over the body and rich in amastigotes, a negative Montenegro skin-test, and a frequent failure to respond to conventional antimony treatment. After its discovery, new cases of ADCL were recorded in Venezuela and, subsequently, in other countries of the Americas [Bolivia, Brazil, Colombia, the Dominican Republic, Honduras, Mexico, United States of America (southern Texas) and Peru]. *L. (L.) pifanoi*, *L. (L.) mexicana*, and *L. (L.) amazonensis* are the causative agents involved (Lainson & Shaw 1998).

In Brazil, *L. (L.) amazonensis* is considered to be the only species causing ADCL (Lainson 1983, Silveira et al. 1997a, Lainson & Shaw 1998). The disease is clinically characterized by a diffuse infiltration of the skin, on which appear a large number of nodules, papules, tubercules, and infiltrated plaques that rarely become ulcerated (Fig. 1g). In older cases of the disease, disseminated lesions may cover much of the body, but are predominantly on the extremities and rarely involve the nasopharyngeal mucous membranes (Convit et al. 1972, 1993, Barral et al. 1995). In the dermis the histopathological feature is a severe infiltration of macrophages containing abundant amastigotes: lymphocytes and plasma cells are rare, giving the infiltration the aspect of a macrophagic granuloma (Bittencourt & Guimarães 1968, Silveira et al. 1990, Bittencourt & Barral 1991, Moraes & Silveira 1994) (Fig.1h).

In terms of immunology, the DTH to *Leishmania* antigen and the lymphocyte proliferation assay are always negative in ADCL cases, indicating that in these patients the cell-mediated immune mechanisms are incapable of specifically controlling the leishmanial infection (Petersen et al. 1982, Barral-Netto et al. 1998, Silveira et al. 1998). In support of this conclusion an immunocytochemistry assay of five ADCL patients from Amazonian Brazil, showed the lowest level of CD4+ and CD8+ T cells seen in all forms of ACL studied (Fig. 2). This, together with the demonstration of the weakest expression of mRNA to IFN-γ and the strongest expression of mRNA to IL-4 (4 times more in some cases) in cutaneous lesions of the same patients (Fig. 3), clearly confirms that the CD+4 Th2 immune response is predominant in ADCL disease (Ribeiro-de-Jesus et al. 1998, Barral-Neto et al. 1998). Moreover, unlike LCL and MCL patients infected with *L. (V.) braziliensis* [in which there was demonstrated a much higher expression of mRNA to IFN-γ than that to IL-4 in the *L. (V.) braziliensis*- stimulated PBMC], ADCL patients showed a reverse situation of the cytokines; i.e., two times more expression of mRNA to IL-4 than that to IFN-γ (data not shown). These findings reinforce the argument that in ADCL patients due to *L. (L.) amazonensis* there is likely to have been a very high antigen-specific CD4+ Th2 immune response activation at the lymph nodes, resulting in a proliferation of CD4+ T cells primed to operate as Th2 cytokine-producing cells (mainly IL-4 and IL-10) in the peripheral blood as well as in the cutaneous lesions. As a result, conventional therapy is very frequently accompained by relapses of cutaneous lesions of ADCL patients (Bonfim et al. 1996), due to the very poor CD4+ Th1 immune response in these cases. Although specific anti-*Leishmania* IgG antibodies are present in great concentrations (mean titre 20.480 by IFAT) in the serum of these patients (unlike individuals with LCL and MCL due to *L. (V.) braziliensis*), there is no evidence of their influence in controlling *L. (L.) amazonensis* infection (Chagas et al. 1999, 2001).

BORDERLINE DISSEMINATED CUTANEOUS LEISHMANIASIS

Together with cases of LCL, between the two poles of MCL and ADCL, a few patients may present disseminated forms of infections that have been referred to as BDCL, and in which it has been possible to determine the location of the primary skin lesion(s) and the secondary ones (Silveira et al. 1997b). In those cases with infection due to *L. (V.) braziliensis* or other species of the subgenus *Viannia*, the process of dissemination is relatively rapid. It may take place in two or three months, when a hundred or more erythematous-papules (acneiform lesions) and/or ulcerated cutaneous lesions may appear (Fig. 4a). The histology of this picture normally shows a nodular infiltration of lymphocytes and plasma cells in the dermis, with rare macrophages and parasites (Fig. 4b). This is the commonest situation seen in the acute phase of infection but, in cases of delayed evolution (over 1 year), some untreated patients may present with simultaneous cutaneous and nasopharyngeal mucosal lesions. This indicates that active cutaneous infections may persist for prolonged periods and that, in these cases, mucosal lesions represent the final result of infection. Among three of our patients with this condition acquired in Pará, a 62 years old man presented with disseminated ulcerated and infiltrated cutaneous lesions of about 15 years duration and a nasal mucosal lesion which appeared during the last 2 years of his disease (Fig. 4c). In these cases, cutaneous lesions may be accompained by an epithelioid granuloma in the dermis (Fig. 4d).

During dissemination of the parasite, which is a critical stage of the disease, the DTH to *Leishmania* antigen and the lymphocyte proliferation assays are generally negative, reflecting some inhibition of the cell-mediated immune mechanisms (CD4+/CD8+ T cells) in these patients. This has prompted adoption of the term "borderline" in an attempt to characterize an *incomplete failure* of the cellular immune response in controlling the leishmanial infection. Moreover, there is evidence from an immunocytochemistry analysis that among the memory T cells present in the cellular infiltrate of cutaneous lesions in these cases there is a significant amount of CD4+ and CD8+ T cells subsets at an intermediate level between LCL and MCL (CD4+: LCL<BDCL<MCL, CD8+: LCL>BDCL<MCL) (Fig. 2). Although the profiles of Th1/

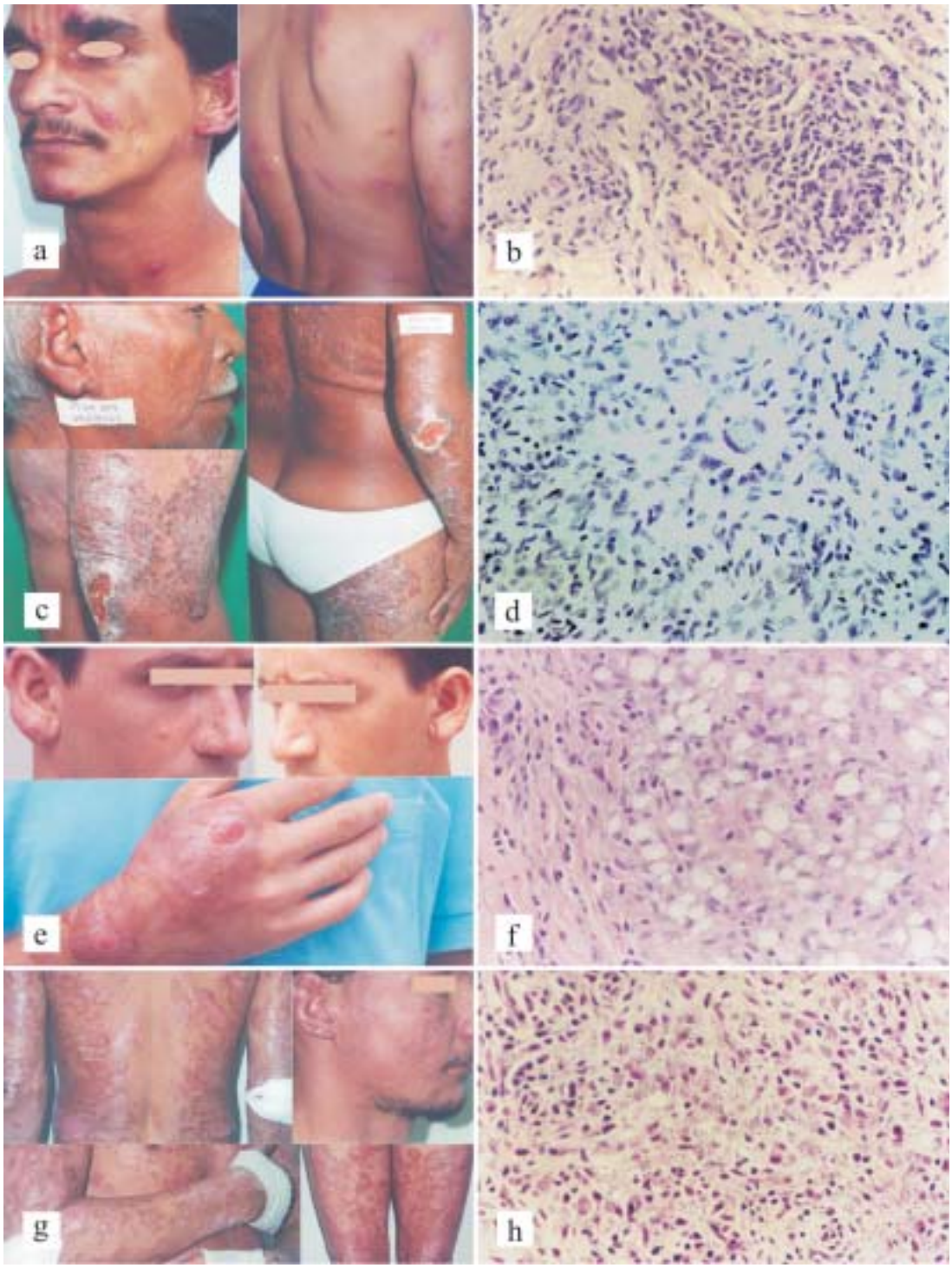

Fig. 4a: typical acneiform lesions disseminated during three months on the head, trunk and arms in a case of acute borderline disseminated cutaneous leishmaniasis (BDCL) due to *Leishmania (Viannia) braziliensis*; b: a histological section of a lesion from the same patient, showing a nodular infiltration of lymphocytes and plasma cells in the dermis, with rare macrophages and parasites (x 400); c: a 15 year-old cutaneous infiltration, principally on the trunk, arms and legs, disseminated from primary ulcerated lesions on left knee and right elbow of an individual with chronic BDCL infection due to *L. (V.) braziliensis*; d: a section of a lesion from the same patient exhibiting an epithelioid granulomatous reaction in the dermis (x 400); e: a primary infiltrated cutaneous lesion, of about 18 months' evolution, on the right hand of a BDCL patient infected with *Leishmania (Leishmania) amazonensis*. Secondary lesions can be seen on the right and left ears and right wing of the nose; f: a section of the lesion on the hand of the same man, showing large collections of vacuolated and heavily parasitized macrophages in the dermis with surrounding groups of lymphocytes and plasma cells (x 400); g: a 7 year-old eritematous infiltration of the skin, affecting almost the whole body, in a case of BDCL due to *L. (L.) amazonens*. There are some nodular lesions at the extremities, originating from two primary infiltrated ulcerative lesions on the right hand and elbow; h: a section of an ulcerated and infiltrated lesion of the same patient, showing the extensive infiltration of heavily parasitized macrophages in the dermis and some surrounding groups of lymphocytes and plasms cells (x 400).

Th2 cytokines from PBMC or from cutaneous lesions of these cases have not yet been studied, it is very likely, therefore, that the presence of these two types of T cells in the inflammatory infiltrate of cutaneous lesions, together with antimony therapy, may result in a resolution of the disease equal to that obtained by a restoration of the cell-mediated immune responses (e.g.positive DTH and lymphocyte proliferation tests) of these patients. It would appear that in these BDCL patients the CD4+ Th1 immune response is at least partially preserved, and functions in such a way as to overcome the opposite CD4+ Th2 immune response. Supporting this hypothesis, is the fact that the serum of these patients shows a low to moderate level (mean titre 640) of specific anti-*Leishmania* IgG antibodies by IFAT (Silveira, unpublished observation), confirming the presence of a weak antibody response.

In the case of BDCL caused by *L. (L.) amazonensis*, differences can readily be indicated in relation to the dissemination of the parasite and the outcome of disease. Firstly, we have recorded cases in which the dissemination of *L. (L.) amazonensis* has taken place only after 6 months following the appearance of the primary cutaneous lesion, and during this period it was limited to a maximum of six detectable metastatic lesions seen in eight male, adult patients examined, almost all of them with 1 to 2 years of disease (Fig. 4e). In the dermis of these cases it is possible to find large collections of vacuolated and heavily parasitezed macrophages, surrounded by groups of lymphocytes and plasma cells (Fig. 4f). The exceptional case was a 27 years old man who had been mistakenly treated for lepromatous leprosy during the past 7 years. The patient had large area of skin compromised by an erythematous infiltration and some nodular lesions at the extremities (Fig. 4g). The histology of his lesions was marked by an extensive infiltration of heavily parasitized macrophages with some surrounding groups of lymphocytes and plasma cells in the dermis (Fig. 4h). This case and the 3 cases of BDCL due to infections of *L. (V.) braziliensis* with a long period of evolution represent patients with the major characteristic of this form of disease, as they were on the verge of converting to ADCL and MCL, respectively. This suggests that without the intervention of therapy these leishmanial infections had been in a continuous process of interaction with the host immune response for a long time. Secondly, lymphatic dissemination of infection was demonstrated in 7 of our 8 patients, with *L. (L.) amazonensis* recovered in the culture of material from enlarged lymph nodes in Difco B45 culture medium. This is an interesting finding which seems not to have been recorded in LCL or ADCL forms of *L. (L.) amazonensis* infection. The explanation of this remains uncertain. It might be regarded as an attempt of the host cell-mediated immune mechanisms to change the repertoires of the antigen-specific CD4+ T cells' activation at the lymph node for a beneficial CD4+ Th1 immune response or, on the contrary, an evasive mechanism of *L. (L.) amazonensis* in maintaining the pathogenic (CD4+ Th2) profile of the cellular immune response. The lesions of BDCL patients may be principally in the form of infiltrated plaques, localized at the extremities but absent in the nasopharyngeal mucous membrane.

In terms of cell-mediated immunity, it has been noted that the immune mechanisms of patients with BDCL due to *L.(L.) amazonensis* are more intensely inhibited than in the cases of infection due to *L. (V.) braziliensis*, resulting in a totally negative response of the DTH to *Leishmania* antigen and to the lymphocyte proliferation assay in all cases. This severe, *but incomplete*, inhibition of the cell-mediated immune mechanisms is also evidenced by immunocytochemistry assay. This shows a smaller amount of CD4+ and CD8+ T cells in the dermal infiltrate of these patients than in those infected with *L. (V.) braziliensis*, but also at an intermediate level between LCL and ADCL (CD4+: LCL>BDCL>ADCL, CD8+: LCL>BDCL>ADCL) (Fig. 2). As a result, these patients have been cured after a period of nearly six months of conventional antimony therapy. Even without precise information on the cytokine (Th1/Th2) profiles, both for PBMC and for cutaneous lesions, this suggests to us that, in part, the CD4+ Th1 immune response must have been preserved among the memory T lymphocytes infiltrating the cutaneous lesions of these patients. Favouring this hypothesis is the histopathological characteristic of the BDCL lesion. It differs from that of the seemingly incurable ADCL patients by the marked presence of lymphocytes and plasma cells in the dermal infiltrate, even though there may be a significant number of parasitized macrophages. In contrast to BDCL caused by *L. (V.) braziliensis*, which normally produces low to moderate levels of anti-*Leishmania* IgG antibodies (mean titre 640), there have been demonstrated higher levels of these antibodies (mean titre 5.120) shown by IFAT in patients with BDCL due to *L. (L.) amazonensis* (Chagas et al. 1999, 2001). This indicates a lesser CD4+ Th1 immune response activation in these patients.

DISCUSSION

The spectrum of clinical and immunopathological manifestations of ACL has been the subject of many investigations in attempts to fully understand the host immune mechanisms that are playing a crucial role in the pathogenesis of the disease. However, most of these investigations have neglected two very important features: (1) the accurate identity of the specific leishmanial parasite that is stimulating the host immune response and, (2) the quality and the magnitude of the host immune response stimulated by this specific leishmanial parasite. The spectrum of ACL proposed here is, therefore, based on characterization of the leishmanial parasites responsible for ACL and the subsequent cellular and humoral immune responses elicited by these parasites, with special reference to the disease in the Amazon region of Brazil.

With rare exceptions, cases of LCL occupy the middle of this spectrum, and this form of the disease may be caused by any one of the species of *Leishmania* within the subgenera *Viannia* and *Leishmania*. However, although LCL is regarded as having a well balanced cellular immune response with a very high level of resistence to infection (CD8+>CD4+ and Th1>Th2), there is not yet a general agreement as to which type of T cell (either CD4+ or CD8+) is predominant in the infiltrate of the cutaneous lesions of patients. For example, Barral et al. (1987), Pirmez et al. (1990), and Esterre et al. (1992) found a higher

predominance of CD4+ T cells but, in contrast, Modlin et al. (1985), Martinez-Arends et al. (1991), Isaza et al. (1996) and Vieira et al. (2002) showed that CD8+ T cells are the most frequent type of lymphocyte. In Amazonian Brazil, however, our results of immunocytochemistry analysis (Fig. 2) have conclusively shown that CD8+ T cells were present at a higher level in all forms of the disease (with the one exception of MCL) including cases of LCL due to both *L. (V.) braziliensis* and *L. (L.) amazonensis*. These results, then, seem to confirm the role of CD8+ T cells in a well balanced immune response to LCL and, probably, in the process of cure: this has also been confirmed by Coutinho et al. (1998). There is experimental evidence indicating the participation of CD8+ T cells in the process of cure of murine leishmaniasis, as well as the role of these cells in the production of IFN-γ (Chan 1993, Conceição-Silva et. al. 1994). In addition, a semi-quantitative RT-PCR (Fig. 3) has shown that the CD4+ Th1 immune response was more intensely associated with patients with LCL due to parasites of the subgenus *Viannia* (especially *L. (V.) braziliensis*) than with patients infected by *L. (L.) amazonensis*. These results, together with previous clinical and cell-mediated immune evaluations (Silveira et al. 1991, 1998), have confirmed that *L. (V.) braziliensis* has a greater ability to stimulate a CD4+ Th1 immune response than has *L. (L.) amazonensis*, and that the latter parasite is a very high stimulator of a CD4+ Th2 immune response. Supporting this is recent experimental evidence showing that amastigotes of *L. (L.) amazonensis* are able to condition dendritic cells of BALB/c mice to promote a CD4+ Th2 immune response activation (Qi et al. 2001). When considering all these findings, dichotomy of the clinical and immunopathological spectrum of ACL proposed here is more readily understood: i.e., from the middle of this spectrum, and depending on the species of *Leishmania* involved, there are some patients in which the infection escapes from the cell-mediated immunity mechanisms and evolves to one or other of the two poles of disease. In cases of infection due to *L. (V.) braziliensis* and, more rarely, other species of the subgenus *Viannia*, this infection generally leads to the cellular hypersensitivity pole represented by MCL (CD4+>CD8+ and Th1>Th2). On the opposite side of the spectrum, in cases of infection due to *L. (L.) amazonensis* and some other species of the subgenus *Leishmania*, infection generally leads to the cellular hyposensitivity pole represented by ADCL (CD8+>CD4+ and Th1<Th2).

In the case of MCL, there are at least two aspects of major interest to discuss here regarding the immunology of this form of ACL. Firstly, it has been shown that MCL is characterized by a highly antigen-specific T cell immune response against *L. (V.) braziliensis* and that this strong reaction corroborates with deviation of the immune response of patients to the cellular hypersensitivity pole of the spectrum. This immunological status may be evidenced at a clinical level by an exacerbated DTH, particularly to a homologous antigen of *Leishmania* [e.g. *L. (V.) braziliensis*] and, at in vitro evaluation, by the lymphocyte proliferation assay (Cástes et al. 1983, Carvalho et al. 1985, Convit et al. 1993, Silveira et al. 1998). Secondly, an immunocytochemistry assay among our ACL patients from Amazonian Brazil has indicated that MCL was the only clinical form of disease in which CD4+ T cells were more predominant than the counterpart CD8+ T cells. We feel it must be more than coincidental that this occurs only in MCL, which is closely associated with the cellular hypersensitivity pole within the ACL spectrum. Considering the pronounced cellular hypersensitivity immune response in MCL, one would expect to find high levels of CD4+ Th1 cytokines (IFN-γ, IL-2, and TNF-α, mainly) in mucosal lesions of these patients. On the contrary, however, there is evidence that a "mixture" of CD+4 Th1 and Th2 immune responses occur in MCL patients from Venezuela (Cáceres-Dittmar et al. 1993, Castes et al. 1993) and Southeast of Brazil (Pirmez et al. 1993). This differs from our results in Amazonian Brazil where a typical CD4+ Th1 immune response was found in MCL patients; i.e., a high expression of mRNA to IFN-γ and a complete lack of mRNA to IL-4 in mucous membrane lesions of these patients. In fact, these results were not very different from those of Cáceres-Dittmar et al. (1993), who found a high expression of mRNA to IFN-γ and a low expression of mRNA to IL-4 in their cases of MCL. They differed very much, however, from those of Pirmez et al. (1993), whose demonstrated the highest level of mRNA expression of IL-4 in cases of MCL, approximately 3 times more than was indicated by Cáceres-Dittmar et al. (1993). Moreover, as Pirmez et al. (1993) also found an increased mRNA expression of IL-10 in their samples of MCL, they concluded that there is a mixture of Th1 and Th2 immmune responses in MCL. In addition, it may be emphasized that in our study in the Amazon region of Brazil we also demonstrated both cytokines, IFN-γ and IL-4, in the *L. (V.) braziliensis* stimulated PBMC from MCL patients. Although this, too, we regard as "a mixture" of CD4+ Th1 and Th2 immune responses, the level of mRNA expression of IFN-γ was very much higher (≥ 6 times) than that of IL-4, and this does characterize a functional CD4+ Th1 immune response. Supporting this suggestion is the finding of Barral et al. (1998) of a very much higher level of IFN-γ, which was over 10 times that of IL-10, in supernatants from cultures of *Leishmania*-antigen stimulated cells of MCL patients. Our conclusion, remains, therefore, that in MCL patients there is probably a very highly antigen-specific CD4+ Th1 immune activation at lymph nodes by antigen-presenting cells (e.g. cells of Langerhans) primed by *L. (V.) braziliensis*, and a minimal CD4+ Th2 immune activation: this results in high levels of gene expression of IFN-γ and minimal levels of IL-4 in PBMC. In consequence, T cells recruited from the peripheral blood to the inflamatory infiltrate of mucosal lesions (the focus of leishmanial infection) are preferentially primed to operate as Th1 cytokines (IFN-γ, IL-2 and TNF-α, principally). In this respect, Costa et al. (2003) have recently demonstrated the role of adhesion molecules (CD11a, CD11b and CD62L) in determining the preferential address of T cells to inflamatory sites of leishmaniasis lesions, especially their effect on the highest expression of CD11a in CD4+ T cells. This raises speculations regarding the possible role of adhesion molecules in promoting a CD4+ Th1 immune activation by *L. (V.) braziliensis*.

In the case of ADCL, there seems to be a less conflict-

ing situation regarding its immunology. It has been shown that there is "mixture" of Th1 and Th2 cytokines in PBMC and cutaneous lesions of ADCL patients from the Amazon region, resulting in very low levels of mRNA expression of IFN-γ and very high levels of mRNA expression of IL-4, respectively. There is general agreement, however, that there is a high predominance of CD4+ Th2 immune response in these patients, which has resulted in ADCL being regarded as a polar form of ACL. The view that has been expressed in most situations is that MCL is an intermediate form between LCL and ADCL and, consequently, the disease has been linked to a mixture of Th1 and Th2 immune responses (Convit et al. 2004). We feel it more correct, however, to regard MCL and ADCL as polar forms of ACL, in the hypersensitivity pole (high Th1 immune activation) and the hyposensitivity pole (high Th2 immune activation), respectively, with LCL (moderate Th1 immune activation) occupying the middle of this spectrum. Accepting the existence of an immunological mixture of Th1 plus Th2 immune responses in the spectrum of ACL this would represent BDCL. In this way, then, between LCL and the extreme pathogenicity poles MCL and ADCL, there are a few patients presenting disseminated forms of infections due to parasites of the subgenera *Viannia* and *Leishmania*, respectively, which we regard as the intermediate form, BDCL. This form of the disease is characterized by an incomplete inhibition of the cellular immune response but with clinical, histopathological and immunological evidence suggesting that, at least partially, the CD4+ Th1 immune response of these patients is preserved (CD8+>CD4+ and probably Th1≥ Th2) (Fig. 5).

A number of previous publications have discussed some clinical features which may be related to BDCL. Thus, Bryceson (1969) was the first to use a clinical and histopathological classification, similar to that applied in leprosy, in order to study 33 cases of ADCL disease caused by *L. (L.) aethiopica* in Ethiopia. He considered five of these as being of the "intermediate histopathological pattern II" (i.e. a "borderline" form). Two of them gave a positive DTH to *Leishmania* antigen, and two others had early evolution of the disease confined to the face. These 5 cases might be considered analogous to the cases of BDCL caused by *L. (L.) amazonensis* in Brazil.

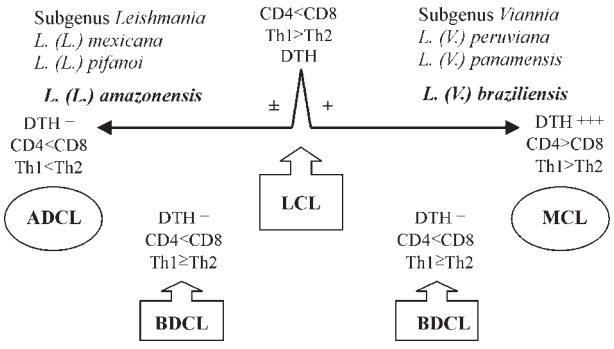

Fig. 5: American cutaneous leishmaniasis: clinical and immunopathological classification according to the species of *Leishmania*. ADCL: anergic diffuse cutaneous leishmaniasis; LCL: localized cutaneous leishmaniasis; MCL: mucocutaneous leishmaniasis; BDCL: borderline disseminated cutaneous leishmaniasis; DTH: delayed type hypersensibility; CD4: T lymphocyte CD4; CD8: T lymphocyte CD8; Th1: Th1 immune response; Th2: Th2 immune response.

Moriearty et al. (1978) first used the term "borderline" when they described a case of leishmaniasis from a region with a high incidence of the mucocutaneous disease caused by *L. (V.) braziliensis* in the state of Bahia, Brazil. The patient had multiple nodular, papular and ulcerovegetative skin lesions as well as a lesion in the nasal septum mucosae and, on the basis of Bryceson's work, these authors regarded the case as borderline, between MCL and ADCL, because the patient had a negative DTH to leishmanial antigen (as in ADCL). The test was also negative using other antigens, including 2,4 dinitrochlorobenzene (DNCB), indicating, most likely, the existence of a nonspecific immunodeficiency which might have been interfering with the patient's immune response.

Convit et al. (1989) used the term "intermediate form" to designate a chronic (1 to 30 years of evolution) and cell-mediated hypersensitivity form of ACL recorded in 11 patients from Venezuela, most of them presenting with multiple verrucose and ulcerovegetative skin lesions caused by *L. (V.) braziliensis* (10 cases) and *L. (V.) panamensis* (1 case). After a treatment schedule using immunotherapy alone (heat-killed *L. (L.) amazonensis* promastigotes plus BCG) for 6 cases, and immunotherapy plus chemotherapy (conventional antimonial Glucantime) for the other 5, all patients were clinically cured. Later, Convit et al. (1993) presented a review of the clinical and immunological spectrum of ACL in which they considered LCL and DCL as immunologically reactive and nonreactive polar forms of the disease, respectively, and mucosal leishmaniasis (ML) as a hipereactive and intermediate form between the LCL and DCL forms in this spectrum.

Costa et al. (1986) described some clinical and immunological aspects of 8 cases of disseminated cutaneous leishmaniasis from the state of Bahia, in Northeast Brazil. Isolates of the parasites were made from 5 patients, with 4 identified as *L. (V.) braziliensis* and only 1 as *L. (L.) amazonensis*. These authors noted that all the patients, with one exception, had a negative DTH to *Leishmania* antigen before the treatment and converted to positive after cure of disease. All of them had presented moderate levels of circulating anti-*Leishmania* IgG antibodies and 5 responded well to pentavalent antimonial therapy, which suggested a functioning cellular (Th1) immune response.

Finally, Carvalho et al. (1994) also presented some clinical and immunopathological aspects seen in 8 cases of disseminated cutaneous leishmaniasis from the northeast state of Bahia. Contrary to the patients of Costa et al. (1986), however, 5 of these individuals were infected with *L. (L.) amazonensis*. There were some interesting clinical aspects which differed from those seen in BDCL due to the same parasite in Amazonian Brazil. These included a very high number of cutaneous lesions (as many as 75 to 800), which appeared in only 1 to 6 months; papules and acneiform lesions, which were the most frequent cutaneous manifestations; dissemination of infection, in 4 cases, occurring in a period as short as only 2 days; 3 patients

presented mucosal lesions and 5 of the 8 cases had no adenopathy. Some features, however, did resemble BDCL due to *L. (L.) amazonensis*. Thus, in 4 cases the DTH to *Leishmania* antigen and the lymphocyte proliferation assay were negative, and there was also a decreased level in CD4+ T cell markers. These immunological deficiencies were restored, after antimony therapy, in a manner similar to that seen in our BDCL patients from Amazonian Brazil.

In summary, based on the clinical and immunopathological aspects of ACL, BDCL would seem a logical term to clinically classify those forms of disseminated infections, with parasites of the subgenera *Viannia* [e.g. *L. (V.) braziliensis*] and *Leishmania* [e.g. *L. (L.) amazonensis*] which appear to be intermediate between LCL and the extreme pathogenicity poles of MCL and ADCL respectively. Such infections have the potential to evolve, however, to either one of these poles, depending on the species of *Leishmania* causing the disease.

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