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The clinical and immunological spectrum of American cutaneous leishmaniasis

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Abstract

American cutaneous leishmaniasis is characterized by a spectrum of clinical manifestations. These include localized, often self-healing single lesions, intermediate forms which frequently produce mucosal lesions and often show exaggerated delayed-type hypersensitivity (DTH), and the rare diffuse cutaneous leishmaniasis in which no reaction of protective cell-mediated immunity or DTH can be demonstrated. Clinical, pathological and immunological studies have begun to unravel some of the mechanisms associated with different disease manifestations, dependent on complex interactions between the host immune response, measured in terms of indices including lymphocyte subsets and lymphokines *in vitro* and within active lesions, and different species of *Leishmania*.

Introduction

In earlier publications (CONVIT, 1974; CONVIT & PINARDI, 1974), we have described the clinical and immunological spectrum of American cutaneous leishmaniasis (ACL). The resistant polar form of ACL at one extreme of the spectrum is represented by localized cutaneous leishmaniasis (LCL). This form of the disease is characterized by a single or few ulcerated lesions with an immune granulomatous structure consisting, in typical cases, of tuberculoid and epithelioid-type nodules, marked infiltration by lymphoid cells, and few parasites. This typical histological pattern, particularly evident in early non-ulcerated lesions, is altered in older lesions because of chronic secondary bacterial infection of the open ulcers or by the intensity of the necrotic ulcerative process. Antigen-specific cell-mediated immune (CMI) reactions *in vivo* and *in vitro* as well as *Leishmania*-specific serological reactions are frankly positive in essentially all cases of LCL unless an exceptionally early diagnosis is made.

Diffuse cutaneous leishmaniasis (DCL), the opposite polar form of ACL, is usually characterized by numerous non-ulcerated nodules and plaques with a histopathological granulomatous structure formed by undifferentiated macrophages containing large numbers of parasites and few lymphoid elements. Antigen-specific CMI reactions *in vivo* and *in vitro* are absent, but the antibody response to *Leishmania* is conserved and often accentuated. The lesions of DCL are resistant to chemotherapy, and relapse almost invariably occurs after treatment, in spite of transient improvement and apparent healing in some cases. DCL undoubtedly reflects severe antigen-specific T cell deficiency of the infected host (CONVIT *et al.*, 1972). The frequency of this form of ACL is very low, less than 0·1% of the total cases of ACL in Venezuela, where this form of disease was first described (CONVIT & LAPENTA, 1948). Interestingly enough, all isolates of DCL have been classified as members of the *L. mexicana* complex (LAINSON, 1983; personal observations) in spite of the predominance of infection by *L. braziliensis* in Venezuela and essentially all of South America.

We have placed the mucosal and verrucous lesions of ACL in the intermediate area of the spectrum. These lesions are often diagnosed after a long period of development; careful history taking and physical examination often reveal the characteristic scar of an earlier healed cutaneous ulcer, although in some cases the intermediate complications of the mucosal tissues coincide with the presence of the initial cutaneous lesion (MARSDEN, 1986). Exaggerated antigen-specific CMI reactions have been reported in the intermediate area of the spectrum (CASTES *et al.*, 1983; CARVALHO *et al.*, 1985) and levels of circulating antibodies are often high, in spite of modest numbers of parasites in many of these lesions. Relapse is frequent after chemotherapy. 'New World' mucocutaneous leishmaniasis (MCL) associated with a positive CMI response is apparently invariably associated with infection by isolates of the *L. braziliensis* complex.

Studies carried out during the last decade, which include clinical, parasitological and immunological aspects of ACL as well as the application of immunotherapy, have permitted us to make a number of observations which we consider of interest and usefulness in the understanding and management of this disease, which constitutes an important endemic throughout much of Central and South America.

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Patients and Methods

The principal immunological results presented in this paper are based on the results of studies on a group of 211 Venezuelan patients with ACL, including 130 patients with LCL, 20 with DCL and 61 with MCL, diagnosed at the Instituto de Biomedicina, Caracas, Venezuela. Only patients with a confirmed parasitological diagnosis (direct observation of parasites in stained imprints or tissue sections, culture of parasites in artificial media or in hamsters, or a positive *Leishmania*-specific polymerase chain reaction) have been included in this study. Most of the DCL patients in this group had received repeated and prolonged treatment.

Routine immunological tests in all patients included (i) leishmanin intradermal skin tests with 0·1 mL of an autoclaved suspension of *L. mexicana pifanoi* promastigotes ($6{\cdot}25\times10^6$/mL), injected intradermally and read at 48 h (CONVIT *et al.*, 1989) and (ii) enzyme-linked immunosorbent assays (ELISAs) for the measurement of circulating antibodies, using a formalin-treated promastigote antigen prepared from *L. braziliensis braziliensis* strain MHOM/BR/75/M2903 and a polyvalent peroxidase-labelled anti-immunoglobulins G, A and M second antibody (ULRICH *et al.*, 1988). With other procedures which have been applied to limited numbers of patients, the details of methods are included in the appropriate references.

Results and Discussion

Localized cutaneous leishmaniasis

At least 90% of the cases of ACL diagnosed in Venezuela (and in the Americas) can be assigned to the relatively benign LCL polar form of disease, with single, few or occasionally numerous ulcerated lesions. Within this group there is a significant tendency to self-healing of the lesions, variable within different foci of the disease but reportedly as high as 65% in some areas (COSTA *et al.*, 1987). Males are more commonly infected than females in Venezuela, in a proportion of about 2:1. While immunological factors may contribute to this difference, epidemiological and gender-related behavioural patterns such as occupation associated with greater exposure to vectors may be of greater importance. Histopathological examination of very early LCL lesions shows the typical tuberculoid structure described above; the necrosis observed in ulcerated lesions results in considerable disorganization of the histological structure (RIDLEY, 1980).

Leishmanin tests were positive (reaction diameter >10

Table 1. Immunological characteristics of the three principal clinical manifestations of American cutaneous leishmaniasis

Clinical form	No.	ELISA[a] OD±SEM	ELISA[a] Positive (%)	Leishmanin test mm±SEM	Leishmanin test Positive (%)	No.	LTT-*Leishmania*[b] SI±SEM	LTT-*Leishmania*[b] Positive (%)
Localized	130	0·77±0·02	92·3	17·6±0·7[c]	93·1	88	7·49±1·29	55·7[d]
Mucocutaneous	59	0·82±0·05	88·1	28·6±1·9[c]	98·3	42	11·47±1·90	81·0[d]
Diffuse	18	0·65±0·05	88·9	2·4±0·8	5·5	10	1·97±0·25	0
Controls	33	0·24±0·02	6·1	nd[e]			nd[e]	

[a]Enzyme-linked immunosorbent assay; OD=mean optical density, SEM=standard error of the mean.
[b]*Leishmania* antigen-induced lymphocyte transformation; SI=stimulation index.
[c]Difference statistically significant ($P<0·001$).
[d]Difference not statistically significant ($P=0·084$).
[e]nd=Not done.

mm) in the vast majority of cases of LCL (93% in our group of 130 patients) at the time that diagnosis was made, i.e., in the presence of active lesions, even of relatively short development (Table 1). No difference was observed in the average reaction size between males and females (17·7 mm [$n=87$] and 17·8 mm [$n=43$], respectively). Exaggerated DTH reactions, defined arbitrarily as leishmanin reactions greater than 30 mm in diameter, are rather unusual in LCL and were observed in 5·4% of the group of patients mentioned above. Cases of several months development which remain skin-test negative are exceptionally rare and may be early DCL cases. An *in vitro* test of CMI, antigen-specific lymphocyte proliferation, gave a significantly lower percentage of positive reactions in this series of patients (56%, Table 1). Interferon γ (IFNγ) was not measured in this group of patients, but earlier studies have demonstrated significant levels of this lymphokine in antigen-stimulated lymphocyte cultures from LCL patients (RADA *et al.*, 1987; CASTÉS *et al.*, 1988). We found no significant correlation between the size of leishmanin skin tests and the magnitude of stimulation indices in proliferation tests *in vitro*.

Anti-*Leishmania* antibody levels measured by ELISA are variable, but often quite high; positive results (ELISA values greater than the mean plus 2 standard deviations of a group of 33 healthy controls) were detected in 92% of the 130 patients mentioned above in sera taken at the time of clinical diagnosis (Table 1). Antibody levels were not correlated to the intensity of leishmanin reactions. Significant levels of antibodies, similar to or higher than initial levels, persist in some patients after treatment and apparent clinical cure, which suggests the persistence of parasites and may be a useful prognostic criterion for the detection of relapse or secondary involvement. Nevertheless, average levels of antibodies in patients treated with meglumine antimoniate or combined vaccine immunotherapy decrease significantly after clinical cure (M. Ulrich *et al.*, paper submitted for publication).

Immunocytochemical studies in LCL lesions have revealed CD4/CD8 cell ratios of 0·80 and 0·98 in 2 studies and a high proportion of CD4+CD45RA− memory T cells; Langerhans cells in the epidermis are present in significant numbers (MODLIN *et al.*, 1985; MARTÍNEZ-ARENDS *et al.*, 1991), more than in normal skin. Messenger ribonucleic acid (mRNA) signals for IFNγ and tumour necrosis factor α (TNFα) are particularly strong in LCL lesions (G. Cáceres-Dittmar *et al.*, paper submitted for publication).

A high percentage of patients with LCL respond very well to immunotherapy with a combined vaccine containing heat-killed *L. amazonensis* promastigotes and viable bacillus Calmette-Guérin (BCG) (CONVIT *et al.*, 1987, 1989) in association with conscientious local antiseptic treatment. This treatment appears to offer an attractive alternative to the widely-used chemotherapy with pentavalent antimonial compounds, since it is much less expensive and is associated with fewer adverse side effects. Subsequent mucosal involvement has been observed in less than 1% of patients given immunotherapy after a follow-up period of 2–5 years, but longer observation is required.

Some of the more important observations on LCL in the 'New World', with emphasis on our observations on Venezuelan patients, are summarized in Table 2.

Diffuse cutaneous leishmaniasis

In progressive polar DCL, we have observed localized lesions or, in some cases, lesions limited to relatively restricted areas of the body surface. The lesions may be asymmetric, affecting a single limb and leaving the rest of the skin free of clinical lesions, or they may be symmetrical but limited to both legs or to both arms. These clinical manifestations, which seem to suggest some capacity to localize DCL lesions in spite of the absence of demonstrable CMI, may reflect relatively early stages of the disease, or they may be related to particular physiological characteristics of the affected skin.

Diffuse cases have also been observed which begin as an ulcerated lesion which appears to heal normally, with typical scar formation, and subsequently relapses with the formation of nodules at the border of the scar as well as at some distance from it. The apparently localized lesions may persist with few changes for months or years and then slowly disseminate to other areas.

Interestingly enough, 9 of the 20 DCL patients in our series were women, but the possible basis for this difference in sex-related distribution compared to LCL and

Table 2. Summary of principle characteristics of American localized cutaneous leishmaniasis

Lesions. One or multiple; ulcerated.
Histopathology. Typical immune granuloma with epithelioid differentiation, particularly in early non-ulcerated lesions; intense destructive ulcerative reaction and chronic secondary infection, often observed at the time of diagnosis, may produce significant necrosis and other histopathological alterations. Few parasites except in very early lesions.
Immunocytochemistry. Similar numbers of CD4+ and CD8+ T cells; increased Langerhans cells in the epidermis and a high proportion of T memory cells in the granuloma. Strong mRNA signals for IFNγ and TFNα.
Cell-mediated immunity (CMI). More than 90% positive in DTH skin tests and a significantly lower proportion of CMI tests *in vitro*; 5% of skin tests show exaggerated hypersensitivity. Serological tests positive in >90% at the time of diagnosis.
Therapeutic-response. Good; variable numbers of patients heal spontaneously. Immunotherapy offers an attractive alternative to chemotherapy, with similar efficacy.
Causative agents. Apparently all members of the *L. mexicana* and *L. braziliensis* complexes which infect human beings, except *L. mexicana pifanoi*.

MCL patients, about one-third of whom are women, is unknown.

DCL is characterized by negative DTH reactions in the leishmanin skin test (Table 1). Skin-test negativity is almost invariably observed at the time of diagnosis. However, in 2 cases in the early stage of disease which had, nevertheless, lasted for more than a year, we observed positive leishmanin reactions of more than 20 mm diameter, which subsequently became negative. The presence of early DTH in these patients suggest dissociation between protective immunity, severely compromised or completely absent in these patients, and DTH. Nevertheless this dissociation is transient, with the DTH reactions eventually becoming negative. Separate subpopulations of T cells responsible for protection and DTH have not been clearly defined, but the involvement of several subpopulations of lymphocytes as well as the role of macrophages and perhaps other accessory cells undoubtedly leads to a very dynamic situation which may result in dissociation of the 2 phenomena.

In later stages of DCL, invasion of the nasal mucous membranes is frequent, as reported previously (CONVIT & PINARDI, 1974), so that mucocutaneous lesions in ACL are not limited to infections produced by members of the *L. braziliensis* complex. The mucosal lesions of DCL are not accompanied by the strong inflammatory reactions of MCL, in our experience.

In general, the early lesions of DCL initially appear to respond well to chemotherapy. After treatment is suspended, new lesions appear which are progressively less responsive to subsequent chemotherapy. As a consequence of immunotherapy with the combined vaccine described above together with Glucantime® (meglumine antimonate), sub-acute lesions may appear with a histopathological structure characterized by a granuloma with limited epithelioid differentiation and significant infiltration by lymphoid cells. This histological structure is reminiscent of the reversal reactions observed in lepromatous leprosy during the course of immunotherapy with the appropriate combined vaccine, heat-killed *Mycobacterium leprae* and BCG (CONVIT *et al.*, 1986).

The response of patients with DCL to simultaneous immuno- and chemotherapy is highly variable and will be discussed in a separate publication. This variability within the severe polar form of disease is also observed in the treatment of lepromatous leprosy and undoubtedly reflects the multifactorial bases of non-responsiveness in these patients with severe antigen-specific T cell deficiencies.

Tests *in vitro* of CMI responses in DCL in untreated or relapsed patients invariably show an absence of antigen-specific lymphocyte proliferation (CONVIT & PINARDI, 1974; CASTÉS *et al.*, 1983; see Table 1). Untreated patients do not produce IFNγ in lymphocyte cultures stimulated with *Leishmania* antigens (RADA *et al.*, 1987; CASTÉS *et al.*, 1988), although IFNγ production is normal in cultures of lymphocytes from DCL patients stimulated with phytohaemagglutinin (CASTÉS *et al.*, 1988). Suppressor activity mediated by adherent cells (PETERSEN *et al.*, 1982) or demonstrable by *Leishmania* antigen inhibition of mitogenic responses (CASTÉS *et al.*, 1984) has been reported in DCL.

Anti-*Leishmania* antibody levels are high in these patients, with many sera from untreated or relapsed patients having titres of 1:4800 or greater in ELISAs (unpublished data). This observation, also reported by others, has often been cited to support the negligible role of antibody-mediated protective mechanisms in leishmaniasis.

Immunocytochemical studies of DCL lesions are of particular interest because they reveal the dynamic situation in the active lesion. Some of the more relevant observations include a CD4+/CD8+ cell ratio of 0·79 to 0·80, and a marked decrease in the number of CD4+ T helper–inducer cells which produce interleukin 2 (IL2) and of CD4+CD4RA− memory T cells (MODLIN *et al.*, 1985; MARTÍNEZ-ARENDS *et al.*, 1991). Significant numbers of cells with receptors for IL2 are present in DCL lesions. Recently we have demonstrated the presence of mRNA for IL4, IL5, IL10 and IFNγ in DCL granulomas, using the reverse transcriptase-polymerase chain reaction technique (G. Cáceres-Dittmar *et al.*, paper submitted for publication).

A summary of the major characteristics of 'New World' DCL is given in Table 3.

Table 3. Summary of principle characteristics of American diffuse cutaneous leishmaniasis

Lesions. Often innumerable, sometimes few or restricted to limited areas; nodules or plaques, not ulcerated.
Histopathology. Undifferentiated macrophage granulomas with few lymphoid elements; enormous numbers of intracellular parasites.
Immunocytochemistry. Few CD4+ T cells producing IL-2; CD4+/CD8+ ratio <1; few CD4+ memory cells; significant mRNA signals for IFNγ, IL2, 4, 5 and 10.
Cell-mediated immunity. Absent except in very rare early diagnosis; positive reactions, which may be transient, observed after aggressive treatment which includes immunotherapy.
Antibody response. Usually elevated; levels may drop significantly after therapy.
Therapeutic response. Poor; relapse almost invariable after chemotherapy; combined immuno- and chemotherapy has produced prolonged remission in some patients.
Causative agents. Members of the *L. mexicana* complex.

Mucocutaneous leishmaniasis

The intermediate forms of disease in the spectrum of ACL often respond well initially to treatment, but relapse is frequent and these cases often become progressively more difficult to manage. MCL represents the most important form of inter-polar disease, both in frequency and in consequences for the patient. The most severe cases may be associated with mutilation, deterioration of the general state of health and even death when there is profound compromise of the respiratory system. Seventy-two per cent of our MCL patients were males, which is not significantly different from the sex ratio in LCL.

In a study of 61 patients followed for several years, we have identified 11·5% with minimal lesions, localized on the nasal mucous membranes with a single exception. The average time of development of these minimal lesions was 8·5 months; 4 of the 7 cases were hyper-reactive in the leishmanin skin test (reactions >30 mm). The remainder of the patients showed progressive involvement of the oral cavity, pharynx and larynx. Lesions were of variable severity in each of these anatomical regions and the associated inflammatory reaction sometimes resulted in perforation of the nasal septum and extensive destruction of cartilaginous structures. The average developmental time of the lesions in the entire group was 9·7 years (standard error of the mean = 1·75).

The immunological observations in the intermediate forms of ACL are of particular interest. Particularly in MCL, exaggerated hypersensitivity is frequently present, e.g. the presence of leishmanin reactions >30 mm in diameter or of elevated lymphocyte proliferation indices (CASTÉS *et al.*, 1983; CARVALHO *et al.*, 1985). Nevertheless, this has not been a uniform finding in all studies, particularly with regard to lymphocyte proliferation (MARSDEN, 1986; SARAVIA *et al.*, 1989; CONCEIÇAO-SILVA *et al.*, 1990). In our group of 61 patients with MCL, 98% were leishmanin-positive, but only 39% had skin test reactions greater than 30 mm. Leishmanin activity was significantly higher in the MCL group than in the LCL group, but the differences in lymphocyte proliferation *in vitro* were not statistically significant (Table 1). While only 28% of the MCL cases occurred in women, 42% of the hyper-reactors were women.

When we compared antibody levels with the number of years during which the mucosal lesion had developed, clinical severity, and number of areas of the upper respiratory tract affected, the only statistically significant difference was related to the number of areas affected. Interestingly, leishmanin reactivity was weaker with more extensive compromise of the upper respiratory system, in inverse relationship to levels of antibodies to *Leishmania*. This finding suggests that an intense DTH reaction may be associated with some degree of protection in limiting the extension of lesions, though it may contribute significantly to the intensity of the inflammatory reaction. MARSDEN (1986) has reported an association between negative skin tests in 3 patients with mucosal lesions and the presence of long-standing, advanced, multiple lesions. The relationship between antibody levels and DTH in relation to the number of anatomical areas affected (nasal, oral, pharyngeal, laryngeal) is shown in the Figure.

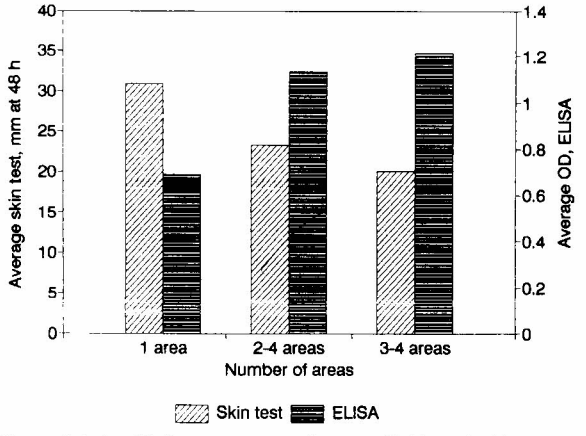

Figure. Relationship between average diameter of leishmanin skin test reaction and antibody level (mean optical density [OD] in enzyme-linked immunosorbent assay [ELISA]) in patients with mucocutaneous leishmaniasis involving one or more areas of the upper respiratory tract.

In vitro tests of CMI, including antigen-induced lymphocyte transformation (LTT) and significant IFNγ synthesis, were positive in 81% (n=42) and 59% (n=17) of the patients tested, respectively. No significant correlation was found comparing leishmanin skin test size, LTT stimulation index and IFNγ production, but the last 2 showed a highly significant correlation (r=0·7274, P<0·001). The lack of correlation between LTT and leishmanin skin tests has been reported by others (SARAVIA *et al.*, 1989; GUTIERREZ *et al.*, 1991).

Levels of antibodies to *Leishmania* are extremely variable in MCL. Of 59 pre-treatment sera studied, 88% gave positive tests (Table 1). Levels of antibodies showed no correlation with the size of leishmanin skin tests nor with severity of clinical lesions in the group as a whole, but were clearly related to the anatomical extension of lesions, as mentioned above.

Immunocytochemical studies of MCL mucosal lesions showed a CD4+/CD8+ cell ratio of 1·41 and a high proportion of T memory cells (MARTÍNEZ-ARENDS *et al.*, 1991). The relatively high CD4+/CD8+ cell ratio in lesions is in marked contrast to the low ratio observed in the peripheral blood of MCL patients (CASTÉS *et al.*, 1988). The most characteristic feature of these mucosal lesions is the lack of epithelial Langerhans cells (MARTÍNEZ-ARENDS *et al.*, 1991). MCL lesions gave strong mRNA signals for IFNγ, TNFα, and IL2, 4, 5 and 10 (G. Cáceres-Dittmar *et al.*, paper submitted for publication).

Early diagnosis is of exceptional importance in the management of patients with MCL, since the therapeutic response is usually much better and more stable in cases with minimal lesions. Apparently complete healing of cutaneous lesions by appropriate therapy with antimonial drugs has been associated with a much lower incidence of secondary lesions of the respiratory mucous membranes in some studies (MARSDEN, 1986). Periodic measurement of antibodies to *Leishmania* together with the application of leishmanin skin tests in the follow-up of LCL, though rather difficult to implement in largely rural populations, might offer important tools for the early detection of mucosal lesions. Serum titres have been shown to correlate with the presence of amastigotes in lesions (GUITIERREZ *et al.*, 1991) in lesions and this may be relevant to the persistence of amastigotes in the apparent absence of lesions in some individuals.

Immunotherapy with combined vaccine, associated with antimonial chemotherapy, may offer the best therapeutic alternative available at the present for MCL (paper in preparation).

Table 4 summarizes the principal characteristics of MCL.

Table 4. Summary of principle characteristics of American mucocutaneous leishmaniasis

Lesions.	Often appear years after a localized lesion and may affect one or more areas of the upper respiratory system (nasal, buccal, pharyngeal, laryngeal).
Immunocytochemistry.	Large numbers of CD4+ T cells, a CD4+/CD8+ ratio >1 and increased T memory cells in lesions, but no epithelial Langerhans cells; high levels of mRNA for several lymphokines.
Cell-mediated immunity and antibody response.	Exaggerated DTH in skin test occurs in <50% of patients in Venezuela. Inverse relationship between the size of the mucocutaneous lesions and the intensity of the hypersensitivity reaction, though more extensive lesions are associated with higher antibody levels.
Therapeutic response.	Response to chemotherapy alone is often poor and characterized by frequent relapse. Therapeutic protocols which combine immuno- and chemotherapy have given very satisfactory results.
Causative agents.	Members of the *L. braziliensis* complex. *L. mexicana* may invade mucous membranes, but does not produce typical mucocutaneous lesions.

BLOOM *et al.* (1992) have recently suggested that human CD4+ and CD8+ T cells can be divided into 2 types; type 1 cells are characterized by a pattern of lymphokine synthesis in which interferon γ predominates, while type 2 cells are characterized principally by synthesis of interleukin 4. The predominance of responses in which type 1 or 2 activity is selectively enhanced depends upon such factors as recognition of different epitopes, restriction elements, presence of other lymphokines in the micro-environment, and differences in antigen-presenting cells. These considerations are highly relevant to the differing host responses in ACL, ranging from a relatively effective response in LCL through the exaggerated DTH in a significant proportion of MCL cases to the peripheral tolerance reflected in the absence of protective CMI as well as DTH in DCL. Enormous advances have been made in the definition of activity of types 1 and 2 helper T cells (Th1 and Th2) in lesions produced by *Leishmania* in experimental murine infections (LOCKSLEY & SCOTT, 1991). Taken together, these concepts can be expected to clarify the immunological bases underlying many of the characteristics of this and other diseases demonstrating a spectrum of host responses.

Conclusions

The clinical and immunological variations in the 3 principal forms of ACL described above reinforce the concept of a clearly defined spectrum of disease manifestations. While some features of the spectrum are related

to the infecting strain of *Leishmania*, the immunological response of the host undoubtedly plays a predominant role in determining spontaneous healing of LCL, development of mucocutaneous complications, isolated cases of DCL and other features of the disease. While some aspects of the spectrum cannot be studied in detail in the human disease, nevertheless ACL offers a unique opportunity for the study of immunological mechanisms, regulation and intervention in a protozoan infection.

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