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In situ characterization of the cellular immune response in American cutaneous leishmaniasis

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SUMMARY

American cutaneous leishmaniasis is a spectrum of granulomatous disease caused by related species of an intracellular parasite. The host response in localized cutaneous leishmaniasis (LCL) is effective in that few organisms can be found in tissue lesions. In contrast, diffuse cutaneous leishmaniasis (DCL) patients mount a poor response with numerous parasites present in multiple skin lesions. Immunopathological correlates were sought in LCL and DCL with immunoperoxidase techniques using monoclonal antibodies directed against T lymphocyte subpopulations and interleukin-2 in tissue lesions. Both LCL and DCL granulomas showed a mixture of T lymphocyte subpopulations with the ratio of helper:suppressor phenotypes less than one. This ratio and localization of cells is more similar to the ineffective lepromatous leprosy granuloma than the effective tuberculoid leprosy granuloma. In contrast, interleukin-2 was identified in equivalent numbers of cells in LCL and tuberculoid leprosy, an order of magnitude greater than DCL and lepromatous leprosy lesions. Cells expressing Tac, the receptor for interleukin-2, were present in approximately equal numbers in all disorders. The immunological effectiveness of granulomas appear to related less to the numbers and location of T cell phenotypes than to the functional aspects of these cells, particularly the ability to generate lymphokines.

Keywords leishmaniasis granulomas T cell phenotypes interleukin-2 interleukin-2 receptor

INTRODUCTION

Leishmaniasis is a group of granulomatous diseases caused by related species of intracellular parasites and with a spectrum of host response (Convit, 1974). Localized cutaneous leishmaniasis (LCL) is the most benign form of the disease spectrum with few limited self-healing skin lesions. In contrast diffuse cutaneous leishmaniasis (DCL) is characterized by extensive unremitant skin involvement. Histologically, LCL may show epithelioid foci or a lymphohistiocytic infiltrate with few or no organisms present, while DCL granulomas are composed mostly of macrophages containing large numbers of organisms.

Castes *et al.* (1983) have shown that LCL patients have positive delayed hypersensitivity skin tests and *in vitro* lymphocyte transformation to leishmanial antigens. These responses were lacking in DCL. In addition LCL patients did not show organism induced suppressor cell activity which was present in DCL patients (Castes, Agnelli & Rondon, 1984).

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In this paper we report our immunopathological studies of LCL and DCL including the examination of T cell phenotypes, interleukin-2 (IL-2) receptors and IL-2 as identified by monoclonal antibodies (MoAb) and immunoperoxidase techniques.

MATERIALS AND METHODS

Patients. Eight patients with LCL and one with DCL were studied at the Instituto Nacional de Dermatologia. One additional patient with DCL was studied at the Los Angeles County Medical Center for a total of two cases of the rare DCL. The diagnosis was made by established clinical, histological, microbiological and immunological criteria (Convit, 1974). Lesions were present for 3–6 weeks and all were untreated except one DCL patient who was relapsing on therapy. All the LCL lesions were ulcerated. Biopsy specimens were obtained at the periphery of the lesions to minimize the likelihood of interference from secondary bacterial infection. Foci of polymorphs were rare in the sections studied. Smears, hamster foot innoculation and Montenegro tests were positive in the LCL patients. The two DCL patients had multiple skin lesions with numerous parasites in macrophages. One patient was Montenegro test negative, the other was lost to follow-up.

Tissue preparation. Scalpel skin biopsy specimens were embedded in OCT medium (Ames Co.) and rapidly frozen in liquid nitrogen. The tissues were then stored at −70°C until sectioning. Cryostat sections were cut to 5 μm in thickness and allowed to air dry. Prior to immunostaining, the sections were placed in reagent grade acetone at 25°C for 10 min, allowed to dry then hydrated in modified phosphate-buffered saline (PBS) (Modlin *et al.*, 1983c).

MoAb. Murine MoAb were used at concentrations predetermined by checkerboard titrations. Each specimen was stained with a panel of MoAb including anti-IL-2 at 1:50 (Dr Steven Gillis, Immunex Corp.; Steinman *et al.*, 1983) and an antibody to the IL-2 surface receptor, Tac, at 1:5,000 (Dr Thomas A. Waldmann, NCI; Leonard *et al.*, 1982). Also used were antibodies against T lymphocyte subpopulations including the pan-T cell marker Leu 4 at 1:60 (Becton–Dickinson), the helper/inducer T cell marker Leu 3a at 1:60, the suppressor/cytotoxic T cell marker Leu 2a at 1:100, the thymocyte marker T6 at 1:50 (Ortho) which is known also to react with Langerhans cells in the epidermis (Fithian *et al.*, 1981) and possibly in the dermis (Murphy *et al.*, 1983) and the group specific antigen HLA-Dr at 1:50 (Becton–Dickinson). Controls consisted of omission of the primary antibody, or the use of an antibody of irrelevant specificity at the same protein concentration.

Immunoperoxidase staining. For anti-IL-2 and Tac antibodies, a modified avidin–biotin–immunoperoxidase technique was used (Modlin *et al.*, 1983a, 1983b) Slides were sequentially incubated for 30 min with primary mouse MoAb, biotinylated horse anti-mouse antibody 50 μg/ml (Vector, Burlingame, California) and avidin–biotin–peroxidase complex (Vectastain kit, Vector). For Leu 4, Leu 2a, Leu 3a, T6 and HLA-Dr an indirect immunoperoxidase technique was used (Modlin *et al.*, 1983c) Slides were sequentially incubated for 15 min with primary mouse MoAb and peroxidase conjugated goat anti-mouse IgG (Tago Inc.) at 1:10. Five minute washes with PBS were performed between all incubations.

A red coloured reaction product was obtained by incubating specimens with aminoethyl carbazole in the presence of hydrogen peroxide for 10 min. Slides were then washed, counterstained with Mayer's haematoxylin, washed again and then mounted with glycerol jelly.

Cells staining positively for IL-2 were few (approximately 100 in LCL lesions and 10 in DCL lesions), and were therefore counted in the entire section under standard light microscopy. The section was measured and the percentage of the section occupied by granuloma was estimated by two independent observers. Dividing the number of positive cells by the area of granuloma was performed to normalize to square millimetre of granuloma. Tac positive cells were also few and counted in three random fields per section on a square millimetre grid, the average obtained. The percentage of cells in granulomas staining with these antibodies was obtained by dividing the number of positive cells per square millimetre of granuloma by the total number of cells per square

milimeter of granuloma and multiplying by 100. The total number of cells per square millimetre of granuloma was found to be 3,600 cells/square millimetre for all tissues studied.

Leu 4, Leu 3a, and Leu 2a positive cells were numerous; the percent of positive cells was directly estimated by two independent observers (whose readings invariably agreed within 5% of each other).

In normal epidermis, Langerhans cells account for 3–5% of all epidermal cells so that interconnection of dendritic extensions is rare (Modlin *et al.*, 1983a). Langerhans cells were said to be increased if frequent interconnection of dendritic extensions from neighboring cells was observed.

Peripheral blood T lymphocytes. T lymphocyte subpopulations were measured in the peripheral blood of six of eight patients with LCL and seven controls by indirect immunofluorescence. Specificities sought included the pan-T cell marker T11 (Coulter), the helper/inducer T lymphocyte marker T4 and the suppressor/cytotoxic T cell marker T8.

RESULTS

T lymphocyte subpopulations

A similar architecture was observed in LCL and DCL granulomas with both Leu 2a and Leu 3a cells randomly scattered among macrophages. Leu 2a cells were more numerous than Leu 3a cells (Table 1 & Fig. 1). Overall, the Leu 3a : Leu 2a ratio in the LCL granulomas (0·8) was similar to the DCL granulomas (0·8). More lymphocytes were found by immunostaining in DCL then seen in paraffin embedded, haematoxylin & eosin stained tissue, similar to previous reports of numbers of lymphocytes observed in other granulomas (Modlin *et al.*, 1983a, 1983b).

Table 1. Percentage of cells in granulomas by phenotype

	LCL	DCL
IL-2	0·22±0·10	0·031±0·011
Tac	2·1±1·1	3·4±0·2
Leu 4	58±13	43±4
Leu 2a	36±11	23±4
Leu 3a	26±4	18±4
Leu 3a/2a	0·8±0·2	0·8±0·3

Anti-IL-2 immunostaining

Anti-IL-2 MoAb identified large cells exhibiting cytoplasmic staining in all both LCL and DCL. Previous studies employing double immunolabelling have suggested that these may be IL-2 producing cells (Modlin *et al.*, 1984). Enumeration of positive cells showed that almost an order of magnitude greater numbers of cells were observed in the LCL granulomas as compaed with the DCL granulomas (Table 1 & Fig. 2)

Tac immunostaining

Tac MoAb identified small lymphoid cells in equivalent numbers in both LCL and DCL granulomas suggesting that there was no lack receptors for IL-2 on the infiltrating cells in any condition (Table 1 & Fig. 3)

T6 and HLA-Dr immunostaining

T6 identified dendritic cells in the epidermis. T6 also identified numerous large cells, occasionally with dendrites, in the dermis of LCL. In six of eight LCL lesions, $T6^+$ cells were increased in the epidermis, as compared with normal and DCL epidermis, as assessed by the numerous interconnections of dendritic extensions.

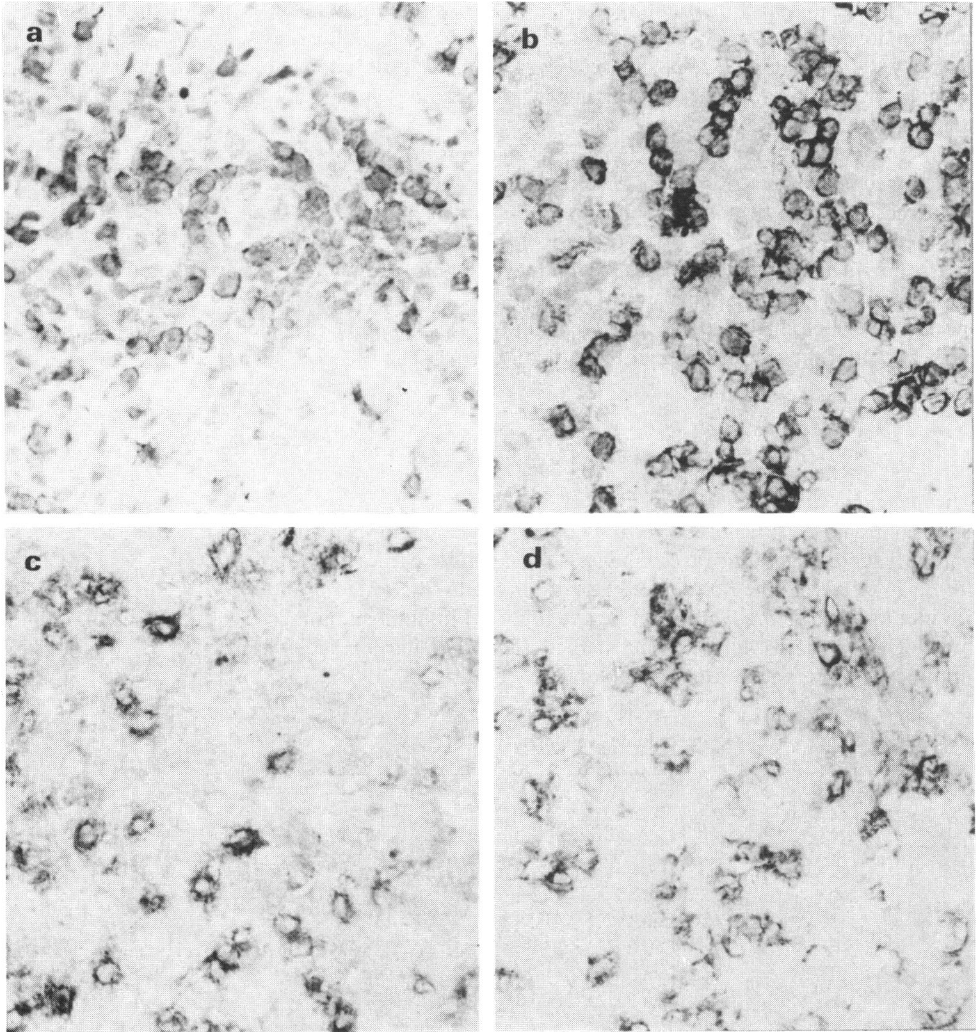

Fig. 1. T lymphocyte subpopulations in leishmaniasis. Both Leu $3a^+$ (a) and Leu $2a^+$ (b) cells are randomly scattered throughout a granuloma of localized cutaneous leishmaniasis. A similar admixture of Leu $3a^+$ (c) and Leu $2a^+$ (d) cells is observed in a diffuse cutaneous leishmaniasis granuloma. In both condition, the Leu $2a^+$ cells are present in equal or greater numbers than Leu $3a^+$ cells (see Table 1) (immunoperoxidase with Leu 2a and Leu 3a, haematoxylin, magnification × 170).

In LCL epidermis, the HLA-Dr antigen was expressed on both Langerhans cells and keratinocytes giving an intense staining reaction. In DCL epidermis, the pattern of staining was not clear. In all granulomas the HLA-Dr antigen was expressed on virtually every cell including macrophages.

Peripheral blood studies

The peripheral blood helper : suppressor ratio was $1{\cdot}4 \pm 0{\cdot}6$ in LCL as compared with the laboratory control ratio of $1{\cdot}5 \pm 0{\cdot}5$.

DISCUSSION

The effectiveness of the host response in LCL is readily apparent in the rarity of organisms in tissue

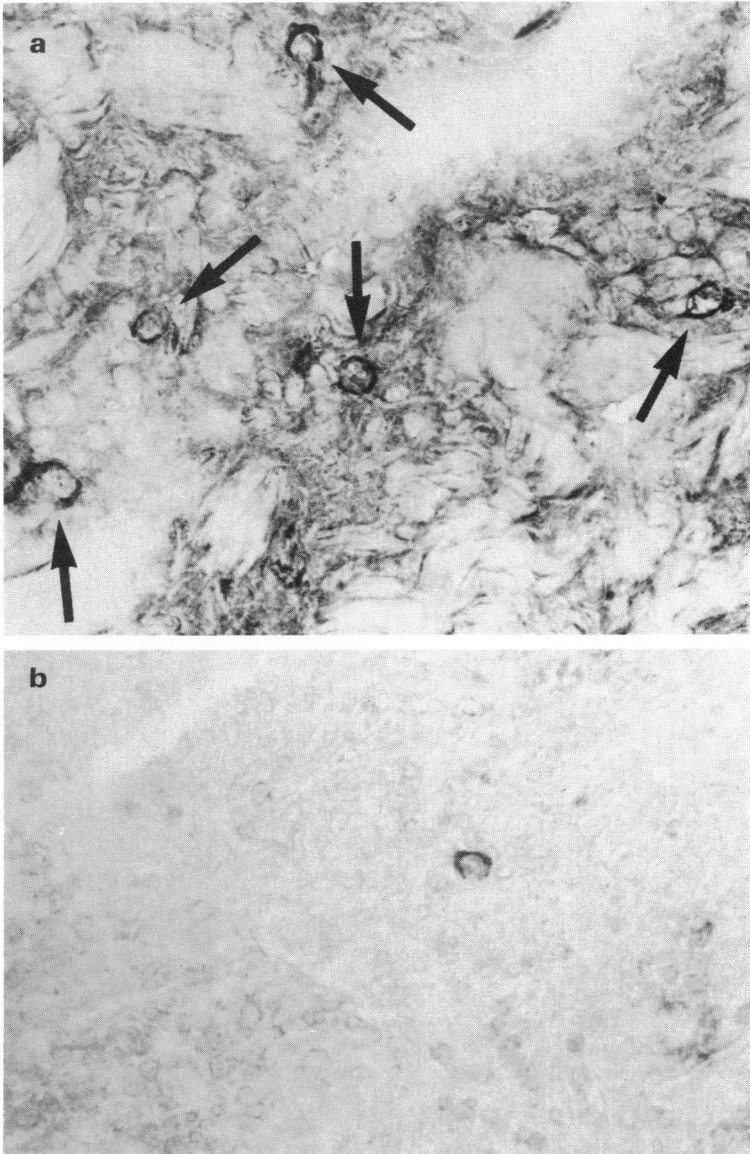

Fig. 2. IL-2 in leishmaniasis. Cells reacting with antibody to IL-2 are large and show cytoplasmic staining. They are more numerous in localized (a) than diffuse (b) cutaneous leishmaniasis (immunoperoxidase with anti-IL-2, haematoxylin, magnification × 270).

lesions and the spontaneous healing of skin lesions (Convit, 1974). In contrast, the host response in DCL is defective or ineffective in that numerous parasites are present in multiple skin lesions. The present study indicates that an effective granulomatous response does not require an excess of T cells with the helper/inducer phenotype as compared with the suppressor/cytotoxic phenotype (Van Voorhis *et al.*, 1982) or a particular microanatomic location of T cell subsets in granulomas (Modlin *et al.*, 1983a, 1983b, 1983c; Narayanan *et al.*, 1983), but instead may be more closely related to lymphokine production.

Convit (1974) has found similarities between the spectra of leishmaniasis and leprosy. Both LCL and tuberculoid leprosy may present with limited skin lesions with few or no organisms present and

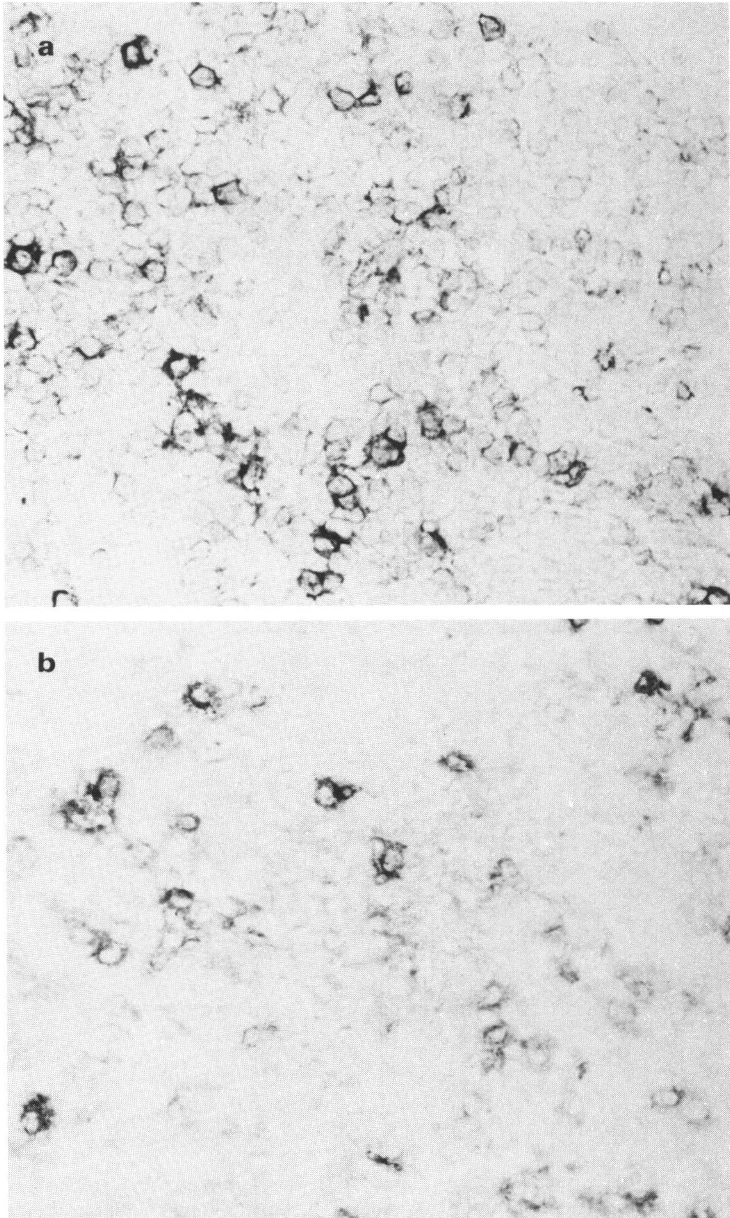

Fig. 3. Tac$^+$ cells in leishmaniasis. Equivalent numbers of cells expressing Tac, the IL-2 receptor are present throughout localized (a) and diffuse (b) cutaneous leishmaniasis granulomas (immunoperoxidase with anti-Tac, haematoxylin, magnification × 170).

have both positive organism specific skin tests and *in vitro* lymphocyte transformation tests (Castes *et al.*, 1983; Myrvang *et al.*, 1973). Unlike tuberculoid leprosy; however, the lesions of LCL are self-healing. In contrast, both DCL and lepromatous leprosy patients have disseminated skin lesions with organisms parasitizing macrophages, negative skin tests, *in vitro* anergy to organism specific antigens but do show organism specific suppressor cell activity when studied by similar protocols (Mehra *et al.*, 1979, 1982; Castes *et al.*, 1984). Aspects of the *in situ* cellular immune response have previously been reported in leprosy (Van Voorhis *et al.*, 1982; Modlin *et al.*, 1983a,

1983b, 1983c; 1984, Narayanan *et al.*, 1983). Because of the similarities between leprosy and leishmaniasis it is tempting to compare the immunopathological finding between the two spectra.

The LCL granulomas studied showed a tissue helper: suppressor ratio of 0·8, in contrast to the ratio of 2·1 reported in tuberculoid leprosy (Modlin *et al.*, 1984). This cannot be due to simple filtration of blood cells as the peripheral blood ratio was 1·4. Despite a tissue suppressor/cytotoxic phenotype excess, the parasite is ultimately eliminated. Furthermore, no separation of T cell subsets with suppressor/cytotoxic phenotypes in the mantle surrounding the granuloma was observed in LCL as in the relatively effective epithelioid granulomas of tuberculoid leprosy, sarcoidosis and tuberculosis (Modlin *et al.*, 1983a, 1983b, 1983c; Narayanan *et al.*, 1983). In fact, the ratio and disorganization of cells observed in LCL (0·8) is similar to that observed in the ineffective granulomas of DCL (0·8), lepromatous leprosy (0·5) and rhinoscleroma than tuberculoid leprosy (2·1). This apparant discrepancy may be explained by at least four considerations. First, the suppressor/cytotoxic cells are being identified by surface phenotype with the two immune functions, suppressive and cytotoxic not separated. If these Leu $2a^+$ cells observed in LCL were in fact cytotoxic cells, it is unlikely that they would affect lymphokine production. Second, suppressor cells in LCL are not activated by leishmania organisms as in DCL (Castes *et al.*, 1984) so that if the Leu $2a^+$ cells observed in LCL lesions are suppressor cells they may not be active as those in DCL lesions. Third, T cells of suppressor phenotype have been shown to be capable of gamma interferon production (Kasahara *et al.*, 1983), which would be useful in activating macrophages for killing of intracellular parasites (Nathan *et al.*, 1983). Fourth, recent studies have shown that lymphocytes may have functions opposite of their phenotype (Thomas *et al.*, 1984). Therefore, we believe that the functioning of the cellular response in tissue lesions cannot be determined solely by counting and observing locations of phenotypes.

Previous studies have indicated that the large cells staining for IL-2 observed in leprosy granulomas may be producer cells (Modlin *et al.*, 1984). Of interest was the finding of similar numbers of these cells in tuberculoid leprosy (0·46) and LCL (0·22) in contrast to DCL (0·031) and lepromatous leprosy (0·028) which contained an order of magnitude fewer cells. This finding further suggests that the phenotypic excess of suppressor/cytotoxic phenotypes (Leu $2a^+$) in LCL may have little functional consequence in that the elaboration of lymphokines critical to T cell and macrophage activation are not impeded. Despite the evident suppressor cell excess, a cell-mediated immune response with elaboration of IL-2 and IL-2 receptor cells (Tac) and virtual elimination of the parasite appears to have taken place in LCL. Therefore the functional attributes of the tissue infiltrating cells, such as lymphokine production, is likely to be more significant than the distribution or ratios of cell phenotypes. The exact mechanism of killing of leishmania organisms in tissue is unknown. We interpret our finding of IL-2 producing cells in LCL to support the role of T lymphocytes and cell-mediated immune processes in the host response.

In contrast to LCL, the *in situ* IL-2 production in DCL appears to be inhibited although the potentially responsive T cells (Tac^+) are present in apparently adequate numbers. This strengthens the similarity between DCL and lepromatous leprosy and suggests a common immunological mechanism of CMI unresponsiveness. There is evidence in both lepromatous leprosy and DCL that antigen-induced T suppressor cells and suppressor macrophages can be found in the blood of these individuals (Mehra *et al.*, 1979, 1982; Castes *et al.*, 1984). It is not unreasonable to speculate that one of the functions of suppressor cells may be to block the production of IL-2 (Bloom, 1983), and in the present experiments this may be happening locally. Since exogenous IL-2 may partially restore lymphocyte responses in lepromatous leprosy patients (Haregewoin *et al.*, 1983) and since IL-2 has permitted the production of gamma-interferon in such patients (Nogueira *et al.*, 1983), it is likely that the immunological failure in DCL, as in lepromatous leprosy, is related to the activity of suppressor cells in the lesions.

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