Influence of *Mycobacterium leprae* and its soluble products on the cutaneous responsiveness of leprosy patients to antigen and recombinant interleukin 2

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**ABSTRACT** Experiments were carried out in the skin of patients with leprosy to examine whether suppressor cell populations either exist in the skin of multibacillary lepromatous leprosy patients, can be activated with antigen, or are induced to emigrate into a cutaneous site from the circulation. For this purpose, purified protein derivative of tuberculin, a delayed-type antigen that generates a cell-mediated immune response, was introduced into the skin alone or with nonviable *Mycobacterium leprae* bacilli. Areas of induration and the resulting numbers and phenotypes of emigratory cells were not influenced by *M. leprae* and its products. Further studies examined the ability of *M. leprae* and its soluble products to modify the cutaneous response to intradermal injection of recombinant interleukin 2 (IL-2), a lymphokine that mimics a cell-mediated response. Neither the simultaneous injection of *M. leprae* and IL-2, nor the prior injection of *M. leprae* followed in 2 days by IL-2, nor the prior administration of IL-2 followed in 4 days by *M. leprae*, into the same skin site, modified the zone of induration generated by IL-2. In addition, the immunocytochemical and histopathological evaluation of biopsy specimens of skin sites showed no difference between sites injected with IL-2 and sites injected with IL-2 and *M. leprae*. We conclude that suppressor T cells, if they exist, do not influence the gross or microscopic responsiveness of a cell-mediated skin reaction to antigen and IL-2. IL-2 did, however, enhance the responsiveness of skin-test-positive tuberculoid patients and family contacts to *M. leprae* antigens by a synergistic effect on the zone of induration and local cell accumulation.

Patients with lepromatous leprosy fail to respond to *Mycobacterium leprae* antigens and do not generate a cell-mediated immune reaction. This defect stems from the inability of T cells to undergo blastogenesis in the presence of specific *M. leprae* epitopes and to secrete important lymphokines such as interferon γ and interleukin 2 (IL-2) (1–4). Although many authors have commented on the nature of this selective anergy, the underlying mechanism(s) is still unclear (5). Based upon *in vitro* experiments, the presence of T cells that inhibit thymidine incorporation of lectin-stimulated peripheral blood T cells has been reported (6–8). Such inhibitory cells were reported to be present in the blood of patients with lepromatous leprosy and absent from patients with the tuberculoid form of the disease and from normal individuals not infected with *M. leprae*. More recently, the isolation of T-cell clones with inhibitor activity was reported by Modlin et al. (9, 10), who emphasized the possible suppressor role of such cells in leprosy.

However, the presence of specific suppressor T cells in lepromatous patients has not been proven. The activity expressed in culture systems that inhibits responses to lec-tins, bacillus Calmette–Guérin, and *M. leprae* is not specific for lepromatous patients and is also seen with cells from tuberculoid patients, normal individuals, and contacts (11). In some cases it was clear that the products of *M. leprae* were themselves nonspecifically suppressive in these *in vitro* systems (11, 12). In addition, a role for monocyte/macrophage suppressor products induced by *M. leprae* has been suggested by many investigators (13–16).

We have therefore carried out *in vivo* experiments within the confines of leprosy lesions to explore the inhibitory properties of (i) preexisting cells of the infiltrate, (ii) particulate *M. leprae* and its soluble products, and (iii) newly emigrated blood cells in the presence or absence of exogenous antigens. We have quantitated suppression by virtue of the ability of these agents to modify the area of induration evoked by a reaction to the purified protein derivative of tuberculin (PPD) (17) or by the injection of recombinant IL-2, a lymphokine that mimics a cell-mediated immune reaction (18).

**MATERIALS AND METHODS**

**Patient Population.** Eighteen leprosy patients, 18–60 years old, 6 with polar lepromatous leprosy (LL) and 12 with borderline lepromatous leprosy (BL) (19), were selected for intradermal administration of PPD at the Leprosy Outpatient Unit, Fundacao Oswaldo Cruz, Rio de Janeiro, Brazil. Thirty-seven leprosy patients, 14–72 years old, 30 with LL, 5 with BL, and 2 with borderline tuberculoid leprosy (BT), as well as 6 family contacts without any overt disease, were selected for the intradermal administration of recombinant IL-2 (Cetus) at the Clinical and Epidemiological Branches of the Leonard Wood Memorial Center for Leprosy Research, Cebu City, Philippines. Leprosy patients in reaction or on steroid treatment were excluded. Written consent was obtained from all participants.

Patients had been treated for <1 month to 7 years and had received multidrug therapy with rifampin (600 mg/day) and dapsone (100 mg/day); about half of them had received clofazimine (50–100 mg/day and/or 300 mg/month) as well. All chemotherapy was continued during the trial. Slit smears for bacterial index (B.I.) were performed within 6 months.

**Abreviations:** B.I., bacterial index; BL, borderline lepromatous leprosy; BT, borderline tuberculoid leprosy; LL, polar lepromatous leprosy; IL-2, interleukin 2; PPD, purified protein derivative of tuberculin.

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before IL-2 and PPD injection. The B.I. of patients tested ranged from 0 to 5+.

**PPD Administration.** Leprosy patients received “simultaneous” injections of PPD and *M. leprae* or PPD and saline as follows. Five units of PPD (Connaught Laboratories) in 0.1 ml was injected into two skin sites on the back. After 5 min, 0.1 ml of isotonic saline or lepromin A (3–4 × 10⁸ M. leprae bacilli per ml; lot J 15-3, National Hansen’s Disease Center, Carville, LA) was injected into one of the sites injected previously with PPD. Erythema and induration were evaluated at time intervals from 1 to 14 days and 4-mm punch biopsy samples were taken.

**IL-2 Administration.** Lyophilized recombinant human IL-2 (3 × 10⁶ units/mg of protein) was reconstituted in pyrogen-free sterile water, diluted in 5% dextrose to a concentration of 100 µg/ml, and used within 2 hr of reconstitution. Injections of 100 µl (10 µg) were given into the skin of the back; patients and contacts received either 10 or 2 such injections. Within a few minutes they received 100 µl of either soluble *M. leprae* antigens (Rees antigen or leporin; World Health Organization) or lepromin A (3–4 × 10⁸ M. leprae bacilli per ml; lot LV-9). This represented “simultaneous” injections. In other experiments either antigen or IL-2 was administered first and was followed 1–4 days later by the complementary antigens or IL-2 into the same site. Appropriate control sites for both antigens and IL-2 alone were injected nearby. All injection volumes were 100 µl.

Local erythema and induration of the injected sites were evaluated daily. Selected sites of IL-2, antigen, and IL-2 plus antigen injection and matched control sites were biopsied (4-mm punch) 1–7 days after the first injection.

**Histopathology.** A part of each biopsy specimen was fixed in neutral buffered 10% formalin overnight, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histological diagnosis and acid-fast staining for enumeration of *M. leprae*.

**Immunocytochemistry.** Biopsy specimens were fixed in phosphate-buffered saline containing 3% paraformaldehyde, 75 mM lysine, and 10 mM sodium metaperiodate (20) and were processed as described (18). Frozen sections were stained with mouse monoclonal antibodies as described (17) to identify cell types and phenotypes.

**Monoclonal Antibodies.** Mouse monoclonal antibodies were used for the identification of specific cell types. Anti-Leu-4, anti-Leu-2a, and anti-Leu-3a (CD3, CD4, and CD8, anti-T cells) (21, 22) and anti-Leu-M5 (CD11c, anti-monoctye/macrophage) (23) were obtained from Becton Dickinson. OKT6 (24) (CD1, anti-Langerhans cells) was obtained from Ortho Diagnostics. Antibody 9.3F10 (anti-major histocompatibility complex class II antigen) was produced in this laboratory (25).

**RESULTS**

The possible modulating influence of the lepromatous state and the administration of *M. leprae* and its products was first examined in the presence of a delayed-type antigen-mediated event. For this purpose injection of PPD alone or in the presence of nonviable *M. leprae* was carried out. As noted previously, the response to PPD in sensitized human skin includes an emigratory event (17), the generation of primary and secondary cytokines (26), and complex responses of endothelium, fibroblasts, Langerhans cells, and keratinocytes (27, 28).

**Influence of M. leprae Antigen on the Cutaneous Response to PPD.** Fig. 1 shows a comparison between PPD plus *M. leprae* and PPD plus saline injection in 3 patients (A) and in all 18 patients (B). The rate of generation and the intensity of the cell-mediated immune response induced by PPD were not influenced by the presence of *M. leprae*. This is consistent with our former studies (17, 27) in which the responsiveness of PPD-sensitized lepromatous patients was virtually the same as compared to normal controls and tuberculoid patients.

**Emigating Blood Cells Accumulating in Skin Sites Injected with PPD or PPD and M. leprae.** Cellular infiltrates in response to PPD contained predominantly T cells and monocytes that differentiated into epithelioid and multinucleated giant cells. The simultaneous injection of *M. leprae* and PPD did not evoke any difference in response. Neither the kinetics of infiltration, nor the cellular composition of the reaction, were modified (data not shown). However, the sites injected
with *M. leprae* and PPD contained larger numbers of intact and fragmented bacteria as a result of the injected *M. leprae*.

The nature and phenotype of emigratory cells were evaluated. Injection of PPD with or without *M. leprae* gave rise to the same increase in dermal cellularity, keratinocyte HLA-DR antigen expression, and dermal CD1+ cells. The majority of the emigratory T cells were of the CD4+ phenotype and the dermal CD4+/CD8+ T-cell ratios were similar in the presence or absence of *M. leprae* (Fig. 1C).

We conclude that endogenous and/or newly administered exogenous *M. leprae* neither enhances nor suppresses the panoply of responses generated by the PPD antigens.

**Responsiveness of Lepromatous and Tuberculoid Patients to IL-2.** The initial study examined the basal reactivity of the skin of multibacillary and paucibacillary patients to the injection of IL-2. Lepromatous patients contain within their dermis heavily parasitized macrophages and a sparse T-cell infiltrate in which cells of the CD8+ 'suppressor/cytotoxic' phenotype predominate. In contrast, BT patients exhibit a more exuberant, cell-mediated, granulomatous reaction with few bacilli and many T cells, largely of the CD4+ 'helper' phenotype. The skin responses that resulted when IL-2 (10 μg) was introduced into these disparate milieus are shown in Fig. 2A. Within 5 hr and peaking at 24 hr the injected site demonstrated mild erythema and firm induration with a slightly raised surface. By 48 hr the zone lacked erythema and consisted of a firm nodule that gradually receded in diameter over the next 4 days. The responsiveness of LL and BT patients to IL-2 was virtually identical, suggesting that the endogenous cellular infiltrate, number of bacilli, and expression of cell-mediated immunity failed to modify responsiveness to this lymphokine.

**Effect of Simultaneous Injection of *M. leprae* and its Soluble Products on Responsiveness to IL-2.** Lepromatous patients with a high B.I. (3+ or more), in various stages of treatment, were injected with 3–4 × 10⁶ nonviable *M. leprae* bacilli. This quantity is routinely employed for evaluating skin reactivity in the Mitsuda reaction. Within 15 min after the deposition of bacilli, 10 μg of IL-2 was introduced into the same area, often via the same needle track. At the same time, but into different sites, control injections of leprosy bacilli or IL-2 alone were made. Zones of induration were measured over the next 7 days (Fig. 2B). Both the maximum response at 24 hr and the rate of reduction in induration up to 7 days were not influenced by the presence of intact, extracellular *M. leprae*. None of the lepromatous patients responded to the bacilli during the 7-day period. The experiment was repeated in a group of lepromatous patients with low B.I. (1.5+ or less) who had been treated for over 2 years. These experiments employed intact *M. leprae* as well as the soluble *M. leprae* antigens. Zones of induration were measured after simultaneous injection of bacilli or soluble antigens plus IL-2 or injection of IL-2 or soluble antigens alone (Fig. 2C). Again, peak responses and clearance were uninfluenced by the intact or soluble *M. leprae* antigens.

We conclude that neither particulate nor soluble *M. leprae* antigens influence the intrinsic and emigratory cells of the microenvironment and in turn the responsiveness to IL-2.

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**Fig. 2.** Effect of *M. leprae* on the dermal response to recombinant human IL-2. (A) Response to intradermal injection of IL-2 in 30 lepromatous (○) and 2 tuberculoid (■) patients. No difference in the area of induration was observed in the two types of leprosy patients. (B) Induration induced by IL-2 with (○) or without (■) simultaneous injection of *M. leprae* in 6 high-B.I. (3–5+) lepromatous patients. Injection of *M. leprae* alone (△) produced no induration. (C) Induration induced by IL-2 in the absence of *M. leprae* (○), with soluble *M. leprae* antigens (□), or with intact *M. leprae* (○) in 12 low-B.I. (0–1.5+) lepromatous patients. Injection of soluble antigens alone (△) produced no induration. (D) Effect of intact *M. leprae* on the response to IL-2 in 7 lepromatous patients. Bacilli were injected into one of the IL-2 sites at 4 days as indicated by arrow. No difference in response was observed when *M. leprae* was injected with or before IL-2. Results are expressed as mean area of induration in mm² (A–D) ± SD (A and B).

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Response to *M. lepra* Antigens in Cutaneous Sites Preinjected with IL-2. In this experiment we wished to evaluate whether prior injection of IL-2 could modify the cutaneous responsiveness to *M. lepra* antigens. For this purpose 10 μg of IL-2 was injected intradermally and 4 days later *M. lepra* was injected into the same cutaneous site. A second *M. lepra* injection was given into a separate site, and the response at this site was compared to that at the IL-2-prepared *M. lepra* site (Fig. 2D). The prior injection of IL-2 did not lead to early cutaneous *M. lepra* responsiveness, nor did it modify the cutaneous responsiveness to a second injection of *M. lepra*. This is in keeping with *in vitro* studies in which IL-2 failed to enhance thymidine incorporation into lymphocytes from lepromatous leprosy patients either after addition *in vitro* (4) or following cutaneous administration (unpublished data).

Effect of *M. lepra* on the Response to IL-2 in Antigen-Norreactive Individuals. Nonviable *M. lepra* bacilli were injected intradermally into lepromatous patients and allowed to influence the preexisting cell populations and/or induce the accumulation of cells emigrating from the peripheral blood. At 48 hr, 10 μg of IL-2 was introduced into the same site. Control sites on other areas of the back received either *M. lepra* or IL-2 alone. The study was conducted with patients who did not give an immediate immune response to *M. lepra* (negative Fernandez reaction). Fig. 3A shows that the prior injection of *M. lepra* had no influence on the cutaneous response to a subsequent injection of IL-2. The nature of the immediate response and the regression of induration were essentially similar in the IL-2-injected site and in the *M. lepra*-primed IL-2-injected site.

We conclude that during the 48 hr after the introduction of *M. lepra* into unresponsive lepromatous patients' skin, cells with the capacity to inhibit the dermal response to IL-2 were not observed.

Effect of *M. lepra* on the Response to IL-2 in Antigen-Reactive Individuals. To examine whether skin-test reactivity to *M. lepra* modulated IL-2 responsiveness, a similar experiment was carried out in skin of leprosy patients and family contacts who exhibited positive Fernandez and Mitsuda reactions. *M. lepra* (3–4 × 10⁶ bacteria) was injected into two sites on the back of family contacts and examined at 24 and 48 hr. At this time IL-2 was injected directly into one of the *M. lepra* sites and at another, distant site. Zones of induration were evaluated over the next 48 hr (Fig. 3B). Only small, but definite, zones of induration occurred at the sites injected with *M. lepra* alone. Injection of IL-2 alone gave the expected induration pattern noted previously. However, a significant enhancement in the extent of induration occurred when IL-2 was placed into a developing *M. lepra* reaction.

The simultaneous injection of soluble *M. lepra* antigens and IL-2 into the skin of two BT patients is depicted in Fig. 3C. IL-2 alone gave the expected induration pattern noted previously. Soluble antigens gave a typical Fernandez response, which persisted for up to 4 days and then subsided. A significant enhancement in the extent of induration was observed at 68 hr when the two mediators were injected into the same site.

These findings are consistent with the ability of IL-2 to enhance the cellular reactions occurring in response to an antigen-mediated event. Such experiments again failed to provide any evidence for the generation of an active suppressor cell population under these conditions.

Immunocytochemical Analysis of the Effect of *M. lepra* on Numbers and Phenotype of the Emigratory Cells in Response to IL-2. The skin of lepromatous patients is characterized by an inflammatory infiltrate involving 10–15% of the dermis. No infiltrate is observed in the skin of family contacts. After injection of IL-2, the percentage of the dermis infiltrated was enhanced similarly in both groups, to 40–50% and 30–40%, respectively. The addition of *M. lepra* to IL-2 did not affect the infiltration of cells into the dermis in lepromatous patients (40–50%) but enhanced the extent of infiltration in the responder contacts to 70–80%.

The predominance of CD8⁺ T cells in the infiltrates of lepromatous patients (CD4⁺/CD8⁺ ratio of 0.9) was reversed in response to both IL-2 and IL-2 plus *M. lepra*. Forty-eight hours after injection, lepromatous patients showed CD4⁺/CD8⁺ T-cell ratios of 3.1 ± 0.6 and 2.2 ± 0.5 in response to IL-2 and IL-2 plus *M. lepra*, respectively. The same ratios were observed in contacts. *M. lepra*-responsive sites in these individuals demonstrated a CD4⁺/CD8⁺ T-cell ratio of 2.3.

Keratinocyte HLA-DR and dermal accumulation of CD1⁺ Langerhans cells were induced similarly by IL-2 and by IL-2 plus *M. lepra* in lepromatous patients as well as family contacts.

**DISCUSSION**

One of the difficulties in accepting a role for suppressor cells as an explanation for the anergy of LL patients is the disparity...
between their expression in the host and in the test tube. Those investigators who have considered the matter have reported only partial inhibition of thymidine incorporation with high effector cell ratios. This fact, when added to (i) a significant direct inhibitory role of M. leprae macromolecules on monocyte–T-cell stimulation and (ii) a lack of specificity of the suppressor populations (11), does not make a compelling case for T suppressor cells in the regulation of the disease process.

We have attempted to define an experimental situation that utilizes the location of bacillary replication. It employs the cutaneous infiltrate as it exists in the lepromatous patient and utilizes emigratory cell populations that are evoked either by antigen or by lymphokine. A number of parameters at both the gross and microscopic level serve to characterize the expression of cell-mediated immunity in the skin. Induration is a sensitive index of cell infiltration and has been used effectively in both murine and human systems to characterize cell-mediated reactions. It encompasses a variety of reactions including chemotraction, endothelial cell transmigratation, and the stimulation of the secretory repertoire of both macrophages and lymphocytes. Microscopically this is characterized by the presence of a predominant CD4+ T-cell infiltrate, expression of interferon-γ-induced protein (IP-10), keratinocyte major histocompatibility complex class II determinants, Langerhans cell differentiation, and the production and function of cytokotoxic effectors (17, 18, 26–30).

We use this wide variety of immunological events to evaluate the possible suppressor role of T cells. For the first experiments we chose an antigen-driven event because it also incorporated a recognition phase and the clonal expansion of small T-cell populations. Subsequently we employed IL-2, a major growth factor for T cells whose presence is required for the expression of other lymphokines and cytokines. In both instances neither the cells involved in the lepromatous state nor those (i) migrating into cutaneous sites, (ii) activated by M. leprae antigen, or (iii) stimulated by crossreacting mycobacterial antigens (PPD) had a suppressive effect on the above parameters of cell-mediated immunity. We consider this strong evidence that suppressor T cells play no major role in down-regulating the essential features of cellular immunity in the skin of lepromatous patients.

It has recently come to our attention that the influence of M. leprae products on a tuberculin response had been investigated by Sengupta et al. (16). Those authors mixed PPD and soluble molecules of M. leprae prior to injection and reported a decided reduction in induration. Such data conflict with those reported here, and we suspect that mixing of these disparate molecules led to a reduction in tuberculin and leprosin activity.

An understanding of the T-cell unresponsiveness of lepromatous patients to M. leprae antigens is essential for a rational approach to any form of immunotherapy. Such mechanisms must consider findings in which LL/BL patients exhibit either a complete absence of in vivo T-cell reactivity or a spectrum of hyporesponsiveness (4). The present data do not support a role for suppressor T-cell populations in explaining this anergy and point toward various defects in T-cell activation caused by ablation in the neonatal or postnatal period or defective antigen presentation. Numerous publications dealing with this subject in murine systems have implicated the thymus in the process. Exploration of the mechanisms of antigen presentation, T-cell receptor selectivity, and extent of T-cell deletions in patients with lepromatous leprosy promises to yield important information.

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