Efficacy of a cell-mediated reaction to the purified protein derivative of tuberculin in the disposal of *Mycobacterium leprae* from human skin

(leprosy/cell-mediated immunity/granuloma/T lymphocytes/mononuclear phagocytes)

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ABSTRACT   The purpose of this study was to evaluate the effects of a delayed-type cell-mediated immune response to *Mycobacterium tuberculosis* antigen on the *Mycobacterium leprae* load in the skin of leprosy patients. Twelve patients with the lepromatous form of leprosy have been injected intradermally with 5 units of the purified protein derivative of tuberculin (PPD). Ten individuals responded with areas of induration ranging from 12 to 21 mm in diameter, and two were unresponsive (<10 mm). Twenty-one days thereafter, the injected and control sites were biopsied, and the histology, number of acid-fast bacilli, nature and phenotype of the emigrant cells, and ultrastructural characteristics of the lesions were evaluated. Eight of the 10 responding patients showed reductions in the number of acid-fast bacilli by factors ranging from 5 to 10,000. Two responders and both nonresponders exhibited no discernible decline in the number of organisms. The reduction in bacillary load was correlated with an intense mononuclear cell infiltrate, the maintenance of a high $CD4^+$ T-cell/$CD8^+$ T-cell ratio, the formation of granulomata, and the extensive destruction of previously parasitized macrophages.

Lepromatous leprosy is characterized by a selective inability of T cells to respond to *Mycobacterium leprae* antigens and to generate a cell-mediated immune response (reviewed in ref. 1). In association with this defect, helper T cells fail to migrate and to accumulate in the dermal lesions, and adequate amounts of lymphokines are not synthesized and released locally (2, 3). This absence of local lymphokines leads to a failure of macrophage activation, and these cells serve as permissive hosts for the bacilli (4). *M. leprae* then multiply within the secondary lysosomes of the vacuolar apparatus to levels as high as 10^9 organisms per g of skin (reviewed in ref. 5).

Patients with lepromatous leprosy do react, however, to other mycobacterial antigens, including those contained in the purified protein derivative of tuberculin (PPD) with a delayed-type cell-mediated response (6). We suspected that the generation of a tuberculin reaction in the dermal leprosy lesions might supply the deficient cell subsets and cytokines and have a beneficial effect on the clearance of organisms.

Our initial observations on the response to PPD in the skin of lepromatous leprosy patients have been reported. These include the emigration of large numbers of helper T cells into the dermis (7), the local production of the macrophage-activating lymphokine γ interferon and its induced product IP-10 (8), the induction of HLA class II (Ia) antigens on the cells of the dermis and epidermis (7), and the accumulation of dermal Langerhans cells (9)—an important accessory cell for

T-cell responses (10). We now have extended these studies and have evaluated the effectiveness of this second-party, cell-mediated reaction to influence the disposal of *M. leprae*. Our results indicate a striking reduction in the local bacillary load, which may be by a factor as large as 10,000.

MATERIALS AND METHODS

Patient Population. Twelve patients with lepromatous leprosy or borderline lepromatous leprosy, as defined by the Ridley–Jopling classification (11), were seen in the Dermatology clinic, SMS Medical College, Jaipur, India, and at the Leprosy Mission Hospital, Shahdara, New Delhi, India, and were tested for their response to PPD. Patients had been treated for leprosy for 0–8 months with multidrug therapy [pulse therapy with one 600-mg tablet of rifampin per month, 100 mg of Lamprene every second day, and 100 mg of dapsone per day (patients 1, 2, 8, 9, and 11); 600 mg of rifampin per day and 100 mg of DDS per day (patients 3–7); and 100 mg of DDS per day only (patient 10)]. Medication was continued during the study.

Tuberculin Testing. Five units of PPD (0.1 ml) was injected into leprosy lesions on the lower back, and the site was evaluated for induration at 48 hr. The injected site and a similar adjacent site were biopsied (4-mm punch) 21 days after PPD administration. The biopsies were divided into three parts and fixed for histopathology, immunohistology, and electron microscopy as described below. The processed biopsies were transported back to the United States for further evaluation.

Histopathology and Enumeration of Bacilli. Biopsies were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin for histological diagnosis. Acid-fast stained sections were used for the enumeration of *M. leprae*. A logarithmic index of bacilli in the biopsies (12, 13) was used to express bacterial numbers in the control and the PPD-injected site (see Table 1).

Immunohistology. Biopsy specimens were fixed in 3% paraformaldehyde/0.075 M lysine/0.01 M sodium *m*-periodate/phosphate-buffered saline (PLP/PBS; PBS, phosphate-buffered saline) for 3–4 hr at 4°C as described by McLean and Nakane (14). This fixative preserves structural details without inhibiting the binding of monoclonal antibodies to their antigens. The biopsies were washed in PBS containing 10% sucrose and 50 μM digitonin and then serially suspended in graded solutions of sucrose (15–25%). The tissue was stored in PBS containing 25% sucrose and 5% glycerol until frozen.

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Table 1. Diagnosis, treatment, and histological evaluation of lesions before and after PPD administration

Patient	Diagnosis	Treatment,* mo	Induration, mm	Infiltrate†, % of area		BI‡		Granuloma formation§	*M. leprae* reduction in BI‡
				Control	PPD	Control	PPD		
1	LL	3	17 × 17	5	5	5+	5+	−	0
2	LL	8	21 × 11	10	40	5+	5+	+	−/+¶
3	BL	5	12 × 12	15	30	4+	3+	+	1+
4	LL	0.3	14 × 14	5	20	5+	1+	+	4+
5	LL	8	21 × 20	70	75	5+	5+	−	−/+¶
6	LL	1	16 × 16	15	40	5+	4+	+	1+
7	BL	1	14 × 14	15	30	5+	5+	−	0
8	BL	1	18 × 18	15	40	5+	2+	+	3+
9	BL	0.1	16 × 16	20	40	5+	2+	+	3+
10	BL	3	18 × 18	10	25	1+	0	+	1+
11	LL	3	Negative	40	40	5+	5+	−	0
12	LL	None	Negative	35	40	5+	5+	−	0

LL, lepromatous leprosy; BL, borderline lepromatous leprosy.
*Patients were treated with multidrug therapy in addition to PPD administration.
†The percentage of the area of the dermis infiltrated by inflammatory cells was estimated.
‡Logarithmic index of bacterial numbers (BI) was evaluated as described (12, 13). The reduction in BI was calculated as the difference in index units between the control lesion and the PPD lesion.
§Granuloma formation is considered positive when epithelioid and multinucleated giant cells are clearly present in the biopsies.
¶No reduction in BI units was observed, but a reduction in the total numbers of bacteria per unit area was observed by three independent investigators.

Biopsies were embedded in OCT compound and frozen at −20°C; 6- to 8-μm sections were cut on a cryostat and applied to gelatin-coated multiwell slides (Carlson Scientific, Peotone, IL). The sections were dried overnight at 37°C, rehydrated in PBS, and incubated with mouse monoclonal antibodies followed by incubation with biotinylated horse anti-mouse Ig and then avidin-biotin–peroxidase complexes (Vector Laboratories, Burlington,CA). The reaction product was developed with 3-amino-9-ethylcarbazole at 0.8 mg/ml and 0.015% H_2O_2. Sections were counterstained with hematoxylin.

Monoclonal Antibodies. Mouse monoclonal antibodies were used for the identification of specific cell types. Leu-1, Leu-2a, and Leu-3a (15, 16) (anti-T cells and their subsets) and Leu-M5 (anti-monocyte/macrophage) (17) were obtained from Becton Dickinson Monoclonal Center.
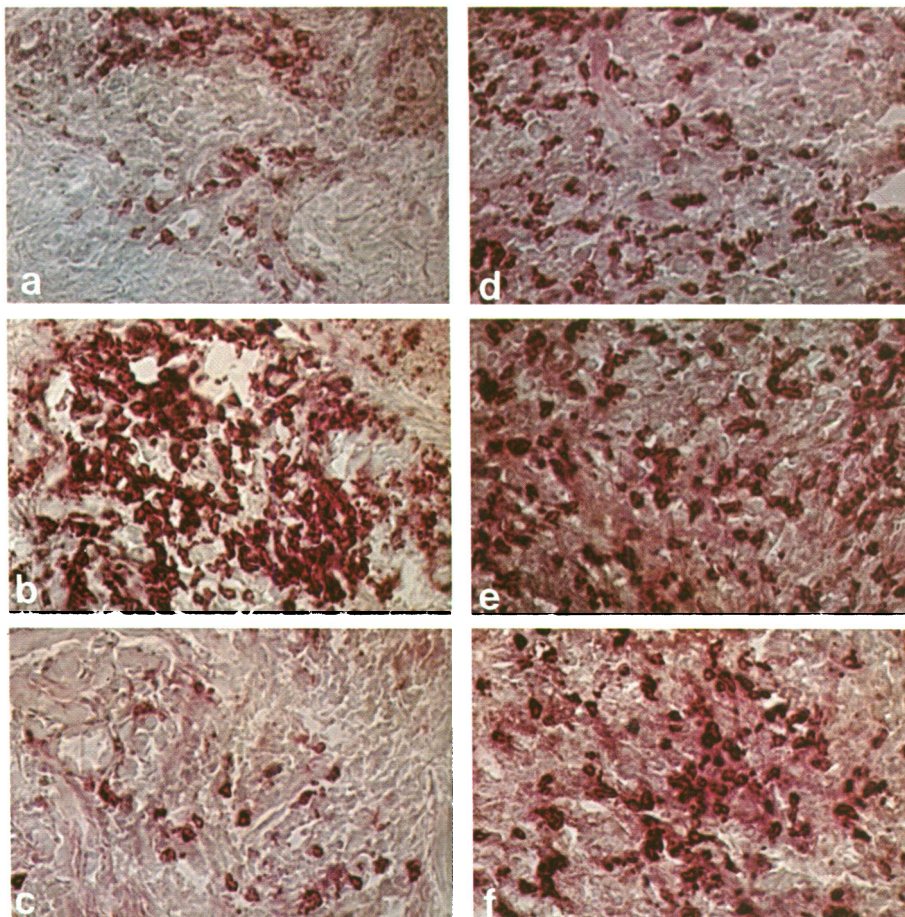

FIG. 1. Infiltrating T cells in the dermis of control and PPD sites 21 days after antigen injection. (*a–c*) Patient 2. (*d–f*) Patient 5. Immunoperoxidase staining used the monoclonal antibody to helper T cells (Leu-1) to stain sections from control sites (*a* and *d*) and PPD-injected sites 21 days after injection (*b* and *e*) and used the monoclonal antibody to T cells of the suppressor/cytotoxic phenotype (Leu-2a) to stain sections from control sites (*c*) and PPD-injected sites (*f*). In patient 2, where a persistence of granuloma was observed, we found a predominance of T helper cells (see Table 1). The absence of granuloma persistence is associated with a predominance of T suppressor/cytotoxic cells (patient 5). (×340.)
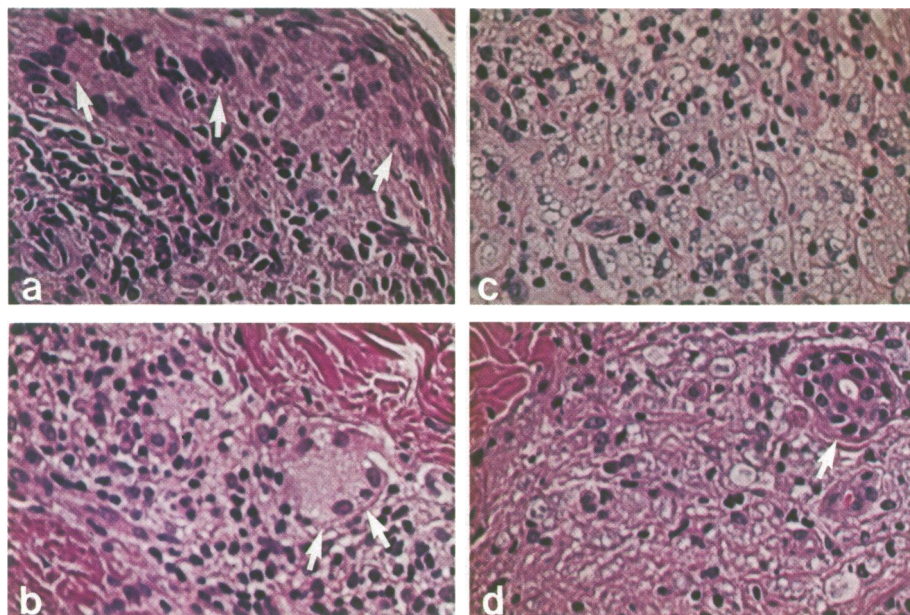
FIG. 2. Nature of the cellular infiltrate in PPD responders 21 days after antigen administration. (*a*) Patient 9 shows persistent granuloma with lymphocytes and epithelioid cells (arrows). (*b*) Patient 3 shows persistent granuloma with a multinucleated cell (arrow) and many lymphocytes. (*c* and *d*) Patients 5 and 7 show a loose infiltrate of foamy macrophages and lymphocytes. The arrow in *d* indicates a sweat gland duct. (Hematoxylin and eosin; ×170.)

Electron Microscopy. A part of each biopsy was processed for transmission electron microscopy studies. Biopsies were washed in saline at 4°C, cut into 1- to 2-mm pieces, and fixed in 2.5% glutaraldehyde/0.1 M cacodylate buffer/0.1 M sucrose, pH 7.4, for 16 hr at 4°C. The tissues were cut to 1 mm or smaller and postfixed in 2% OsO$_4$ for 6 hr at 4°C. The tissue was then stained *en block* for 2 hr with 0.25% uranyl acetate, dehydrated in increments with ethanol, and embedded in Epon blocks. Semithin sections were stained with methylene blue–azur–basic fuchsin and examined for areas containing infiltrating cells. Sections were stained with uranyl acetate and lead citrate and examined with a Jeol JEM 100CX transmission electron microscope. At least 200 cells from each patient were examined and photographed on Kodak electron-imaging film.

RESULTS

The purpose of this study was to evaluate the efficacy of a delayed-type cell-mediated immune response in the reduction of *M. leprae* in the dermis of patients with the multibacillary form of leprosy. Accordingly, patients who had re-

ceived multidrug therapy for a relatively short time and who still had high bacillary indices were injected intradermally with 5 units of PPD. Twenty-one days thereafter, 4-mm punch biopsies of the injected site and an adjacent area were obtained, and segments were processed for (*i*) histology and acid-fast staining, (*ii*) immunocytochemistry for mononuclear cell subset identification, and (*iii*) transmission electron microscopy as described. Slides stained for acid-fast bacilli were coded and examined by three independent observers whose results were collated. The findings on 10 patients who gave positive responses to PPD and 2 unresponsive patients are described in Table 1.

Each of the responsive patients with lepromatous leprosy or borderline lepromatous leprosy demonstrated areas of firm induration ranging from 12 × 12 to 21 × 20 mm at the 48-hr reading. Patients 11 and 12 were unresponsive, having indurations of <10 mm. After 21 days, and at the time of biopsy, the indurated areas of the positive reactants were 10 × 10 mm or less. Upon retrospective analysis, the extent of the initial area of induration was unrelated to the nature of the 21-day site and to its histology and cytochemistry. Induration was associated with extensive infiltration of the dermis by
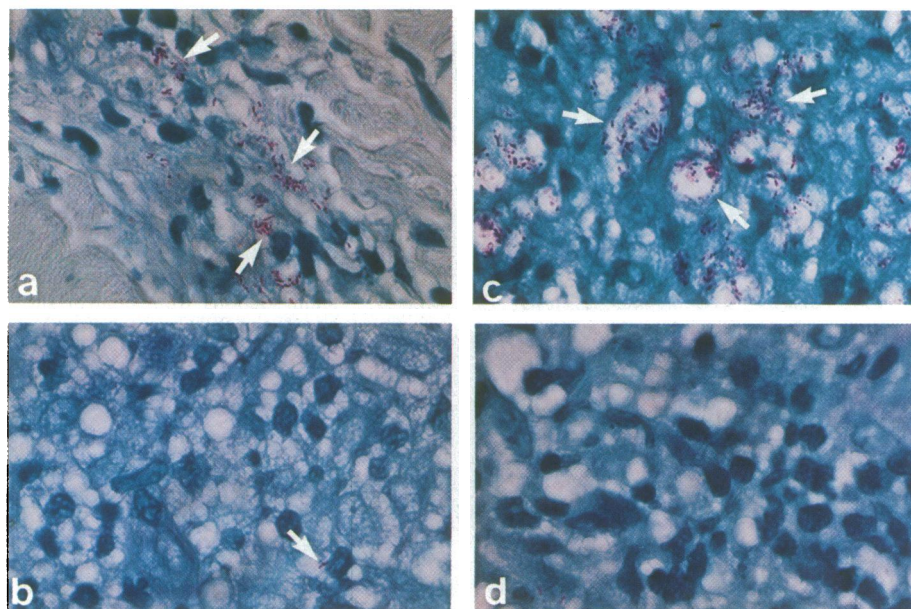

FIG. 3. *M. leprae* load in the control and PPD-injected sites 21 days after antigen administration. (*a* and *c*) High logarithmic bacterial index number (5+) in a control site (patients 8 and 9). (*b* and *d*) Reduced bacterial load (2+) in the PPD responsive site (patients as above). (Fite stain; ×850.)

mononuclear leukocytes. As will be reported in more detail elsewhere, the early infiltrate (48–92 hr) was composed predominantly of T cells, the majority of which were of the "helper" phenotype, and blood-borne monocytes. These cells most often were localized to and expanded areas of the underlying lepromatous infiltrate. In some cases (e.g., patient 4), the preexisting leprosy infiltrate involved only 5% of the upper dermis. Twenty-one days after the delayed reaction, the area of involved dermis was 4-fold higher (20%), with a dense accumulation of mononuclear cells. In certain cases (patient 5), 70% of the dermis was initially involved, and this was only slightly increased by the response to PPD treatment. It should be noted, however, that the cell density of the post-PPD infiltrated area was considerably higher. In the unresponsive patients, no enhancement of cellularity was apparent. We believe that the increase in the area of involved dermis, following the delayed reaction, is a rough estimate of the number or quantity of newly emigrating cells.

A rather striking qualitative distinction occurred in responsive patients in terms of their ability to form and/or maintain granulomata in the 21-day sites (Table 1). By our definition, granulomata represent the accumulation of epithelioid cells and multinucleate giant cells with an organized structure (6). In the absence of granulomata, the sites contained a loose association of T cells and mononuclear phagocytes. This is illustrated in the photomicrographs of Fig. 1.

It was of particular interest that the presence or absence of granulomata was clearly associated with the phenotype of the T cells present in the 21-day dermis. Biopsies that demonstrated granulomata had a strong predominance of the CD4$^+$ "helper" T-cell phenotype, whereas the absence of granulomata was associated with the CD8$^+$ "suppressor/cytotoxic" phenotype. Examples of this correlation are shown in Fig. 2.

Careful enumeration of the number of acid-fast *M. leprae* was conducted in each of the control sites and sites that had undergone a cell-mediated immune reaction. The results obtained for each of the 12 patients are shown in the right-hand side of Table 1. It should be remembered that a 1+ change in the bacterial index represents a 10-fold (1 order of magnitude) modification in the numbers of leprosy bacilli. Of the 10 PPD-responsive patients in Table 1, 6 had a reduction by 1–4 orders of magnitude in the number of bacilli in their skin lesions. Another 2 patients had less than 1-order-of-magnitude reduction (i.e., had no reduction in bacterial index units) but showed a 50% decrease in total bacilli [(−/+) in Table 1]. In the remaining two patients (1 and 7), no apparent decrease in numbers of organisms could be discerned. An example of the decline in acid-fast bacilli in the 21-day PPD site compared with the control uninjected site is seen in Fig. 3.

Careful examination of the PPD and control sites was also carried out by means of transmission electron microscopy. Control sites showed multibacillary vacuoles in the cytoplasm of viable dermal macrophages. Many of the *M. leprae* contained in these organelles appeared intact. Other nearby vacuoles contained organismal remnants and lipoid inclusions, presumably the result of bacterial death and degradation.

The appearance of the PPD sites was strikingly different. Here, the parasitized macrophages were undergoing cytolysis in close proximity to newly emigrated mononuclear phagocytes and lymphoid cells (Fig. 4b). Intact *M. leprae* were rare, and bacterial remnants were the only evidence of *M. leprae* infection. The reduction in structurally intact bacilli in macrophages was in keeping with the reduction in acid-fast bacilli reported at the light microscope level (Table 1).

Although the bacilli were rare in macrophages, they were, however, present in selected areas of the dermis. In particular, small peripheral nerves showed occasional multibacillary vacuoles within Schwann cells (Fig. 4c). No significant lymphoid infiltrate was associated with myelinated fibers, and Schwann cells were structurally intact. We suspect that
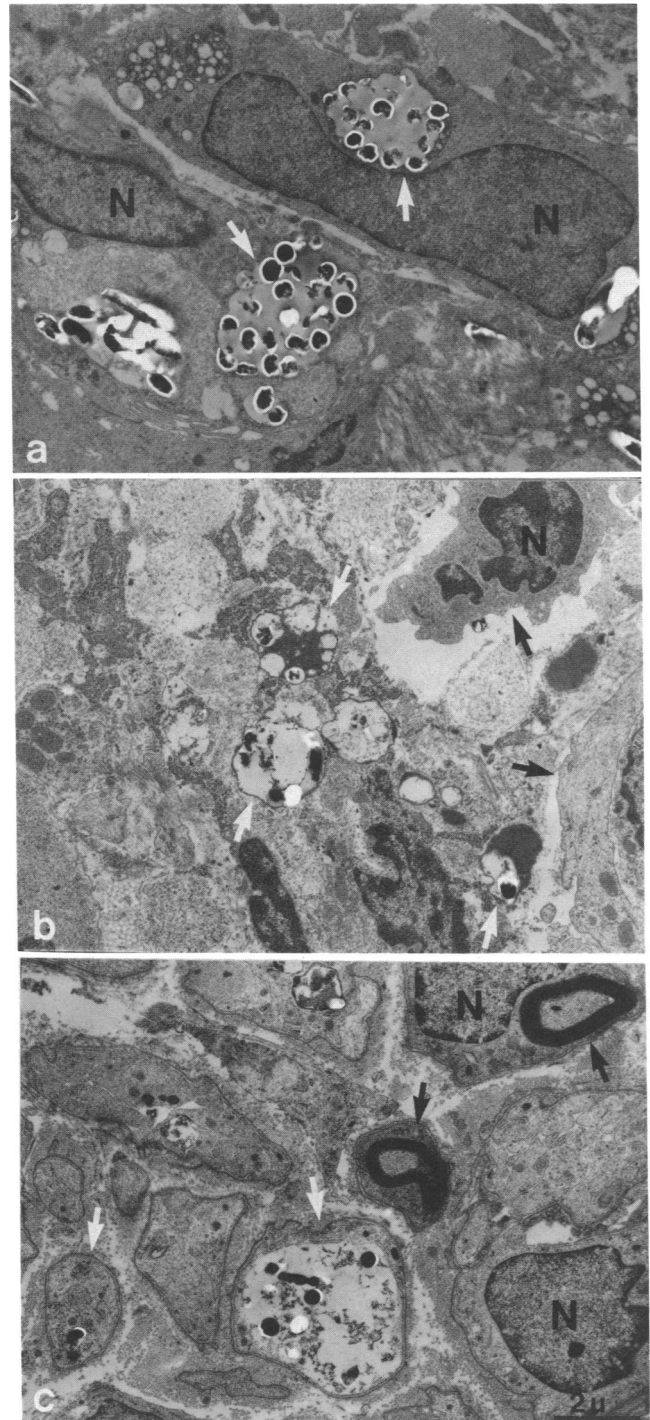

FIG. 4. Electron micrograph of *M. leprae* in the control and PPD-injected site 21 days after antigen administration. (*a*) Large numbers of bacilli, many of them intact, in the vacuole of two macrophages (arrows). N, macrophage nucleus. (*b*) Damaged partially digested *M. leprae* (white arrows) in the vacuoles of a dead macrophage of a PPD-injected site. An intact T cell and endothelial cell can be seen (black arrows). N, macrophage nucleus. (*c*) Intact *M. leprae* in a vacuole of a peripheral nerve Schwann cell (white arrow) of a PPD-injected site. Myelinated nerves can be observed (black arrows). N, Schwann cell nucleus.

these neural elements are not involved in the cell-mediated response and may continue to serve as bacterial reservoirs.

DISCUSSION

We have been able to modulate within a period of 3 weeks the bacterial load of a most static and chronic form of multiba-

cillary leprosy. This has occurred as the consequence of a new and effective cell-mediated response initiated by the antigens of *Mycobacterium tuberculosis*. It is now important to dissect the components of this delayed immune reaction and to identify the effector cells and molecules involved.

Certain correlates emerge from this study of which three are particularly pertinent. First, for a reduction in bacterial numbers to occur, exposure to a single dose of antigen must lead to the persistence of CD4$^+$ helper T cells in the lesion. Those sites in which CD8$^+$ suppressor/cytotoxic cells predominated failed to show significant bacterial clearance. It may be that the ability of patients to mobilize and maintain either CD4$^+$ or CD8$^+$ populations is a reflection of the severity of their underlying immunological defect. Second, the maintenance of an organized granuloma with epithelioid and giant cells is directly correlated with the ability to clear organisms. This may be a reflection of the ability of CD4$^+$ helper cells, or a subset thereof, to produce interleukin 4, a molecule recently shown to induce giant cells from human monocytes and macrophages (18); the presence of interleukin 4 or the appropriate messenger RNAs in such lesions should be investigated. Finally, although not detailed in this manuscript, it is clear that *M. leprae* clearance requires the destruction of initially parasitized macrophages. This is associated with the presence of newly emigrated lymphoid cells of unknown phenotype and is also observed in the lesions of tuberculoid leprosy (4). Such killer cells may represent lymphokine-activated killer cells, natural killer cells, and/or specific cytotoxic T lymphocytes. We suspect that the destruction of macrophages and the liberation of bacteria and their products is followed by cycles of ingestion, killing, and digestion carried out by newly emigrated and oxidatively capable monocytes (19).

New monocytes from the circulation are probably confronted with a variety of microbial substrates in the extracellular space following host-cell death. We assume, since the patients have been on bactericidal drugs for some time, that many of the organisms would be nonviable. The few viable organisms could be ingested and killed by monocytes capable of generating toxic oxygen intermediates. Subsequently nonviable bacteria would be exposed to the spectrum of intralysosomal acid hydrolases, and some or all of their constituents would be degraded. In fact, we know little about the capabilities of mammalian hydrolases to dismantle the unusual molecules of *M. leprae*. The bacterial fragmentation seen by electron microscopy and the loss of acid fastness suggest that some local digestion is taking place. However, disposal of bacteria and their products also could result from the migration of monocytes into afferent lymphatics and eventually to the local nodes.

Our results may be related to the therapeutic use of bacillus Calmette–Guérin and leprosy antigens reported by Convit and his colleagues (reviewed in ref. 20). In this instance, after multiple injections via parenteral routes, the authors claim general reductions in microbial loads (21). Similarly, Klein many years ago described the anti-tumor efficacy of intradermal PPD administration and topical dinitrochlorobenzoic acid sensitization (reviewed in ref. 22). In both instances, antigen-induced delayed-type cellular immune responses resulted in limited anti-tumor and anti-microbial responses.

The use of antigens to induce a cell-mediated immune response requires the prior sensitization of the recipient. If considered as a therapeutic modality, in conjunction with effective chemotherapy, only a portion of any population would be appropriately susceptible. Nevertheless, one might consider this reversible, easily controlled system for selected lesions and cosmetic reasons. Perhaps, recombinant lymphokines also could be used to carry out this form of immunotherapy.

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