# THE NATURE AND KINETICS OF A DELAYED IMMUNE RESPONSE TO PURIFIED PROTEIN DERIVATIVE OF TUBERCULIN IN THE SKIN OF LEPROMATOUS LEPROSY PATIENTS

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Leprosy is a chronic granulomatous disease of man caused by the intracellular bacterium *Mycobacterium leprae* (1). The clinical manifestations of the disease range from single paucibacillary lesions (tuberculoid) to disseminated multibacillary involvement of almost the entire skin (lepromatous). The polarity of the disease depends in large measure on the ability of the host to mount a cellular immune response to the infectious agent (reviewed in reference 2). To study the mechanisms underlying these delayed-type cell-mediated reactions, we have selected the response to a purified protein derivative of tuberculin (PPD)[1] in skin of the naturally sensitized hosts. We have already commented on the stimulation of keratinocyte proliferation and the induction of MHC class II antigen (3), the local production of macrophage-activating lymphokines, the induction and localization of γ IP-10 (4), and the influence of the delayed-type hypersensitivity reaction on the distribution of $T6^+$ Langerhans cells (5). More recently we have reported that the cellular response to PPD leads to the clearance of *M. leprae* from the skin of lepromatous patients (6).

In these studies we now present a detailed temporal examination of an effective tuberculin reaction in the skin of patients with lepromatous leprosy and comment on the determinants associated with the disposal of *M. leprae*.

## Materials and Methods

*Patient Population.* 154 patients with lepromatous leprosy (LL), borderline lepromatous leprosy (BL), or tuberculoid leprosy (BT and TT), as defined by the Ridley-Jopling classification (7), were seen in the Dermatology clinics, Safdarjung Hospital, New Delhi, SMS Medical College, Jaipur, and at the Leprosy Mission Hosipital, Shahdara, New Delhi, and tested for their response to PPD. Patients had been treated for leprosy for 0–6 yr with multidrug therapy. Medication was continued throughout the study.

*Tuberculin Testing.* 5 U of PPD (0.1 ml) were injected into apparently "normal" skin or

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[1] *Abbreviations used in this paper:* BL, borderline lepromatous leprosy; LL, lepromatous leprosy; PPD, purified protein derivative of tuberculin.

leprosy lesions on the lower back and the site evaluated for induration at 1–21 d. The injected site and a similar adjacent uninjected site were biopsied (4-mm punch) 1–21 d after PPD administration. The biopsies were divided into three parts and fixed for histopathology, immunohistology, and EM 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 were paraffin embedded. Sections were stained with hematoxylin and eosin for histological diagnosis. Acid-fast stained sections were used for the enumeration of *M. leprae* (8, 9).

*Immunohistology.* Biopsy specimens were fixed in paraformaldehyde (3%), lysine (0.075 M), and sodium-*m*-periodate (0.01 M) in PBS for 3–4 h at 4°C as described by McLean and Nakane (10). This fixative preserves structural details without inhibiting the binding of mAbs to their antigens. The biopsies were washed in PBS containing sucrose (10%) and digitonin ($5 \times 10^{-5}$ M) and then serially suspended in graded solutions of sucrose (15–25%). The tissue was stored in PBS with sucrose/glycerol (25% and 5%, respectively) until frozen.

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

*mAbs.* Mouse mAbs were used for the identification of specific cell types. Leu-1, Leu-2a, and Leu-3a (11, 12) (anti-T cells and their subsets) and Leu-M5 (anti-monocyte/macrophage) (13) were obtained from Becton Dickinson & Co., Mountain View, CA.

*Determination of Epidermal Thickness.* Epidermal thickness was evaluated by direct examination of hematoxylin-stained sections using a computer-based image digitizing system (Southern Micro Instruments, Inc., Atlanta, GA). The distance from the epidermal-dermal junction to the outer surface of the epidermis was measured at equal intervals along the length of the sections. At least 25 measurements were made per section. Results were expressed as the ratio of mean thickness of the PPD-injected site vs. the control site for each patient.

*EM.* A part of each biopsy was processed for transmission EM studies. Biopsies were washed in saline at 4°C, cut into 1–2-mm pieces, and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer with 0.1 M sucrose, pH 7.4, for 16 h at 4°C. The tissues were cut to 1 mm or smaller and postfixed in 2% $OsO_4$ for 6 h at 4°C. The tissue was then stained en block for 2 h with 0.25% uranyl acetate, dehydrated in increments with ethanol, and embedded in epon blocks. Semi-thin 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 transmission electron microscope (model JEM 100CX; JEOL USA, Peabody, MA). At least 200 cells from each patient were examined and photographed on Kodak electron image film.

## Results

*PPD Responsiveness of Leprosy Patients and Nonleprosy Controls.* The local reaction to the intradermal injection of 5 U of PPD into leprosy patients and control volunteers is outlined in Table I. 154 patients with different forms of leprosy and treatment regimens were examined as well as 50 control subjects. Approximately 70% of the control population responded positively to the injection of PPD. Areas of induration that were 10 mm or more in diameter at 48 h were considered positive. Patients with the BT and TT paucibacillary forms of the disease were more responsive (84%). Lepromatous patients were as a group less responsive with 58% demonstrating reactivity. Within the lepromatous group, those under treatment for <18 mo showed lower reactivity than those patients with more than 18 mo of therapy. Many of the

TABLE I

*PPD Response in Leprosy Patients and Nonleprosy Controls*

| Diagnosis | Number tested | Induration > 10 mm* |
|---|---|---|
| | | % |
| LL | 63 | 37 (58) |
| Rx less 18 mo | 44 | 22 (50) |
| Rx 18 mo or more | 19 | 15 (73) |
| BL | 32 | 25 (78) |
| BT/TT | 59 | 50 (84) |
| Nonleprosy controls | 50 | 34 (68) |

Rx, therapy

* Number of responders and percent of patients in the test group.

untreated LL/BL patients with a very high bacterial index (6+) failed to respond to PPD.

*The Course of Induration.* Macroscopically, the positive lesions showed areas of induration, often with a slightly erythematous skirt, which were well developed by 24 h (Fig. 1 *A*). The peak area of induration occurred by 96 h and ranged in size from 10-25 mm in diameter. These were slightly raised, firm nodules. Thereafter, the indurated areas gradually were reduced in size but in most cases were still palpable after 21 d. The erythematous responses occurred during the earlier days of the reaction.

*Epidermal Thickening.* Microscopic evaluation of the thickness of the epidermis overlying the site of PPD administration revealed enhanced epidermal thickness as compared with the matched control site in all patients biopsied from 1-14 d after antigen administration (3-5). The kinetics of thickening corresponded with that observed for induration (Fig. 2). Maximum thickening occurred by 3-4 d and ranged from 1.4- to 2-fold normal thickness. Epidermal thickness returned to normal in all patients tested by 21 d, largely as the result of keratinization and sloughing of the upper epidermal layers.

Associated with the enhanced thickening of the epidermis was a significantly enhanced healing of the site of punch biopsy taken from PPD-responsive skin lesions. By day 2, the PPD sites were dry and exhibited a base of granulation tissue and keratinocyte movement into the wound. The control site showed similar gross characteristics at day 3-4. By day 5-6 no apparent differences in the sites were noted.

*The Cellular Response to PPD.* To evaluate the extent of cellular infiltration, the nature of the subsets and the sequellae of cellular interactions, we evaluated the PPD reactions from 1-21 d in the skin of LL and BL patients. 4-mm punch biopsies of the skin served as the source of material for histopathology, immunocytochemistry, and transmission EM. In each instance biosies were taken from the center of the PPD-injected site as well as an adjacent, uninjected area of the skin. It should be pointed out that all LL and BL patients in the study had at least sparse infiltrates of inflammatory cells even in so called uninvolved "normal skin." These consisted of perivascular aggregates of macrophages, some of which were *M. leprae* infected, with occasional T cells, largely of the $CD8^+$ subset (14). These accumulations were

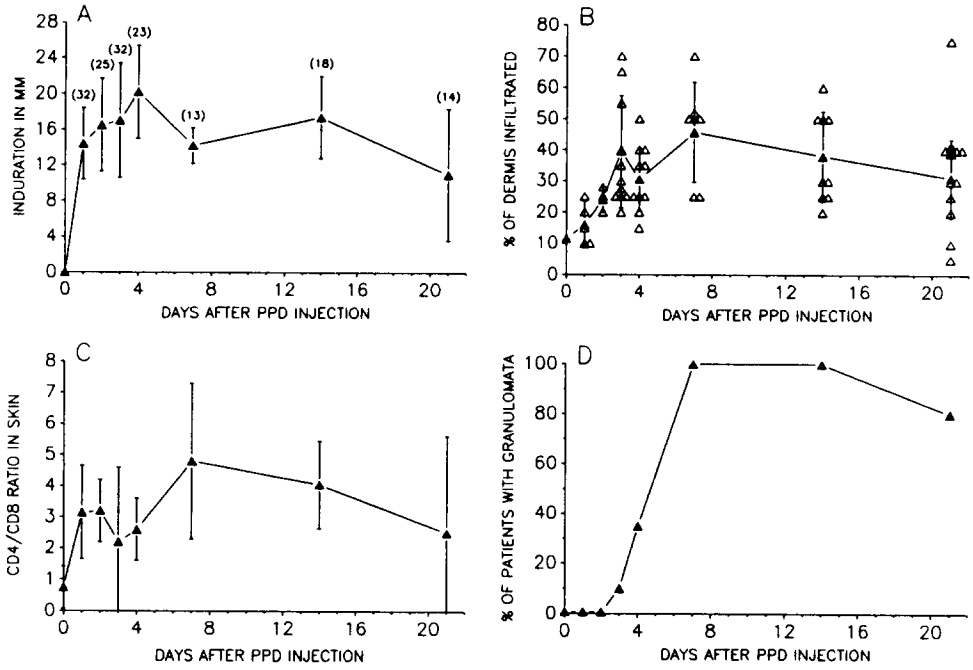


FIGURE 1. Kinetics of the local delayed-type hypersensitivity response to intradermal administration of PPD. (*A*) Induration. (*B*) Percent of the dermis infiltrated by mononuclear cells. (*C*) T cell subsets expressed as $CD4^+/CD8^+$ ratio. (*D*) Percent of the patients with a granulomatous reaction containing epitheloid and multinucleated giant cell. Results are expressed as mean ± SD in *A*-*C*. Number in parenthesis denotes the number of patients evaluated at each time point.

often concentrated in the upper dermis and were separated from the epidermis by a clear acellular zone.

Zones of induration were no doubt the result of the accumulation of both cells and fluid. We therefore required an independent estimate of the cellularity of the responses as a baseline for the phenotypic evaluations. For this purpose we calcu-

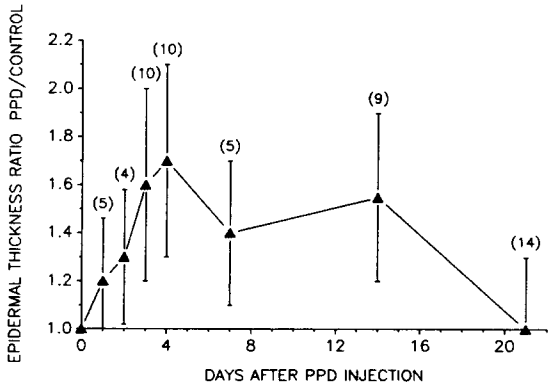


FIGURE 2. Kinetics of changes in epidermal thickness in response to intradermal administration of PPD. Results are expressed as means ± SD of ratio of epidermal thickness of PPD site over control of all patients biopsied at each time point.

lated the percentage of the dermis that was infiltrated with inflammatory cells and this information is shown in Fig. 1 *B*. The majority of skin biopsies from control non-PPD sites showed that 10–15% of the dermis was already involved. After PPD injection the rate of dermal infiltration was almost linear for the first 72 h and reached peak values only at 1 wk. Photomicrographs of this process are seen in Fig. 3. Clearly induration precedes infiltration during the first 2–3 d of the response and this suggests a significant extravasatory element. At 4–7 d as much as 70% of the dermis is heavily infiltrated with newly emigrated cells, the majority of which accumulate about areas of preexisting cells. As the reaction proceeds, these enlarge and coalesce

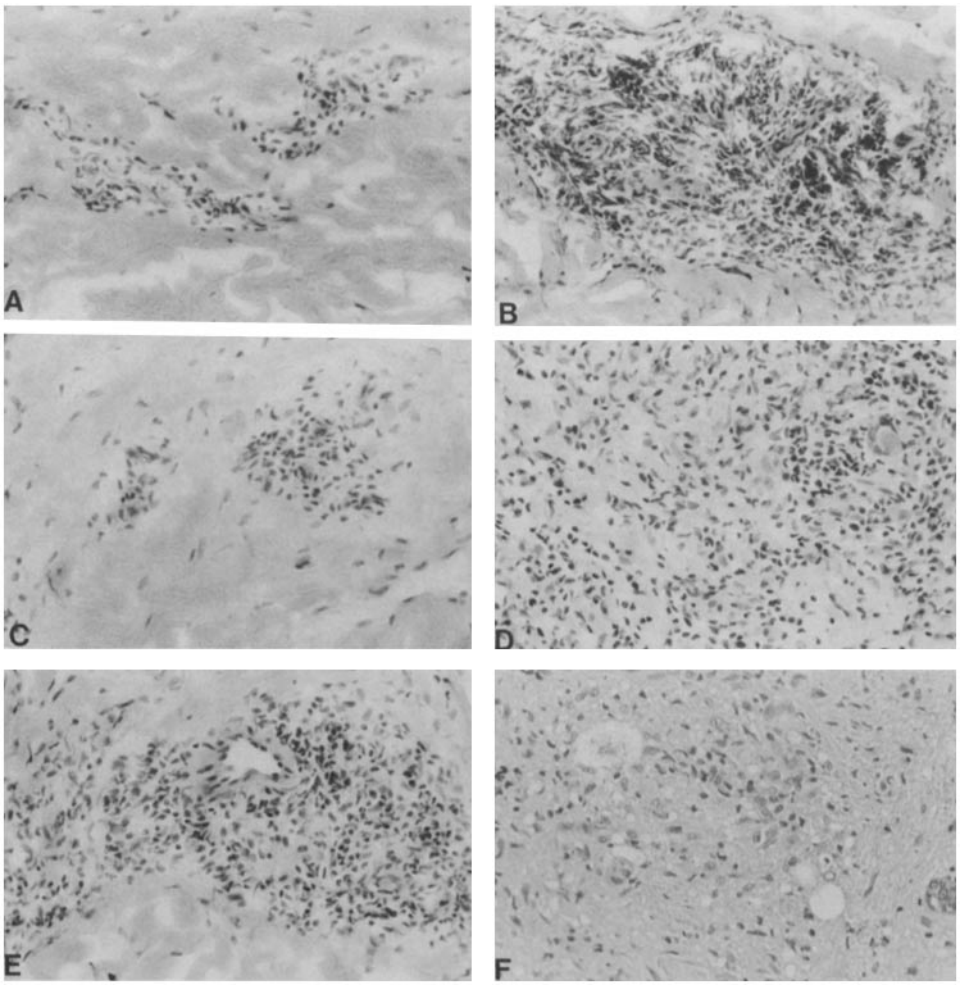


FIGURE 3. The extent of dermal infiltration during a PPD response. Biopsies were taken from patient 102 at time 0 (*A*), 18 h (*B*), and 14 d (*E*), and from patient 126 at time 0 (*C*), 7 d (*D*), and 21 d (*F*). Enhanced dermal infiltration is seen after PPD administration. The cellularity persists for at least 21 d (×100).

to form solid sheets. With the passage of time the cellularity of the dermis decreases but even after 21 d is still three times above the control values.

*The Accumulation and Persistence of T Cell Subsets.* At the earliest time interval after antigen injection (18 h) many lymphocytes had already entered the dermal site and were arranged in a perivascular fashion. These increased in number and plateaued by 7 d, thereafter, gradually disappearing over the next 2 wk in keeping with the overall size of the infiltrate. The vast majority of the newly emigrated cells were of the $CD4^+$ "helper" phenotype but large numbers of $CD8^+$ were also present. This is in striking contrast to the existing, chronic lepromatous leprosy infiltrate (Figs. 1 *C* and 4). The relative preponderance of the helper phenotype showed two distinct peaks at days 2 and 7 and persisted up to 21 d in a majority of patients. There were some, however, who demonstrated inversions of this ratio with a $CD8^+$ preponderance. It is of interest, that these individuals lost the typical character of the responsive site, lacked granulomas at 21 d, and failed to clear their dermal load of *M. leprae* (6).

*The Granulomatous Response.* Subsequent to the accumulation of mononuclear cells, differentiation and organization of cells takes place to form granulomas. These areas consist of macrophage-derived epitheloid cells exhibiting large areas of pale cytoplasm and multinucleated giant cells resulting from the fusion of macrophages (Fig. 5). At the ultrastructural level epitheloid cells illustrate tightly interdigitated plasmalemmal extensions. Certain evidence suggests that this process is under lymphokine control (15). Small numbers of patients exhibited granulomas at 72 h and 30–40% of biopsies were positive by 96 h (Fig. 1 *D*). At 7 d essentially all the PPD sites exhibited granulomas and these were still apparent at 2 wk. By 21 d ~75% of the biopsy sites remained positive and the remainder exhibited a looser, less-organized appearance without epitheloid cells or giant cells. Granulomata were never seen in the underlying lesions of LL/BL patients. The appearance of such granulomata at the electron microscope level is seen in Fig. 5. These sites contain in addition to epitheloid and multinucleated giant cells many lymphocytes closely associated with the mononuclear phagocytes. Many of the lymphocytes have complex membranous projections (Fig. 5 *B*) similar to the cells described in tuberculoid lesions (16).

*The Selective Destruction of Foamy, Parasitized, and Epithelioid Macrophages.* We had reported earlier that within the lesions of paucibacillary tuberculoid leprosy there was extensive destruction of epitheloid cells and what appeared to be constant turnover of the cells of these granulomatous lesions (16). This occurred in association with lymphoid cells, characterized by extensive projections of their plasmalemma. A similar situation was taking place in the developing PPD reaction with some notable differences. First, many more *M. leprae* were initially present within the multibacillary vacuoles of the resident macrophages. At 1 wk the transmission electron micrographs of these sites showed extensive death of parasitized macrophages, cellular dissolution, and the extracellular discharge of the organisms. Examples of these events are shown in the electron micrographs of Fig. 6. On occasion, newly emigrated monocytes contained bacilli and bacillary products similar in structure to the contents of foamy macrophages. The phagocytic vacuoles of such monocytes usually contained only one or two organisms in contrast to the clusters present in the preinjection population. Numerous lymphoid cells were often apparent in direct contact with the dead and dying macrophages and epitheloid cells. Other than their surface projections and relatively electron dense cytoplasm, these cells had not other identifying features.

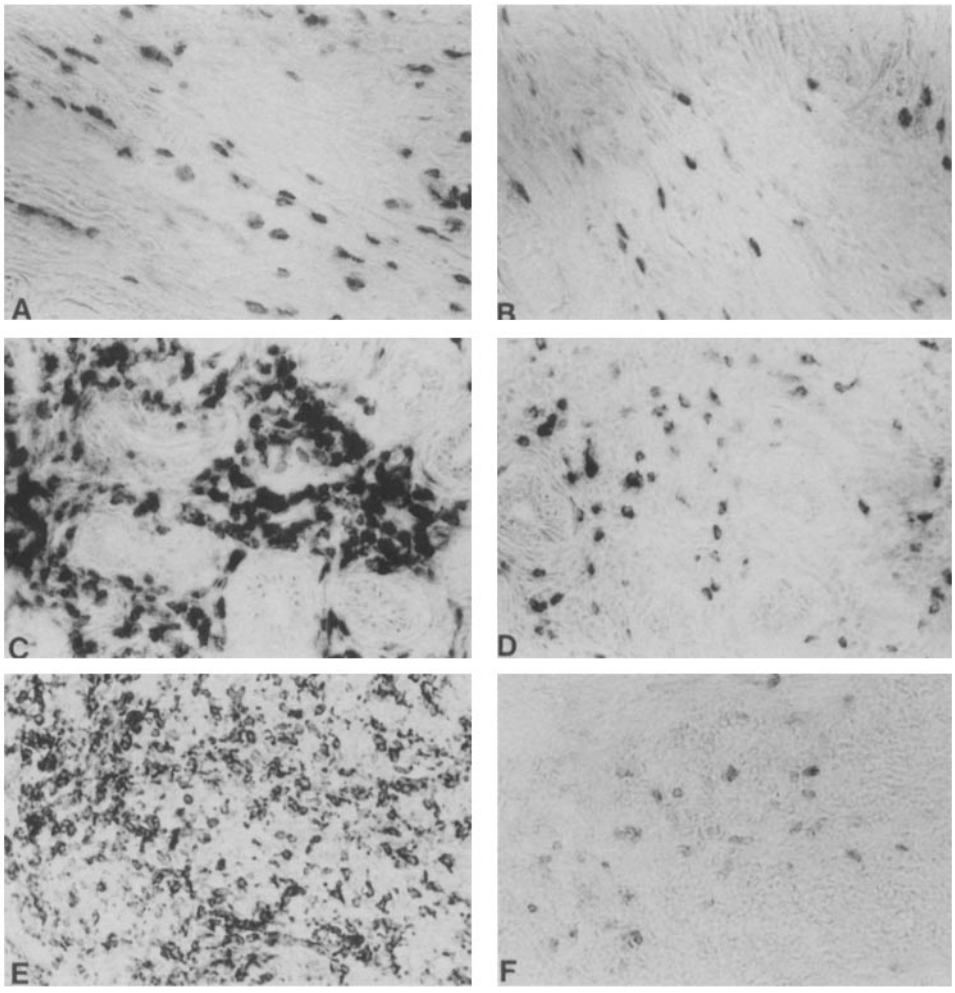


FIGURE 4. T cell subset distribution during a PPD response. Immunohistologic staining of cryostat sections with mAbs that recognized all T cells (Leu-1) (*A, C,* and *E*) and the $CD8^+$ subset of T cells (Leu-2a) (*B, D,* and *F*). Biopsies were taken at time 0 (control lepromatous lesion) (*A* and *B*), 24 h (*C* and *D*), and 16 d (*E* and *F*). In the control biopsy $CD8^+$ T cells account for at least half the T cells stained. At 24 h and 16 d $CD8^+$ T cells account for the minority of cells staining (×200).

No other cell type in the enviroment, including fibroblasts, endothelial cells, sweat glands, or epidermal elements appeared to be injured by the infiltrating lymphoid cells.

*Survival of the Peripheral Nerve.* The extensive destruction of bacilli-laden macrophages and their derivatives did not extend to other cells of the dermis. Such non-macrophages rarely contained microorganisms, with the exception of endothelial cells. There was, however, another cell of the dermis that was heavily parasitized and this was the Schwann cell of the peripheral nerve (Fig. 6). Schwann cells often contained huge vacuoles with 25 or more bacilli, many of them intact. In numerous blocks and sections from PPD responders, we did not see injury either to these cells

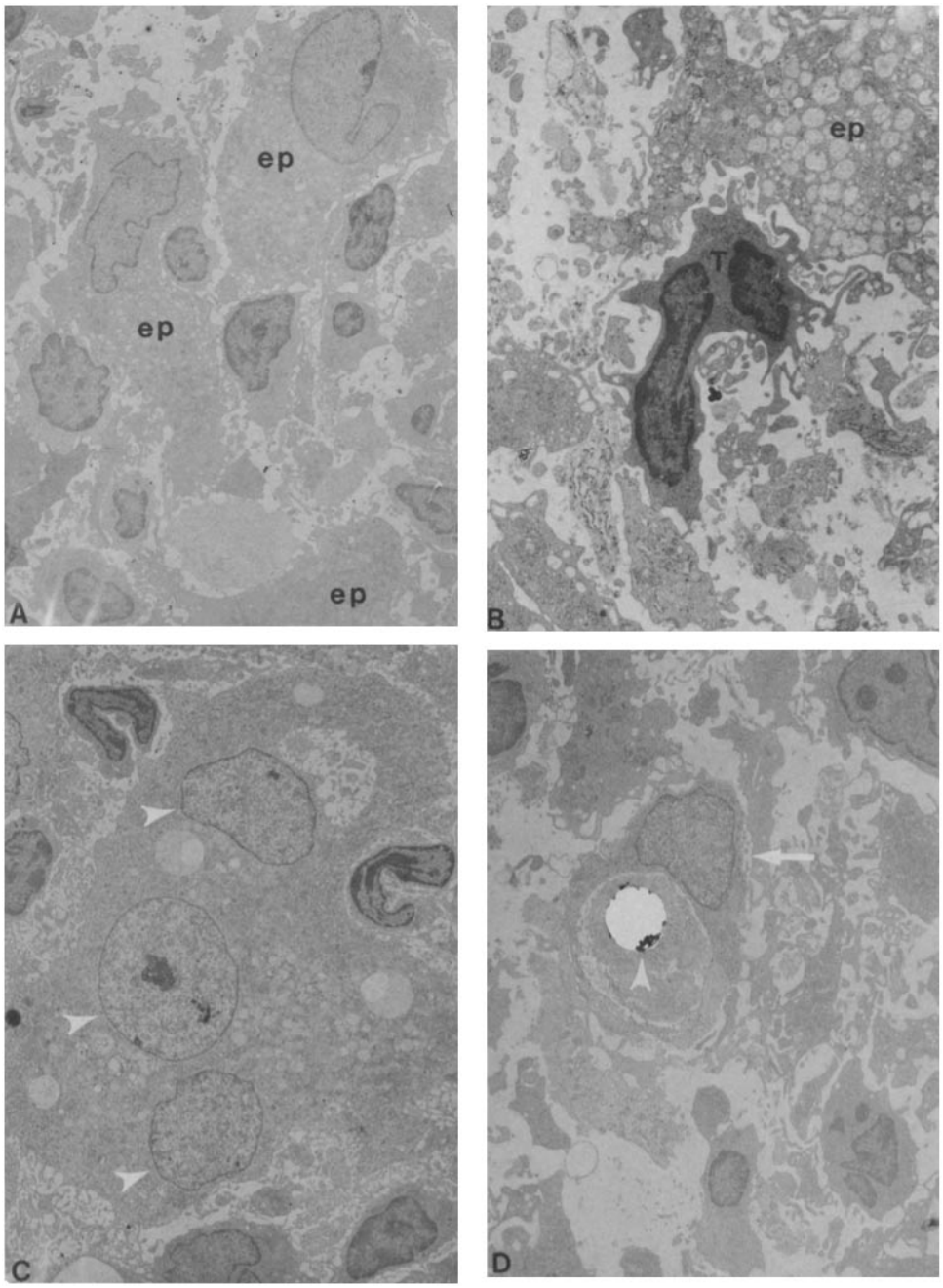


FIGURE 5. Granuloma formation in response to PPD administration. Transmission electron micrographs of PPD responsive sites biopsied at 7 d. (*A*) Epithelioid cells closely associated with T cells are seen. The epithelioid cells (*ep*) contain large numbers of mitochondria, endocytic vacuoles, and RER (×2,000). (*B*) Highly active T cell (*T*) with multiple membrane extensions is seen in association with an epithelioid cell (*ep*) (×5,000). (*C*) Multinucleated giant cell (*arrow heads*) surrounded by T cells (×2,000). (*D*) Within the granuloma intracellular *M. leprae* were found (*arrow heads*) predominantly in Schwann cells of peripheral nerves (*arrow*) (×2,600).

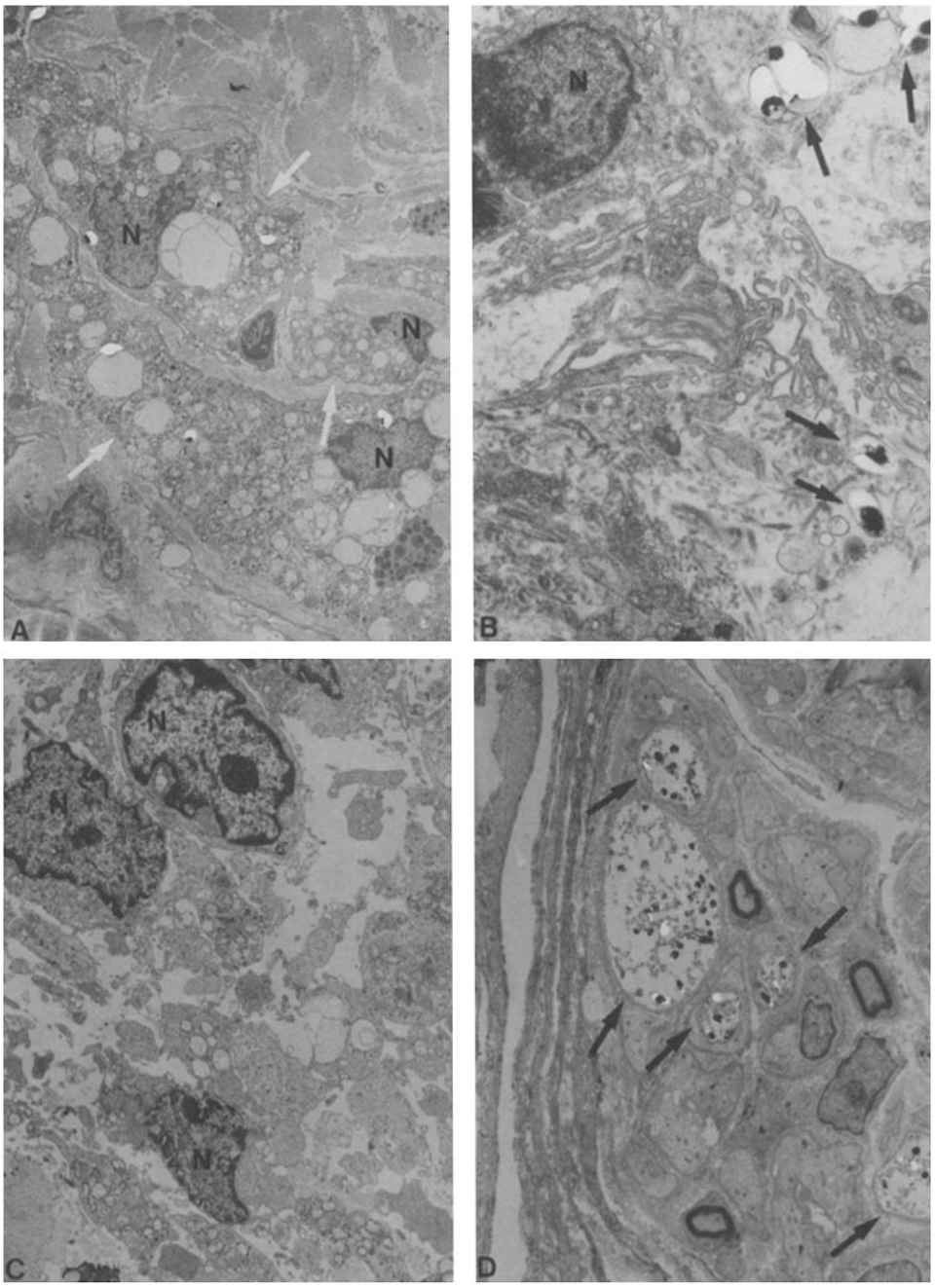


FIGURE 6. The selective destruction of parasitized macrophages and foam cells. (*A*) Control lepromatous leprosy lesion with intact, parasitized foamy macrophages (*arrows*). (*N*) Nucleus of macrophages (×2,600). (*B*) PPD-injected site biopsied 7 d after antigen administration. The parasitized phagocytes are disrupted and extracellular *M. leprae* (*arrows*) and cell debris are observed. (*N*) The nucleus of the damaged macrophage (×8,300). (*C*) Dead and damaged phagocytes in association with intact mononuclear cells at 16 d after PPD administration. (*N*) Nuclei of damaged macrophages (×6,000). (*D*) PPD-injected site at 16 d. Although many of the phagocytes of the dermal infiltrate are dead and damaged, Schwann cells are still heavily infected with *M. leprae* (many of them intact) (*arrows*) and no infiltrating mononuclear cells are seen within the neural sheath (×3,300).

or to other elements of the peripheral nerve. In fact, although extensive cell destruction and infiltration occurred adjacent to the perineural sheath, this barrier was never breached and mononuclear cells were not found infiltrating the nerves.

## Discussion

The introduction of PPD into the sensitized host leads to a complex series of cellular events that may result in the destruction of cellular or microbial targets. The process occurs with a defined sequence, proceeds for a finite time, and persists for at least 3 wk (17). Since this study was carried out in the skin of lepromatous leprosy patients, it is possible that the underlying disease state and the preexisting cells infiltrating the dermis may influence the rate and extent of the process. Bullock and others (18, 19) have commented on a reduced incidence of positive DTH reactions in leprosy patients. In this regard we noted many patients with high bacillary indices were unresponsive to PPD while treated patients were more responsive (20). This suggests that T cell mitogenesis may be suppressed by high concentration of bacillary products and result in a more general form of anergy.

Since the PPD reactions in responder patients showed a similar time course when induced in a lepromatous lesion or in normal uninvolved skin, it seems unlikely that the preexisting lepromatous leprosy infiltrate plays a prominent role in the regulation of the tempo of the positive DTH response. Involvement of microvasculature, endothelium, epidermal lymphoid cells, dermal macrophages, and mast cells in the early signaling events of the DTH response have been suggested to lead to monocyte and T cell accumulation (21).

The nature of signals that initiate the vasculature responses allowing the selected emigration of lymphocytes, monocytes, and Langerhans cell precursors is unclear. During the first 24 h, in addition to the influx of mononuclear cells, one observes extravasation of fluid that is accompanied by an erythematous, vasodilatory phase, but is devoid of granulocytes and other elements of immediate hypersensitivity. Once initiated, mononuclear influx proceeds steadily and reaches a plateau between 4 and 7 d after the administration of antigen. The initial infiltrate seems to localize around the vascular channels associated with the preexisting infiltrate. At the height of the response, either as the result of vessel dilatation or angiogenesis, more vessels are apparent and multiple foci of cell accumulation appear. Although difficulties exist in the enumeration of newly emigrated cells, one can calculate from the depth and diameter of the lesion that many millions of cells enter the lesion and are maintained as a result of a single injection of antigen. From the studies of others (22, 23) it seems unlikely that free antigen exists in the tissues for more than a few hours. This suggests that amplification systems are set into motion, which are largely antigen independent and which sustain the cellularity of the site for a number of weeks.

The predominant cells of the early reaction are T cells of the CD4 or helper phenotype. From studies in small animals, few of the emigrated cells are specifically sensitized to the evoking antigen (24). One suspects, therefore, that a minor fraction of cells initially responds to antigen. As a result of their secretory products and the presence of an increasing number of accessory cells, other lymphocytes are recruited and contribute to the local, secreted lymphokine pool. The biphasic nature of the lymphocyte influx with peaks at 2 and 7 d (Fig. 1) is consistent with two distinct emigratory signals. Indeed, intradermal administration of either human rIFN-$\gamma$ or

IL-2 alone, in the absence of antigens, leads to the infiltration of T cells and monocytes into the injected dermal site (25, 26) (manuscript in preparation). One might also expect that $CD8^+$ T cells of the suppressor/cytotoxic phenotype, lymphokine-activated killer cells, and NK cells have separate emigratory signals, are influenced by the lymphokine rich environment supplied by helper cells, and play a role in the selective and extensive killing that takes place in these sites.

Structural evidence from this and previous examination of tuberculoid leprosy lesions (16) show that the destruction of epitheloid cells and macrophages containing *M. leprae* and *M. leprae* products is associated with the presence of lymphocytes exhibiting extensive cytoplasmic projections. Such cells are in direct contact with dead and dying mononuclear phagocytes. The phenotype of this cytotoxic population is unknown. Possible candidates could be activated CTL cells, LAK cells, or NK cells exposed to local lymphokines. The end result of their cytotoxic activity would be to liberate intact and soluble molecules from fragmented *M. leprae* into the extracellular milieu. Newly emigrated monocytes, oxidatively competent phagocytes (27), then have the opportunity to ingest and kill the organism and accelerate the process of intracellular digestion. We suspect that the older bacilli-laden macrophages of the dermis are oxidatively imcompetent, fail to kill *M. leprae*, and resemble in that respect the hepatic Kupffer cell, resident splenic macrophage, and blood monocyte after long-term culture (27, 28). Numerous cycles of phagocytosis and cell destruction may be necessary to both kill and digest this organism. In any event we believe it is essential to kill the host macrophage and expose its contents to a fresh intracellualr environment. The nature, phenotype, and recognition signals used by the effector cytotoxic lymphocytes are currently unknown. A similar destruction of *L. donovani*-infected Kupffer cells occurs during the development of hepatic granulomas and in the cytotoxicity of viral-infected host cells (29).

Although mononuclear phagocytes of the dermis are destroyed in the developing immune reaction, neural components are spared. Schwann cells often contain large numbers of organisms and peripheral nerve dysfunction is commonly observed during the course of lepromatous disease. Even in paucibacillary disease, neural damage is prominent and the neural sheath shows infiltration with both macrophages and T cells (7). Similarly, in the reactional states, which occur spontaneously in the course of leprosy or following chemotherapy, continued and accelerated neural lesions develop (30, 31). It is therefore of interest why, in the PPD response, the neural sheath remains intact even though cell destruction occurs in close proximity. Answers to this question are not available at this time. However, the apparent lack of nerve involvement with exogenous antigen might be an important feature of this form of local immunotherapy.

## Summary

We have analyzed the nature and kinetics of a delayed, cell-mediated immune response to a purified protein derivative of tuberculin (PPD) in the skin of 154 naturally sensitized patients with lepromatous leprosy. After the intradermal injection of 5 U of PPD, biopsies were taken at 1–21 d and studied for the composition, extent, persistence, and organization of the emigratory cell response by light and electron microscopy.

Induration of positive sites occurred promptly, reached a maximum diameter at

4 d, displayed a major extravasatory element, and was evident for as long as 21 d. The cellularity of the site exhibited a biphasic course, reached a maximum at 7 d, involved as much as 70% of the dermis and millions of new cells, and was elevated threefold above preinjection levels at 21 d. The emigratory cells were limited to T cells and circulating monocytes. T cells were more evident as they entered a preexisting lepromatous lesion containing parasitized macrophages and only occasional T cells many of the $CD8^+$ phenotype. The predominant emigratory T cell was $CD4^+$ although $CD8^+$ cells were in evidence. The CD4/CD8 ratio of the lesions started at less than unity and in two distinct steps reached levels as high as 5:1. In most sites $CD4^+$ cells were in the majority at 21 d. A well-defined granulomatous response with epithelioid and giant cells was apparent at 4 d, reached a maximum at 7 d, and involved all PPD sites at this time point. The generation of these differentiated mononuclear phagocytes from newly emigrated monocytes was never observed in the underlying lepromatous lesion but is a constant feature of the tuberculoid leprosy response. Epidermal thickening and keratinocyte proliferation, sequellae of the dermal reaction, reached a maximum at 7 d and gradually resolved by 3 wk.

A constant feature of the PPD response was the extensive destruction of preexisting macrophages containing *Mycobacterium leprae* bacilli or their products. This was associated with the presence of and intimate contact with highly polarized lymphoid cells of unknown phenotype. Cell destruction did not involve other elements of the dermis and spared parasitized Schwann cells. Newly emigrated T cells and monocytes were never seen within the perineural sheath in contact with neural elements.

It appears that a single antigenic stimulus leads to a very long-term, defined series of events with distinct temporal patterns. It includes waves of emigratory T cells, the maturation and organization of monocytes, the generation of killer cells, and the extensive destruction of parasitized macrophages. We suspect that the chronicity and complexity of the reaction involves antigen-independent amplification signals, the recruitment of other cells in the dermis and epidermis, and the eventual disposal of the intracellular pathogen *M. leprae*.

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