

Local Nerve Damage in Leprosy Does Not Lead to an Impaired Cellular Immune Response or Decreased Wound Healing in the Skin

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This study investigated whether peripheral nerve damage in patients with leprosy impairs local cellular immune responses, thereby reducing wound healing and leading to chronic skin ulceration. Anesthetic and contralateral sensitive skin sites in 42 patients with leprosy were compared for delayed-type hypersensitivity responses to purified protein derivative (PPD) of tuberculin. Leukocyte recruitment, epidermal activation, keratinocyte proliferation, and rates of wound healing after skin biopsy were compared. No significant differences in PPD-induced induration, epidermal activation and thickening or numbers of total T cells, CD8⁺ T cells, CD1a⁺ Langerhans cells, and proliferating Ki67⁺ keratinocytes were observed between anesthetic and sensitive skin sites. Similarly, rates of wound healing over 5 days after skin biopsy did not differ significantly. Thus, local leprosy-associated anesthesia does not appear to contribute to local immune compromise or impaired wound healing. Rather, chronic cutaneous ulceration in leprosy most likely results from repeated trauma associated with loss of sensation.

Leprosy is predominantly a skin disease caused by infection with *Mycobacterium leprae*. The bacilli reside and proliferate in macrophages infiltrating the skin. In addition, dermal nerves are invaded by *M. leprae*, which selectively bind to the laminar surface of Schwann cells [1], gain entry into the cells, and replicate there. Schwann cell proliferation and death, combined with the ensuing host inflammatory response to the mycobacteria, result in damage to the peripheral nerves and lead to functional impairment, including desensitization to temperature, light touch, and pain. Chronic ulcers often develop in these patients, constituting a major clinical problem.

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Written informed consent was obtained from all patients before enrollment. Human experimentation guidelines of the Armauer Hansen Research Institute/All-Africa Leprosy Rehabilitation and Training Research Committee, the national ethical clearance committee of the Ethiopian Science and Technology Commission, and Rockefeller University were followed in the conduct of this clinical research.

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It is not known whether ulceration is due solely to repeated mechanical stress at anesthetic sites or also due to decreased cellular immune response and impaired granulation and tissue repair (wound healing). Improved healing of plantar foot ulcers in patients with foot orthoses supports the hypothesis that ulceration is due to repeated mechanical stress [2, 3]. The alternative hypothesis that nerve damage may lead to an impairment of cellular immune responses is supported by the observation of decreased inflammation at sites of peripheral denervation in experimental arthritis [4]. Similarly, in humans, unilateral paresis in patients with poliomyelitis results in an impaired delayed-type hypersensitivity (DTH) response on the paretic side [5], and patients with rheumatoid arthritis with preexisting central or peripheral motor nerve lesions do not develop joint inflammation in the paralyzed limb [6, 7].

To test whether leprosy-induced nerve damage affects local cellular immune responses, patients with leprosy were tested for their in situ responses to a recall antigen (purified protein derivative [PPD] of tuberculin), injected both into an anesthetic skin site and into a contralateral skin site with intact sensation. In addition, the rate of wound healing after skin biopsy sampling at each site was compared.

Materials and Methods

Subjects. Patients with leprosy ($n = 42$) were recruited to the study from the outpatient and inpatient clinics of the All-Africa Leprosy Rehabilitation and Training Center in Addis Ababa, Ethiopia. A subset of patients ($n = 17$) agreed to have 4-mm punch skin biopsy samples taken from 2 skin sites.

Tuberculin skin test. Sensation to light touch with cotton wool was used to identify an anesthetic skin site on 1 forearm and a

sensitive site at a similar location on the contralateral arm of each patient with leprosy. At each selected skin site, 0.1 mL of tuberculin PPD (RT 23; Statens Serum Institut) was administered intradermally. A positive skin test response was denoted by an induration diameter ≥ 5 mm after 48 h.

Punch biopsy of skin. Patients had biopsy samples taken 72 h after tuberculin skin testing. Lidocaine (Abbott) was injected into the site, and a 4-mm biopsy sample was obtained by biopsy punch (Baker). Biopsy specimens were placed in buffered formalin and embedded in paraffin. Longitudinal skin biopsy sections were placed

on positively charged microscope slides for subsequent immunohistologic and histologic staining with hematoxylin-eosin (H-E).

Immunohistology. Paraffin-embedded skin biopsy sections were deparaffinized and rehydrated through graded alcohol. Antigen unmasking was done by boiling slides in 10 mM citric acid buffer (pH 6.0) for 20 min. Immunohistologic staining was performed in an automated immunostainer (Ventana Medical System) with monoclonal antibodies directed against the human T cell markers CD3 (1:1; Ventana Medical System) [8] and CD8 (1:20; Dako), the proliferation marker Ki67 (1:1; Ventana Medical Sys-

Table 1. Patients with leprosy ($n = 42$) and results of purified protein derivative (PPD) test.

Patient	Sex	Ethnic group	Age, years	Leprosy type	Reactional state	Treatment	DTH/A, mm	DTH/S, mm	DTH result/biopsy status
1	F	Gurage	16	BL	RR/neuritis	MDT/Prd	20	21	Pos/NB
2	M	Amhara	29	BT	RR/neuritis	MDT/Prd	18	17	Pos/NB
3	F	Amhara	15	BT	RR	MDT/Prd	0	0	Neg/NB
4	M	Amhara	23	BL	RR/ENL	Thalidomide/Prd	11	13	Pos/NB
5	M	Amhara	57	BT	Neuritis	NT ^a	0	0	Neg/NB
6	F	Oromo	14	BT	RR	MDT/Prd	0	0	Neg/NB
7	M	Oromo	34	BL	ENL/neuritis	Prd	0	0	Neg/NB
8	M	Gurage	51	BT	Neuritis	Prd	0	0	Neg/NB
9	M	Amhara	44	BL	ENL	Prd	0	0	Neg/NB
10	M	Amhara	15	BL	Neuritis	NT	0	0	Neg/NB
11	M	Amhara	19	BL	RR/neuritis	MDT/Prd	14	10	Pos/NB
12	M	Oromo	14	BT	RR	MDT/Prd	0	0	Neg/NB
13	M	Amhara	NA	BL	RR	MDT/Prd	26	18	Pos/NB
14	M	Amhara	20	BL	Neuritis	MDT/Prd	0	0	Neg/NB
15	M	Amhara	37	BT	RR	MDT/Prd	26	13	Pos/B
16	M	Oromo	25	BL	Acute RR	NT	0	5	Pos/B
17	M	Oromo	24	BL	Acute RR	MDT/Prd	0	5	Pos/B
18	M	NA	23	BL	Neuritis	NT	0	0	Neg/NB
19	F	Oromo	45	BL	Neuritis	NT	0	0	Neg/B
20	F	Amhara	20	BT	RR/neuritis	NT	12	17	Pos/NB
21	M	Amhara	24	BT	NA	NT	0	0	Neg/B
22	M	Amhara	41	BT	RR	Prd	0	0	Neg/B
23	F	Amhara	18	BT	RR/acute neuritis	MDT/Prd	0	0	Neg/B
24	M	Amhara	14	BL	RR/neuritis	MDT/Prd	0	0	Neg/B
25	M	NA	32	LL	Neuritis	MDT/Prd	12	15	Pos/B
26	M	Amhara	58	LL	Neuritis	NT	0	0	Neg/B
27	M	Gurage	50	BT	RR	MDT/Prd	12	5	Pos/B
28	M	Amhara	50	BL	RR	MDT/Prd	0	0	Neg/B
29	M	Amhara	65	BT	RR	Prd	0	0	Neg/B
30	M	Oromo	30	IN	RR	MDT/Prd	13	16	Pos/B
31	M	Oromo	50	BL	RR	MDT/Prd	0	0	Neg/B
32	M	Oromo	20	BT	RR/neuritis	Prd	0	0	Neg/B
33	M	Oromo	25	BL	ENL	Prd	40	40	Pos/B
34	M	Amhara	22	BL	RR/neuritis	MDT/Prd	23	19	Pos/NB
35	F	Amhara	14	BL	Neuritis	MDT/Prd	0	0	Neg/NB
36	F	Amhara	22	LL	RR/ENL	MDT/Prd/Chlor	0	0	Neg/NB
37	M	Amhara	39	BL	RR/neuritis	NT	15	4	Pos/NB
38	M	Amhara	31	BL	ENL/neuritis	NT	0	0	Neg/NB
39	M	Gurage	38	BT	RR	MDT/Prd	0	0	Neg/NB
40	F	Oromo	57	BT	RR/neuritis	Prd	4	0	Neg/NB
41	M	Amhara	25	BT	Neuritis	Prd	15	19	Pos/NB
42	M	Amhara	55	BT	RR	Prd	19	11	Pos/NB
Total Pos/B									7
Total Pos/NB									10
Total Pos									17
Total Neg/B									10
Total Neg/NB									15
Total Neg									25

NOTE. B, biopsy specimen obtained; BL, borderline lepromatous; BT, borderline tuberculoid; Chlor, chloroquine; DTH, delayed-type hypersensitivity; DTH/A, DTH response at anesthetic skin site; DTH/S, DTH response at sensitive skin site; ENL, erythema nodosum leprosum; IN, indeterminate; LL, lepromatous leprosy; MDT, multidrug therapy; NA, not available; NB, no biopsy specimen obtained; Neg, negative; NT, not yet treated; Pos, positive; Prd, prednisolone; RR, reversal reaction.

^a Patient is currently receiving ampicillin.

Table 2. Delayed-type hypersensitivity (DTH) responses in anesthetic and contralateral sensitive sites.

Variable	Anesthetic site	Sensitive site	<i>P</i> ^a
Diameter of induration for study group, mm ^b	0 (0–40)	0 (0–40)	.286
Diameter of induration for DTH-positive patients, mm ^c	15 (11–40)	15 (5–40)	.365
Diameter of induration for patients from whom biopsy specimen was obtained, mm ^c	0 (0–40)	0 (0–40)	.684
DTH microscopic score ^c	1 (0–4)	0.5 (0–3)	.136
Diameter of induration for DTH-positive patients from whom biopsy specimen was obtained, mm ^d	12 (11–40)	13 (5–40)	.684
DTH microscopic score for DTH-positive patients from whom biopsy specimen was obtained ^d	2 (0–3)	1.5 (0–3)	.136

NOTE. Data are median (range).

^a Two-tailed *P* value was calculated with the Wilcoxon signed-rank nonparametric test for paired samples.

^b *n* = 42.

^c *n* = 17.

^d *n* = 7.

tem), and the Langerhans cell marker CD1a (1:1; Beckman Coulter) [9].

Microscopic examination. We assessed the PPD-induced DTH response by using H-E–stained skin biopsy sections. The extent of cellular infiltration in the dermis was evaluated by 2 independent investigators who were unaware of the origin of the biopsy specimens. The extent of cellular infiltration was expressed as a percentage of the dermis occupied by inflammatory cells. A semi-quantitative scale was used to evaluate epidermal activation and thickening [9]. Spongiosis (edema) between keratinocytes in the epidermis and the elongation of epidermal ridges indicated epidermal activation. The extent of epidermal thickening and spongiosis was expressed as the DTH microscopic score and was given a value between 0 and 4, with 4 indicating the highest level of epidermal activation and thickening and 0 indicating none. The number and distribution of CD3⁺ and CD8⁺ T cells were assessed in all sections. Anti-Ki67 nuclear antigen stains the nuclei of proliferating cells. Ki67⁺ keratinocytes and CD1a⁺ Langerhans cells were counted and expressed as the number of cells per high-power field (×40 objective) of the epidermis, as described elsewhere [10]. Micrographs were made with a Nikon Microphot-FX.

Wound healing. Biopsy sites were assessed at postbiopsy days 1, 3, and 5. Differences in the drying of the wound, rounding at the wound edge, and the formation of granulation tissue were expressed qualitatively [9].

Statistical analysis. Nonparametric data were analyzed by using the Wilcoxon signed-rank test for paired samples. *P* < .05 was considered to be statistically significant.

Results

Patient characteristics. In total, 42 patients with leprosy were enrolled into this study: 9 females and 33 males (table 1) with a

median age of 26 years (range, 14–57 years). Of the patients, 18 were paucibacillary (all classified as having borderline tuberculoid leprosy, by the Ridley and Jopling classification) [11], 23 were multibacillary (classified as having lepromatous leprosy and borderline lepromatous leprosy), and 1 was indeterminate. The majority (98%) had been diagnosed with a reactional episode (reversal reaction, erythema nodosum leprosum, or neuritis) at some time prior to the study (table 1). Of the 42 patients, 10 were newly referred to the clinic and not yet treated, 21 were receiving multidrug therapy (MDT) with prednisolone, and 11 had completed MDT and were receiving prednisolone or prednisolone plus thalidomide (1 patient).

Skin induration in response to PPD. Of the 42 patients, 17 showed ≥1 positive skin test response to PPD administration (table 1). Prednisolone is often used for treatment of leprosy reactions and neuritis and may depress T cell responses [12]. However, in the present study, the drug had no significant effect on the induration at either anesthetic or sensitive sites (data not shown). There was no significant difference between the diameters of induration at the PPD injection sites in anesthetic versus sensitive skin in these patients with leprosy (table 1 and table 2).

Histology of DTH response to PPD in the epidermis. Permission for punch biopsies from the sites of PPD administration was obtained for 7 of the 17 skin test–positive and 10 of the 25 skin test–negative patients. H-E–stained skin sections were examined to assess DTH responses by using the DTH microscopic score (see Materials and Methods). We noted that, in biopsy specimens from 7 of the 10 patients who had not displayed induration (skin test negative), there was almost no change from baseline epidermal histology. Patients who were skin test positive displayed epidermal activation and thickening (figure 1). The DTH microscopic score correlated with the extent of induration (*P* = .008, Pearson correlation test for DTH responses in anesthetic skin; *P* = .035, Pearson correlation test for DTH responses in sensitive skin). However the DTH microscopic score did not correlate with presence or absence of anesthesia at the skin site tested (table 2).

Cell composition with dermis at PPD injection site. The extent of cellular infiltration (area of dermis filled with cells) in the PPD injection site was evaluated by 2 independent investigators who were unaware of the nature of the biopsy specimen. No difference in the distribution of extent of cellularity was noted between anesthetic and sensitive sites (figure 2A). One patient with borderline leprosy (patient 28; table 1) had extensive infiltration (~50% of the dermis occupied by cells) of the negative PPD site. The other PPD-negative biopsy specimens showed involvement of 3.5%–20% of the dermis, suggesting that the underlying leprosy disease was of variable cellularity at that particular site.

In patients with a positive PPD response, a mononuclear cell infiltrate occupying >10% of the dermis was clearly evident. Of interest, in the PPD-positive biopsy specimens, there was a cor-

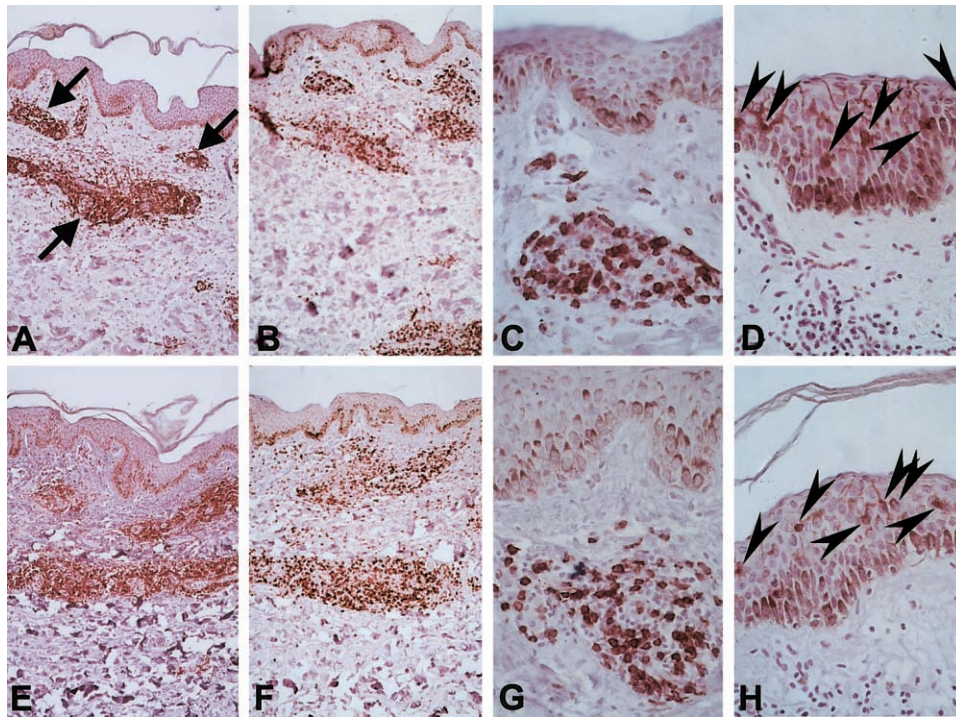


Figure 1. Immunohistology of response to purified protein derivative of tuberculin-positive skin test site biopsy specimens from a patient with leprosy (patient 30; table 1) with skin induration of 13 mm at the anesthetic site (*A–D*) and 16 mm at the contralateral sensitive site (*E–H*). Sections were stained for CD3⁺ T cells (*A* and *E* [magnification, $\times 10$]), CD8⁺ T cells (*B* and *F* [magnification, $\times 10$]; *C* and *G* [magnification, $\times 40$]), and CD1a⁺ Langerhans cells (*D* and *H* [magnification, $\times 40$]). Arrows in panel *A* point to areas of the dermis with CD3⁺ staining cells. Arrowheads in panels *D* and *H* point to CD1a⁺ staining cells in the epidermis.

relation between the extent of induration in response to PPD and the percent of dermis infiltrated (figure 2*B*). However, the area of the dermis occupied by the cellular infiltrate was not related to whether that site was anesthetic or sensitive (figure 1*A* and 1*E* and figure 2*B*).

Immunohistology was used to identify T cells (CD3⁺ and CD8⁺) in the dermis and Langerhans cells (CD1a⁺ cells) and Ki67⁺ proliferating keratinocytes in the epidermis in each biopsy specimen. Some variation in the number and distribution of CD3⁺ and CD8⁺ cells in the dermis was observed among patients—probably due to the underlying leprosy disease. Nevertheless, in the same patient, the extent of cellularity and the cellular distribution were similar in sections obtained from either anesthetic or sensitive skin sites (figure 1). Numbers of Ki67⁺ keratinocytes (anesthetic site: median, 0.3 keratinocytes; range, 0–0.6 keratinocytes; sensitive site: median, 0.2 keratinocytes; range, 0–0.3 keratinocytes; $P = .65$) and CD1a⁺ Langerhans cells (anesthetic site: median, 6 Langerhans cells; range, 1–11 Langerhans cells; sensitive site: median, 5 Langerhans cells; range, 0.5–6 Langerhans cells; $P = .60$) located in the epidermis overlying the DTH site were also similar whether in the presence or absence of anesthesia (figure 1).

Wound healing. The healing of the wound produced by the 4-mm punch biopsy was assessed 1, 3, and 5 days after biopsy.

By 24 h after biopsy, the PPD-injected sites were dry, lacked bleeding points, and were covered with a whitish film. By 3 days, the edge of the wound, which originally had a sharp vertical wall, started to curve, and the wound became shallower. By 5 days, the epidermis started closing the wound. Similar rates of drying of the wound, rounding of the wound bed, and shrinking of the biopsy site were noted for anesthetic and sensitive sites, indicating no discernable difference in the rate of wound healing. This was noted for each patient and for the group as a whole.

Discussion

We examined whether nerve damage in leprosy is associated with impaired cellular immunity and/or delayed wound healing by determining reactivity to PPD and monitoring healing after biopsy of the site. A detailed examination of PPD reactivity and of immune cell composition and epidermal activation at the DTH site failed to demonstrate differences between anesthetic and sensitive skin sites in patients with leprosy. This strongly suggests that nerve damage in leprosy is not associated with decreased cell trafficking into the site of a cellular immune response to soluble antigen. Since there was no impairment of

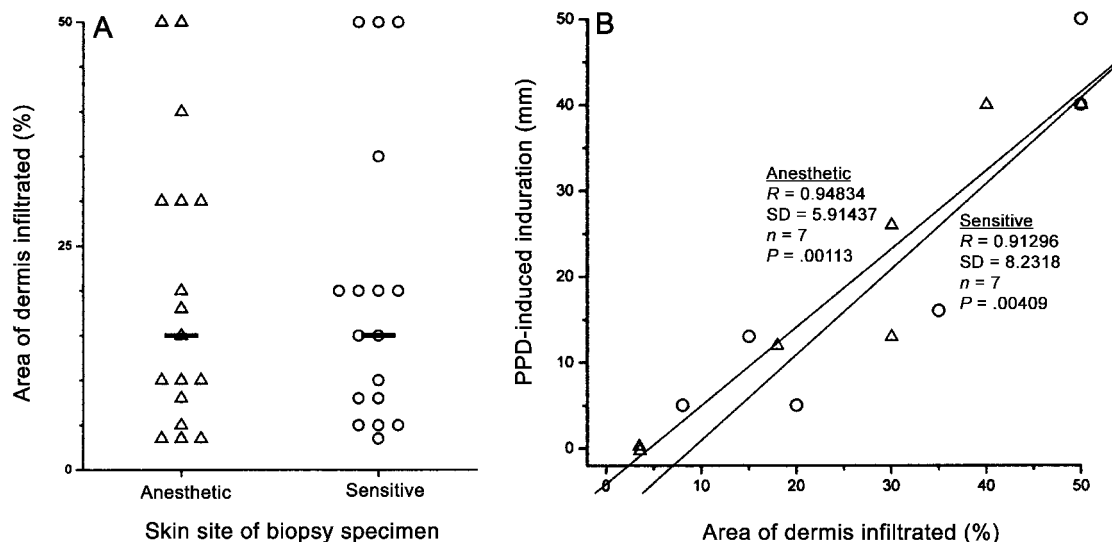


Figure 2. Cellular infiltration in the dermis in purified protein derivative (PPD) skin test sites. *A*, Extent of cellular infiltration expressed as a percentage of dermis infiltrated in anesthetic (Δ) and sensitive (\circ) sites. Bar, median for each group. Each point represents individual patient biopsy sample. *B*, Correlation between extent of induration and percentage of dermis infiltrated in anesthetic (Δ) or sensitive (\circ) PPD-positive skin test sites.

cellular response in the skin, this does not appear to be a cause for the chronic ulcers of leprosy.

Of interest, only 40% of the patients with leprosy in this study had a PPD-induced skin induration >5 mm. The lack of a PPD response in the majority of patients was surprising in a population with a high frequency of exposure to tuberculosis. Corticosteroid treatment did not appear to be the explanation for the low PPD responsiveness in this study group. Anergy to PPD in patients with leprosy has been reported elsewhere and may be due to *M. leprae*-induced cross-reactive T cell anergy [13, 14]. Alternatively, in those persons vaccinated with bacille Calmette-Guérin (BCG) at birth, BCG-induced tuberculin sensitivity may have declined with age, as has been shown to occur in the Karonga District of Malawi [15]. It has been suggested that a decrease in tuberculin sensitivity may be attributed partly to the effect of exposure to environmental mycobacteria [16].

Microscopic evaluation of the cellular response to PPD did not reveal any significant differences in anesthetic or sensitive sites within each person; however, differences were observed between positive PPD and negative PPD sites. For example, as reported elsewhere, an increase in the dermal accumulation of monocytes and T cells and epidermal thickening were observed in the PPD-responsive sites [17]. In addition, a clear increase in CD1a⁺ Langerhans cells and Ki67⁺ proliferating keratinocytes was noted in PPD-positive sites, compared with PPD-negative sites. This observation confirms earlier studies in which keratinocytes were found to proliferate and CD1a⁺ Langerhans cells were observed to accumulate in the skin of patients with leprosy in response to PPD and in response to intradermal administration of granulocyte monocyte colony-stimulating factor (GM-CSF) [9, 10, 17, 18].

Similar rates of wound healing were noted at the anesthetic and the sensitive skin sites. This observation suggested that not only were similar cell populations recruited to the wound bed but also that those soluble mediators required to induce wound healing were produced by cells in both anesthetic and sensitive skin. In previous studies, we showed that activated peripheral blood mononuclear cells produce factors that modify keratinocyte growth and differentiation. Among these factors are interleukin-3 and GM-CSF, which stimulate keratinocyte growth [19]. We have also shown that intradermal administration of recombinant GM-CSF to patients with leprosy leads to increased thickness of the epidermis and activation of keratinocyte proliferation, as indicated by increased expression of Ki67⁺ nuclear antigen [9, 10]. Thus, leukocyte and keratinocyte activation and cytokine production including GM-CSF are probably intact in leprosy-affected skin, even in the presence of nerve damage.

In conclusion, our study results indicate that the local nerve damage associated with *M. leprae* infection does not appear to adversely affect DTH responses to the recall antigen PPD or to inhibit local tissue repair. Therefore, the presence of chronic ulcers in patients with leprosy is likely due to continuing trauma at an anesthetic site, to secondary infections, or both rather than to inadequate cellular trafficking and immune responsiveness.

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