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THE DETECTION OF *MYCOBACTERIUM LEPRAE* PROTEIN AND CARBOHYDRATE ANTIGENS IN SKIN AND NERVE FROM LEPROSY PATIENTS WITH TYPE 1 (REVERSAL) REACTIONS

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Abstract. Type 1 (reversal) reactions are the most common immunological complications of leprosy. These episodes of delayed hypersensitivity produce severe local immunopathology and ultimately nerve damage. To date, the *Mycobacterium leprae* antigens associated with type 1 reactions have not been identified. Using monoclonal antibodies to defined protein and carbohydrate *M. leprae* epitopes (65, 35 and 28 kd and lipoarabinomannan [LAM]) in a two-step immunoperoxidase staining technique, *M. leprae* antigens were demonstrated in skin and nerve biopsies from patients in reversal reaction. Antigen presence and staining patterns were similar in skin and nerve lesions, implying that the pathological processes are similar in the two sites. Antigens were present both in macrophages and Schwann cells but also as a diffuse extracellular infiltrate associated with the inflammatory infiltrate. The 28-kd antigen was present most strongly and may be a potential candidate antigen for initiating type 1 reactions. LAM also stained strongly and persisted after treatment. The possible roles of LAM and 65 kd in the cellular events of type 1 reactions are discussed.

INTRODUCTION

Leprosy is a chronic granulomatous disease caused by *Mycobacterium leprae* and affecting skin and peripheral nerves. The clinical and histological manifestation of disease depends on the infected individual's response to the mycobacterium. The spectrum of responses to *M. leprae* is well recognized, with well-developed cell-mediated immunity, granulomata, and elimination of mycobacteria producing tuberculoid leprosy (TT) at one pole of the spectrum and lepromatous leprosy (LL) with cell-mediated anergy for *M. leprae* and a resulting heavy bacterial load at the other end of the spectrum. Between the two poles is a continuum of disease manifestation with borderline lepromatous (BL), true borderline (BB), and borderline tuberculoid (BT) disease types. The borderline forms of disease are immunologically unstable, and movement along the spectrum occurs; the most common form is upgrading toward the tuberculoid pole in type 1 (reversal) reactions. Type 1 reactions are episodes of delayed-type hypersensitivity localized to skin and nerve and result in mycobacterial elimination and histological upgrading. They manifest clinically as acutely inflamed skin lesions and acute neuritis; the latter is often rapid and severe, resulting in permanent nerve damage, which leaves the patient at risk for deformities.

The histology of the type 1 reaction is that of a delayed-type hypersensitivity response with CD4+ cells, macrophages, and expression of interleukin-2 in lesions. However, it is not clear what provokes the development of a reaction. Reactions frequently develop in the first month after starting antileprosy treatment¹; this may happen because antibacterial treatment results in increased lysis of whole bacteria with release of antigen, which can then be presented by immune cells.

The association of type 1 reactions with increased recognition of whole and sonicated *M. leprae* was demonstrated by Barnetson et al.² in a prospective study measuring peripheral blood lymphocyte responses to whole and sonicated *M. leprae*. Significant increases in lymphocyte transformation responses were found during type 1 reactions; patients with predominantly skin reactions responded preferentially to whole washed bacilli, whereas those with predominantly nerve reactions responded preferentially to sonicated bacilli.

The availability of monoclonal antibodies against the protein and carbohydrate components of *M. leprae*^{3,4} and the use of these antibodies in immunohistological staining techniques have permitted the detection of specific *M. leprae* antigens at sites of active immunopathology. Narayanan et al.,⁵ using monoclonal antibodies, demonstrated 35- and 10-kd antigens in skin lesions of untreated lepromatous and tuberculoid patients. Khanolkar et al.⁶ used a panel of five monoclonal antibodies (against 65-, 36-, 28-, 18-, and 12-kd protein antigens) on skin biopsies from patients across the spectrum of disease and demonstrated positive staining for all five antibodies when the bacterial index was >3+ and in two cases in which no acid-fast bacilli were seen on modified Ziehl-Neelsen staining. Narayanan et al.⁷ used monoclonal antibodies against 65-, 46-, and 17-kd protein antigens and phenolic glycolipid (PGL-1) on biopsies from skin lesions and also a few leprosy nerves. PGL-1 and 17-kd antigens were present in both tuberculoid and lepromatous lesions, the 65-kd antigen stained poorly in skin and was only detected in nerve by antibody III E9.

The presence of specific antigen in skin and nerve type 1 reaction lesions has not been studied. The detection of *M. leprae* antigens in type 1 reaction lesions is particularly pertinent since the lesions are very localized, and it is probable that the expression of local, rather than circulating, antigen provokes the delayed-type hypersensitivity response in leprosy lesion. We modified the currently available techniques for detecting *M. leprae* antigen and identifying the cells associated with antigen, and applied these techniques to skin and nerve biopsies from patients with type I reaction.

The aims of the study were to identify antigens present in skin and nerve at the time of type I reaction, to determine whether skin and nerve diseases are associated with different antigens, and to establish whether any particular antigens are associated with the disease of type I reactions.

MATERIALS AND METHODS

Patient biopsies. Skin and nerve biopsies were taken from patients with type I reactions attending Dhoolpet Leprosy Research Centre, Hyderabad, India. These were taken as part of the diagnostic work-up. The study was approved by the Dhoolpet Ethics Committee through Victoria Hospital, Dich-

TABLE 1
Mycobacterium leprae monoclonal antibodies used*

Monoclonal	Molecular weight	Antigen type	Reference
SL12	65	Protein	Britton et al. ³⁶
IIIE9	65	Protein	Gillis and Buchanan ³⁷
F47-9	36	Protein	Kolk et al. ³⁸
SA1B11H	28	Protein	Young et al. ³⁹
SA1C7F	—	LAM	Khanolkar ⁴⁰

* All these monoclonal antibodies are specific for *M. leprae*. LAM, lipoarabinomannan.

palli, India. A type I (reversal) reaction was diagnosed when a patient presented with a acute skin changes (erythema and/or edema of existing lesions, new skin lesions that were not relapsing leprosy, or erythema nodosum leprosum) or acute neuritis (peripheral nerve tenderness, new sensory symptoms or signs, or new motor symptoms or signs). Patients were graded on the leprosy spectrum according to the Ridley-Jopling classification (TT, BT, BL, and LL).⁸ Patients with a type I (reversal) reaction are designated according to their Ridley-Jopling classification (i.e., BT in RR and BL in RR). Partial-thickness nerve biopsies were taken from the radial cutaneous nerve at the wrist. Skin biopsies were taken from the active edge of lesions. Skin biopsies were also available from new, nonreactive, untreated leprosy patients, from leprosy patients who had completed their treatment, and from normal skin. Normal control nerves were obtained from fresh post-mortem cadavers (<12 h) in the United Kingdom. Nerves from nonreactive patients were not available because there was no clinical indication for a nerve biopsy in these patients. The biopsies were snap-frozen and stored in liquid nitrogen. Cryostat sections, 6 μ thick, were cut and mounted on Poly-L-lysine-coated slides. Sections from each biopsy were stained with hematoxylin and eosin and a modified Fite-Faraco stain for *M. leprae*. These routine stains enabled us to confirm all type I reactions histologically. The bacterial load in each site was counted and expressed on a logarithmic scale as the bacillary index (BI).⁹

Immunohistological staining. *M. leprae* antigens were detected using two-step immunostaining with peroxidase visualization as the final step. Details of the *M. leprae* monoclonal antibodies (MAbs) used are listed in Table 1. Antibodies were diluted in Tris-HCl buffer with 5% normal goat serum. Antibody dilutions were optimized on appropriate leprosy-infected skin sections. Macrophages were stained with a CD68 binding antibody (EMBII)¹⁰ (Dakopatts, Denmark). Peroxidase conjugated goat antimouse immunoglobulin anti-serum (affinity purified H+L) (Biorad, Richmond, CA) was used as the secondary antibody. The secondary antibody showed no cross-reactivity with any human tissue constituent. Sections were fixed in acetone for 15 min. Endogenous peroxidase was blocked using 0.1% H₂O₂ (Sigma) layered on sections for 30 min. After washes in Tris buffer, sections were incubated with 5% normal goat serum to block nonspecific binding of the MAbs to Fc receptors. Incubation with the MAbs was for 1 hour at room temperature. After two washes in Tris-buffered saline at room temperature, the sections were incubated with peroxidase-labelled goat antimouse immunoglobulin G diluted 1:50 in phosphate-buffered saline (PBS, Dulbecco A, Basingstoke) as the secondary reagent. Positive staining was visualized using 3,3'-diaminobenzidine (Sigma)/H₂O₂. Finally, the sections were counterstained with hema-

TABLE 2
Summary of mycobacterial antigen-staining patterns

Patient group	Antigens stained			
	65 kd	36 kd	28 kd	LAM
BT in RR	10/18	10/18	17/20	16/20
BL in RR	10/10	10/11	10/11	11/11
TT/BT untr	0/4	1/4	2/4	3/4
BL/LL untr	3/3	2/3	3/3	3/3
TT/BT tr	0/5	0/5	1/5	5/5
BL/LL tr	0/3	1/3	1/3	3/3

BT, borderline tuberculoid; BL, borderline lepromatous; TT, tuberculoid leprosy; LL, lepromatous leprosy; untr, untreated; tr, treated; LAM, lipoarabinomannan.

toxylin and mounted. The specificity of staining was checked using a mouse monoclonal that had been raised against the egg stage of *S. mansoni* (courtesy Dr. D. Smith). Routine controls included the use of normal serum or omission of primary antibody for the specificity of the staining. Macrophages were identified by staining for the CD68 marker using the antibody PG-M1 (Dako catalog no. M0876) dilution of 1 in 50 and applied for 1 hour at room temperature. Staining was evaluated by two independent observers, who ranked the intensity of staining on a four-point scale.

RESULTS

Skin and nerve biopsies were collected from 34 patients (13 BT in RR, seven BL in RR, eight treated patients, and six untreated patients) together with five normal skin biopsies and two normal nerve biopsies. Table 2 summarizes the staining positivity for each mycobacterial antigen for each patient group. Both skin and nerve biopsies are included, and the degree of staining is not reflected. Antigen was detected in the majority of BT in RR skin and nerve biopsies and in almost all BL in RR biopsies. Protein antigens were present in most type I reaction biopsies but in relatively few of the BT/TT untreated biopsies and both BT/TT and BL/LL treated biopsies.

Tables 3 and 4 give the results of the staining for each antigen in the BT in RR, BL in RR, untreated, and treated patient groups, respectively. The results are given separately for skin and nerve, and the degree of staining obtained is shown.

The 28-kd protein antigen was detected most often and most strongly, being present in 10 of 11 BL in RR cases and in 16 of 20 BT in RR cases. The 65-kd antigen was present in eight of nine BL in RR cases and in 10 of 16 BT in RR cases, but in almost every instance the staining was less strong than for the 28-kd antigen. The 36-kd antigen was present in eight of 11 BL in RR cases and in nine of 16 BT in RR cases, but staining was frequently weak. Staining was strongest for the LAM antigen, which stained positively in all BL in RR cases and in 16 of 20 BT in RR cases.

At least two antigens were detected in all BL in RR cases, and the staining was also stronger, suggesting that more antigen was present. Antigen was detectable in 18 of 20 BT skin and nerve biopsies, of which 18 had a BI of 0 and two had a BI of 1+. Antigen stained strongly in the two cases with a BI of 1+.

In untreated patients, protein antigens were readily detectable in the BL/LL cases; the 65-kd antigen stained strongest.

TABLE 3
Staining patterns obtained for BT in RR patients

Patient number	Biopsy type	Bacterial index	Protein antigens*			LAM <i>C7F</i>
			65 kd <i>L12/IIIIE9</i>	28 kd <i>B11H</i>	36 kd <i>F47</i>	
05	S	0	++	+++	nd	+++
	N	0	-	+	nd	-
08	S	0	+	++	+	+
	N	0	++	++	-	-
18	S	0	+	+	+	+
	N	0	+	++	+	++
22	S	0	+	++	+	++
	N	0	-	-	-	+
28	S	0	-	+	-	++
	N	0	-	+	-	++
43	S	0	nd	++	+	++
	N	0	nd	++	+	++
57	S	0	-	++	+	+
	N	0	-	-	-	-
06	S	0	+	++	-	+
08B	S	0	+	+	+	++
20	S	0	-	-	-	-
59	N	1+	++	+	++	+++
67	N	0	-	++	-	++
68	N	1+	+	++	+	++

LAM, lipoarabinomannan.

* The antibody used to detect each antigen is given in italic type.

N = nerve; S = skin; nd = not done.

In the TT/BT cases, 28 kd was detectable in two of four cases and 36 kd was weakly present in one of four cases. LAM was present in all but one of the new cases. In treated patients, the 28- and 36-kd antigens were detected in only two of eight and one of eight cases, respectively, and in all cases the staining was weak. However, in all treated cases, some LAM antigen was detectable, albeit only weakly in four of those cases.

Staining patterns. Figures 1–3 illustrate the staining patterns obtained. In general, the staining for mycobacterial antigen appears as a diffuse pattern in association with the inflammatory infiltrate. Staining of parallel sections for antigen and macrophages shows that the antigen is associated with macrophages and is located on the macrophage surface. The LAM antigen, however, is also present in specific, darkly staining cells. The sections stained with the monoclonal against the egg stage of *S. mansoni* were negative.

Figure 1 illustrates two skin sections from a BT in RR patient. Staining for LAM is positive within the granuloma and in macrophages (Figure 1B), whereas the conventional Fite-Faraco stain for whole mycobacteria was negative (Figure 1A). Figure 2 shows a granuloma from a BL in RR patient stained for macrophages (Figure 2A), 28-kd antigen (Figure 2B), and LAM (2C). Comparing these figures demonstrates that 28 kd antigen is associated with macrophages and appears to localize on the cell surface as well as being present diffusely throughout the granuloma. LAM staining is also present in a specific and diffuse pattern. Figure 3 illustrates the pattern seen in nerve from a type I reaction. The 28-kd antigen is localized to intraneural granulomas, whereas LAM staining is present diffusely around inflammatory infiltrate and as small, darkly staining areas.

For those cases in which both a skin and nerve biopsy was available (seven BT in RR, four BL in RR), the staining patterns in the two locations may be compared. In the BL in RR cases, there was complete concordance in the antigens detected in skin and nerve; in the BT in RR cases, there was

TABLE 4
Staining patterns obtained for BL patients in RR

Patient number	Biopsy type	Bacterial index	Protein antigens*			LAM <i>C7F</i>
			65 kd <i>L12/IIIIE9</i>	28 kd <i>B11H</i>	36 kd <i>F47</i>	
01	S	2+	++	++	+	+++
	N	5+	++	+	+	+++
07	S	3+	+	++	++	++
	N	5+	+	++	+	+
23	S	3+	+	++	+	+
	N	4+	+	+	+	+++
55	S	4+	++	++	++	+++
	N	4+	++	+++	++	+++
39	S	0	+	-	++	++
40	S	3	+	++	+	+
52	S	0	nd	+	-	+

LAM, lipoarabinomannan.

* The antibody used to detect each antigen is given in italic type.

N = nerve; S = skin; nd = not done.

broad but not complete agreement with no discernible tendency for one particular antigen to be associated with either skin or nerve disease. In the BT in RR patients, all of whom had BIs of 0 for both skin and nerve, there was no evidence of the antigen load being consistently higher in nerve than skin. In all the BL in RR patients, the nerve BI was the same or higher than the skin, but the single antigen staining did not reveal any significant differences between skin and nerve.

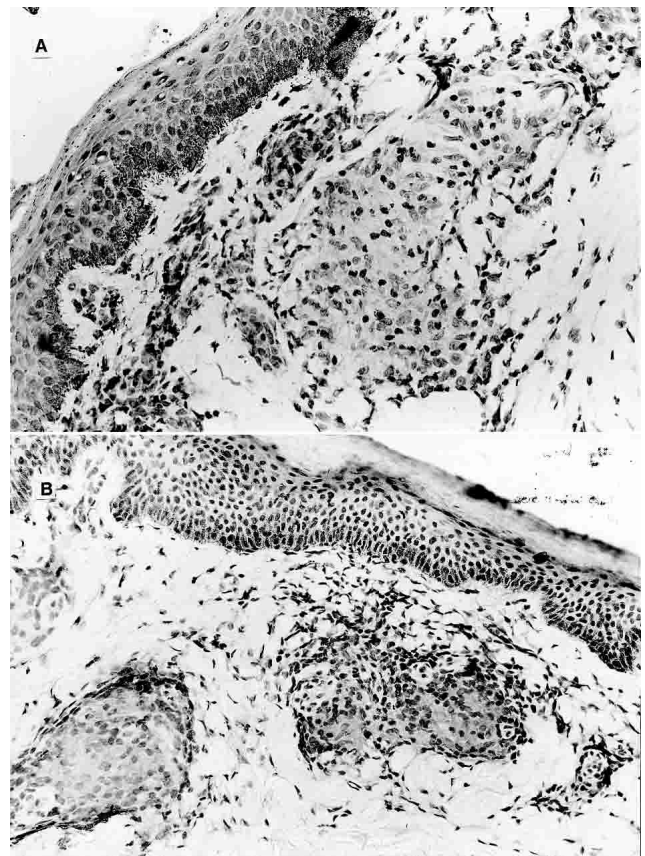


FIGURE 1. Skin sections from a BT in RR patient. **A)** Stained for acid fast bacilli with Fite-Faraco stain. No mycobacteria are present in the granuloma or tissue. **B)** Stained with LAM antibody. Positive staining for LAM is seen in the granuloma.

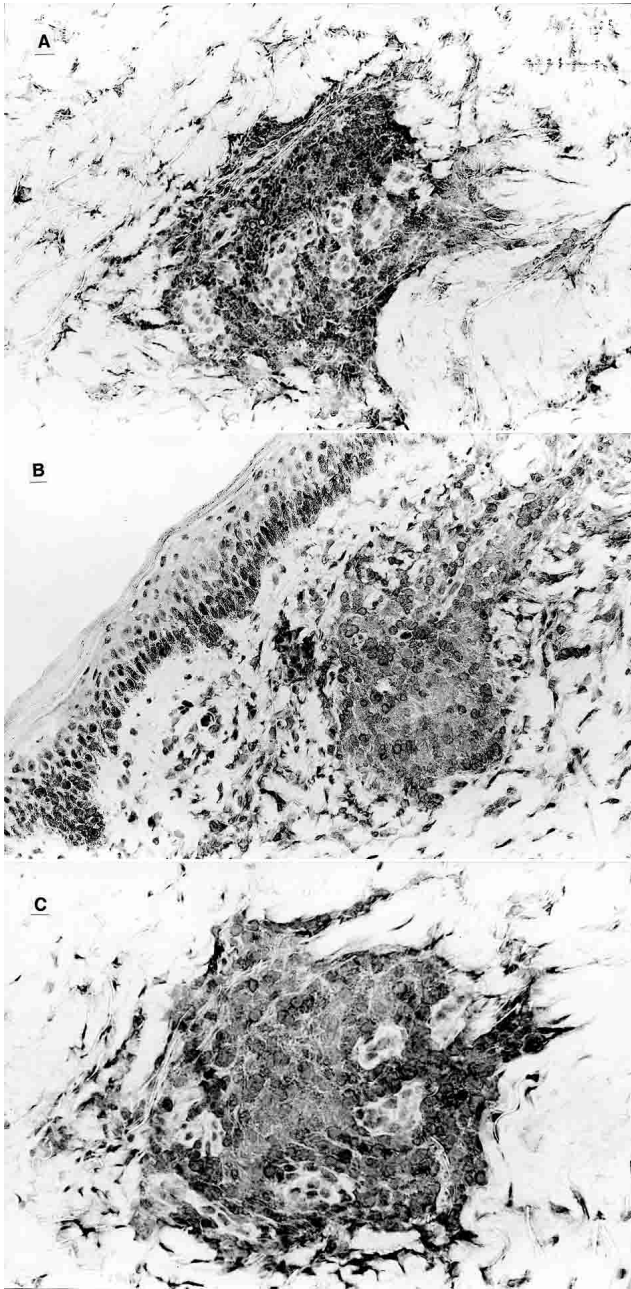


FIGURE 2. Skin sections from a BL in RR patient. **A)** Granuloma stained with the macrophage marker CD68. **B)** Granuloma stained for 28kD. Staining for the 28kD antigen can be seen in macrophages and diffusely through the granuloma. **C)** Granuloma stained for LAM. Heavy staining is seen both as specific dark staining and diffusely through the granuloma.

DISCUSSION

This work demonstrates the presence of individual protein and carbohydrate *M. leprae* antigens in type I reaction skin and nerve lesions. Although previous workers have demonstrated the presence of different antigens in lepromatous and, to a lesser extent, tuberculoid lesions, this is the first study applying a panel of antibodies to type I reaction skin and nerve lesions. Large amounts of stainable antigen are present in both BT and BL type I reaction lesions. The former finding

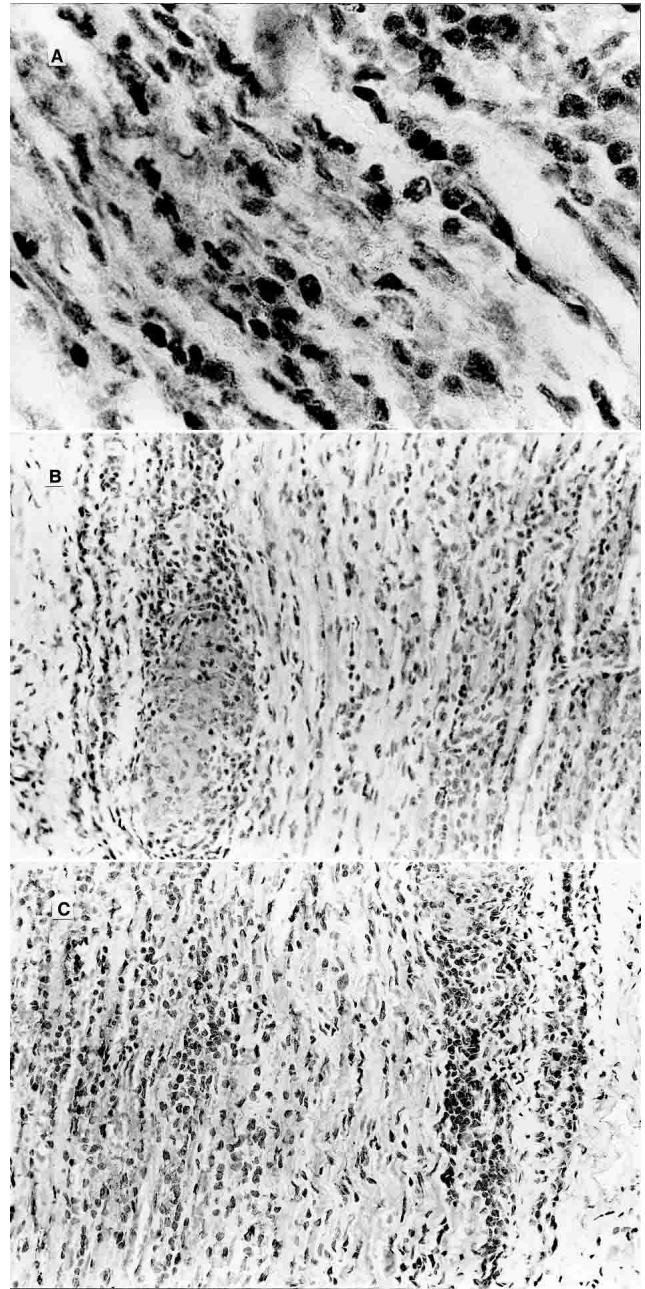


FIGURE 3. Nerve sections from a BL in RR patient. **A)** Stained for macrophages (CD68). A heavy macrophage infiltrate is present. **B)** Stained for 28kD. Positive staining is present in the intra-neural granuloma. **C)** Stained for LAM which is demonstrated in the diffuse inflammatory infiltrate.

is particularly striking because, on Fite-Faraco staining, no bacilli are detectable in the lesions. The finding of antigen within type I reaction lesions argues for a role for *M. leprae* antigens in the pathogenesis of type I reactions. We were not able to demonstrate any location specificity for individual antigens with respect to either skin or nerve during type I reactions. Our work does not support Barnetson et al.'s proposal that different antigens are responsible for skin and neural diseases in type I reactions.

The pattern of antigen staining shows that antigen is confined to inflammatory infiltrates, but there are two patterns of

staining within the infiltrates, intracellular and diffuse. The macrophages were identified by CD68 staining and morphology. Intracellular staining is confined to macrophages in which there are also two patterns of staining: a fine granular pattern and a dense, darkly staining pattern. The latter pattern was seen only with the antibody for LAM. In the nerve biopsies, only a few Schwann cells stained positively for antigens; the majority of antigen staining was associated with macrophages. Shetty et al.¹¹ have also found that antigen staining within nerves occurs predominantly in macrophages and epithelioid cells. The noncellular diffuse infiltrate associated staining pattern we observed has also been reported by Rambukkana et al.,¹² who noted this type of staining in a skin biopsy from a multibacillary patient with a type I reaction. The strength of staining seen in these biopsies suggests that there is a relative abundance of antigen in type I reactions; staining is much stronger than in new paucibacillary patients and all types of treated patient. One possible explanation is that it only requires a small amount of antigen being presented to the immune system to trigger the delayed-hypersensitivity response; however, the influx of inflammatory cells and the cytokines being produced in the granuloma, particularly tumor necrosis factor- α (TNF- α), results in lysis of antigen containing macrophages, thus releasing more antigen at the site of the reaction. This results in amplification of disease and perhaps explains the clinical observation that reactions require prolonged courses of high dose steroids to be terminated.

The identification of separate antigens in reactional lesions is of major interest because one may then postulate a role for individual antigens in the development of immunopathology. Previous work defining immunodominant *M. leprae* protein antigens has focused on either the antibody response or the in vitro response of lymphocytes from leprosy patients and contacts to single antigens. Using these approaches, the 65-, 35-, and 28-kd (among others) antigens have been shown to play an important role in the immune response to *M. leprae*. However, so far, no single antigen has been shown to be solely associated with either the development of disease or a protective response. The 28-kd antigen was most strongly associated with reactional disease. This protein is a superoxide dismutase and a major *M. leprae* cytoplasmic protein¹³ and has been shown to induce T-cell reactivity.¹⁴ The 65-kd protein is both an immunodominant antigen inducing antibody and T-cell responses in leprosy patients^{15,16} and a highly conserved heat shock protein (hsp) with close homology to the 65-kd antigens of *M. tuberculosis* and *M. bovis*.¹⁷ After phagocytosis of *M. tuberculosis* by macrophages, mycobacterial hsp 65 is presented on the cell surface in association with Major Histocompatibility Complex class II antigens. This also induces TNF- α gene expression and secretion.¹⁸ We have shown that there is accumulation of TNF- α mRNA and protein in leprosy skin and nerve from type I reaction lesions.¹⁹ The 65-kd antigen may also have a role in inducing the gamma/delta T cells that accumulate in type I reaction skin lesions.²⁰ Clearly, the 65-kd antigen has an important role in type I reaction disease, although at the moment it is not possible to say whether this role is predominantly concerned with the initiation of disease or the development of disease once the reaction has started. Verhagen et al.²¹ have reported finding LAM in the granulomas of multibacillary cases in reaction.

The strong staining for LAM that persisted in treated cases was surprising. This cell wall-associated glycolipid is excreted in copious amounts by mycobacteria and is probably essential for the intracellular survival of mycobacteria within macrophages.²² It has a wide range of biological effects, such as inhibition of interferon- γ activation of macrophages,²³ induction of the release of TNF,^{24,25} and an inhibition of antigen presentation by antigen-presenting cells.²⁶ Recent work showed that the LAM produced by *M. tuberculosis* induces the transcription of mRNA for the macrophage-associated cytokines TNF- α , granulocyte-macrophage colony-stimulating factor, IL-1 β , IL-18, IL-6, IL-8, and IL-10.²⁷ Thus, the presence of significant amounts of LAM in type I reaction sites may amplify the pathological events already occurring in skin and nerve. In this context, it is interesting to note that LAM was the only antigen present in significant amounts in tissues from treated patients. If potentially immunogenic material is persisting in some sites, this might explain why late reactions can occur up to years after apparently successful antileprosy treatment.¹ However, because LAM does not stimulate T cell-mediated immunity, it could not be the only antigen precipitating a reversal reaction.

Insight into the mechanisms that may be operating in leprosy type I reactions to produce tissue damage comes from a series of experiments examining the Schwartzmann and Koch phenomena. In 1937 Schwartzmann²⁸ showed that a rabbit injected with a suspension of gram-negative organisms developed necrosis only at sites that had been "prepared" 24 hours earlier by an injection of the same organism. The active component of the gram-negative injection has subsequently been shown to be the endotoxin lipopolysaccharide (LPS). In 1891 Koch had demonstrated that skin testing with *M. tuberculosis* preparations in guinea pigs already infected with *M. tuberculosis* produced a massive necrotic reaction.²⁹ The link between these two tissue-damaging phenomena is that one of the major mycobacterial cell wall components, LPS, shares many properties with LAM.³⁰ Furthermore, LPS injected into mice previously infected with bacille Calmette-Guérin (BCG) causes necrosis in the BCG granulomata,³¹ and intravenous sonicated *M. tuberculosis* has a similar effect. That TNF might be the final mediator in this pathway has been demonstrated by Rothstein et al.,³² who showed that injecting TNF directly in to a murine site prepared by a previous inoculation of LPS produced local hemorrhage and necrosis. Mycobacterial sonicates of *M. tuberculosis*, *M. leprae*, and *M. vaccae* may also be used to prime sites for a necrotic reaction (Rook, unpublished, quoted in Rook et al.³³).

Drawing on these data, one may construct a two-step model for the local tissue damage seen in type I reactions. The reaction is initiated when mycobacterial antigens are released and one or maybe several protein antigens are presented in association with class II antigen to T cells. This results in an influx of IFN- γ -secreting CD4 cells, which may activate macrophages. However, this activation is greatly enhanced by the presence of LAM, which has prepared or sensitized the site for the action of TNF- α , and the final outcome is tissue damage. This hypothesis would also partly explain the predilection of borderline patients for developing reversal reactions. In TT patients, IFN- γ is present in lesions, but there is no LAM, whereas in LL patients there is abundant LAM but little potential for generating IFN- γ . Borderline patients have both LAM and IFN- γ and so are at risk for type I reactions.

LAM was present in 27 of the 31 biopsies screened from patients with type I reactions.

This model of tissue damage will almost certainly be refined further as the interactions of other cytokines are documented. The critical role of T cells has been demonstrated in the mouse footpad model, in which the destructive effect of TNF- α could be abrogated by a single dose of anti-CD4 just before TNF- α administration.³⁴ Rook and Hernandez-Pando³⁵ have argued that the tissue-damaging potential of TNF- α is determined by the presence of local T cell cytokines, with tissue damage only occurring where there is either mixed Th1/Th2 or Th0 cytokine activity.

In this study, we have demonstrated tissue localization of defined *M. leprae* antigens in skin and nerve at the time of active immunopathology. We are not able to associate any single antigen with the development of type I reaction disease, although the strong staining for 28-kd antigen with a relative absence of this antigen in new and treated patients makes the 28-kd antigen an interesting potential candidate. This question can only be resolved by performing a longitudinal study of the tissue localization of mycobacterial antigens before, during, and after a type I reaction.

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