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Demonstration of *Mycobacterium leprae* antigen in nerves of tuberculoid leprosy

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Summary. Twenty nerve biopsies of tuberculoid leprosy patients who showed no acid-fast bacilli in their skin smears or in tissue biopsies, were stained for mycobacterial antigens using anti-bacille Calmette-Gûerin (BCG) by the peroxidase-antiperoxidase method. Adjacent parts of some of these nerves were examined for the presence of osmiophilic bacilli under the transmission electron microscope. Eight of the 20 nerves were both clinically and histologically uninvolved. All the 20 involved nerves showed presence of antigen located, mainly intracellularly, in the cytoplasm of epithelioid cells and to a lesser degree in Schwann, endothelial and plasma cells. A few nerves with caseated nerve abscesses showed clusters of antigen deposits in both the caseous mass as well as the wall of the abscess. In six of the nine nerves processed for electron microscopy, electron-dense bacilli were noted within the cytoplasm of Schwann cells but not within the infiltrating cells. The uninvolved nerves showed neither antigen deposits nor osmiophilic bacilli despite fine ultrastructural changes. Our observations indicate that (a) the specificity of the immune response in paucibacillary nerve lesions is probably against bacterial components. (b) There is a differing antigen handling by Schwann cell and the inflammatory epithelioid cell. (c) Plasma cells may play a role in presenting antigen. (d) *Mycobacterium leprae* may be acting as an adjuvant in causing damage to uninvolved nerves at distal sites.

Key words: Leprosy – Nerves – Antigen

Extensive nerve damage is one of the hallmarks of the tuberculoid or paucibacillary form of leprosy, in which the demonstration of intact acid-fast bacteria (AFB) has been infrequent and which even now remains an enigma. Although light microscopy rarely, if ever, detects AFB in inflammatory nerve lesions of tuberculoid patients, one of us (Shetty, unpublished observations), has frequently observed solid osmiophilic bacilli under the electron microscope. Antigenic determinants have also been localised in dermal lesions of leprosy patients [6, 9] on the basis of cross-reactivity observed between antigens of *Mycobacterium leprae* (*M. leprae*) and bacille Calmette-Guérin (BCG) [4]. Technically, the antigen is viewed by light microscopy using anti-BCG sera in the modified peroxidase-antiperoxidase (PAP) technique [12].

The importance of detecting myobacterial antigen in a lesion within the peripheral nerve in tuberculoid patients is obvious. Of additional interest is the detection of antigen, if any, in clinically uninvolved nerves of tuberculoid patients where, despite the absence of inflammatory cells, well-defined morphological changes have been recorded by electron microscopy [10].

We have, therefore, attempted to demonstrate and compare mycobacterial localisation in both clinically involved and uninvolved tuberculoid nerves by three methods, i.e. acid-fast and osmium staining for observation of intact bacteria at light and electron microscopy, respectively and an immunoperoxidase staining for detection of mycobacterial antigenic determinants at the light level. Such a scheme is feasible, since the findings of whole AFB by the standard carbol-fuchsin stain or antigenic determinants (PAP technique) within a nerve can be compared to a scan for bacilli carried out after osmium staining at the electron microscope (EM) level on an adjacent segment of the same nerve.

Material and Methods

Nerve biopsies of 20 tuberculoid leprosy patients who showed no acid-fact bacilli (AFB) in either their skin smears or in biopsies of the skin patch and/or nerve were included in this study (Table 1). Of these, 8 were clinically uninvolved and 12 were involved. The patients were classified on a clinical and histopathological basis by the Ridley-Jopling classification [8]. No patient had any history of tuberculosis. The funicular nerve biopsies of clinically involved nerves were obtained at operations for nerve release.

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Table 1. Summary of cases and results

	Class	Tissue	R_x	Infiltration	Antigen			Antigen-bearing cells			
					Fite	EM	BCG	Schwann	Epithelioid	Plasma	Miscellaneous
1.	L	Skin	Nil	+++	+++	+	+++				
2.	L	Nerve	Nil	+++	+++	+	++++				
3.	T	Ulnar	2 months	+	−	−	++	+	+		
4.	T	I.R.C.	3 years	++	−	+	++	+	+	+	Endothelial
5.	T	I.R.C.	2 months	+	−	−	++	+			Perineurial
6.	T	Ulnar	7 months	++	−	+	++		+	+	Endothelial
7.	T	Ulnar	Nil	++	−	+	++	+		+	Pericytes
8.	T	Ulnar	Nil	++	−	−	++		+	+	
9.	T	S.C.	Nil	++	−	N.A.	+++		+	+	Giant
10.	T	Ulnar	4 months	++	−	+	++		+	−	
11.	T	G.A.	Nil	++	−	N.A.	++		+	+	
12.	T	Ulnar	6 months	++	−	+	++		+		Granules
13.	T	Radial	Nil	++	−	+	+++	+	+	+	Perineurial
14.	T	Sural	Nil	++	−	N.A.	+++		+		
15.	T	I.R.C.	5 months	−	−	N.A.	−	−	−	−	
16.	T	I.R.C.	1 month	−	−	N.A.	−	−	−	−	
17.	T	I.R.C.	Nil	−	−	N.A.	−	−	−	−	
18.	T	I.R.C-	Nil	−	−	−	−	−	−	−	
19.	T	I.R.C.	6 months	−	−	−	−	−	−	−	
20.	T	I.R.C.	Nil	−	−	−	−	−	−	−	
21.	T	I.R.C.	Nil	−	−	−	−	−	−	−	
22.	T	I.R.C.	Nil	−	−	−	−	−	−	−	
23.	N	Ulnar	−	−	−	−	−	−	−	−	
24.	N	I.R.C.	−	−	−	−	−	−	−	−	

L = Lepromatous; T = tuberculoid; N = normal; +, ++, +++ = graded positivity increasing in intensity; N.A. = not available; I.R.C. = index branch of the radical cutaneous; G.A. = greater auricular; S.C = supra clavicular; R_x = treatment

The heavily bacillated tissues from two lepromatous patients and two nerves obtained from healthy individuals undergoing nerve grafting for trauma, served as positive and negative controls, respectively.

All biopsies were fixed in Formol-Zenkar solution and embedded in paraffin wax for routine histology. Five-micrometre-thick nerve sections were stained by the Triff and Fite Farraco method for acid-fast organisms and by the PAP technique for demonstration of mycobacterial antigen [12].

The primary anti-BCG sera (DAKO) was used at a dilution of 1:100 in Tris-HCl buffer and incubated overnight on the sections at 4°C. The reaction was developed with 3.3′ diaminobenzidine tetrahydrochloride and counter stained with haematoxylin and eosin.

Adjacent parts of nine involved and five uninvolved tuberculoid and two lepromatous nerve biopsies were collected in 3% glutaraldehyde, post-fixed in osmium tetroxide and processed for electron microscopy. Ultrathin sections of araldite-embedded blocks were cut and stained with uranyl acetate and lead citrate and observed under a JEOL 100S transmission EM (TEM).

Results

The results are summarized in Table 1.

Controls

Peroxidase staining. The two bacillary-positive lepromatous nerves demonstrated a more intense staining by anti-BCG in the acid-fast-positive, as well as acid-fast-negative, areas as compared to the adjacent sections stained by the Fite technique.

The two nerves of healthy individuals showed no evidence of anti-BCG activity. Controls of the tuberculoid cases, where anti-BCG sera was omitted during staining, revealed the absence of brown precipitate, thus ruling out non-specific staining.

Clinically involved nerves

Triff/Fite staining. All ten nerves showed a characteristic tuberculoid granulomatous infiltrate of epithelioid cells and lymphocytes. Cells, morphologically similar to plasma cells with characteristic cart-wheel nuclei were observed in seven of these biopsies and three cases showed evidence of micro-necrotic abscesses. AFB were absent in all. The presence of plasma cells in (tuberculoid) lesions with caseous necrosis has been previously described [1].

Electron microscopy. Seven nerves were processed for electron microscopy and of these, six showed the presence of typical electron-dense organisms with a characteristic clear transparent zone, typical of *M. leprae.* The bacilli were situated chiefly in the Schwann cell cytoplasm of unmyelinated fibres or in

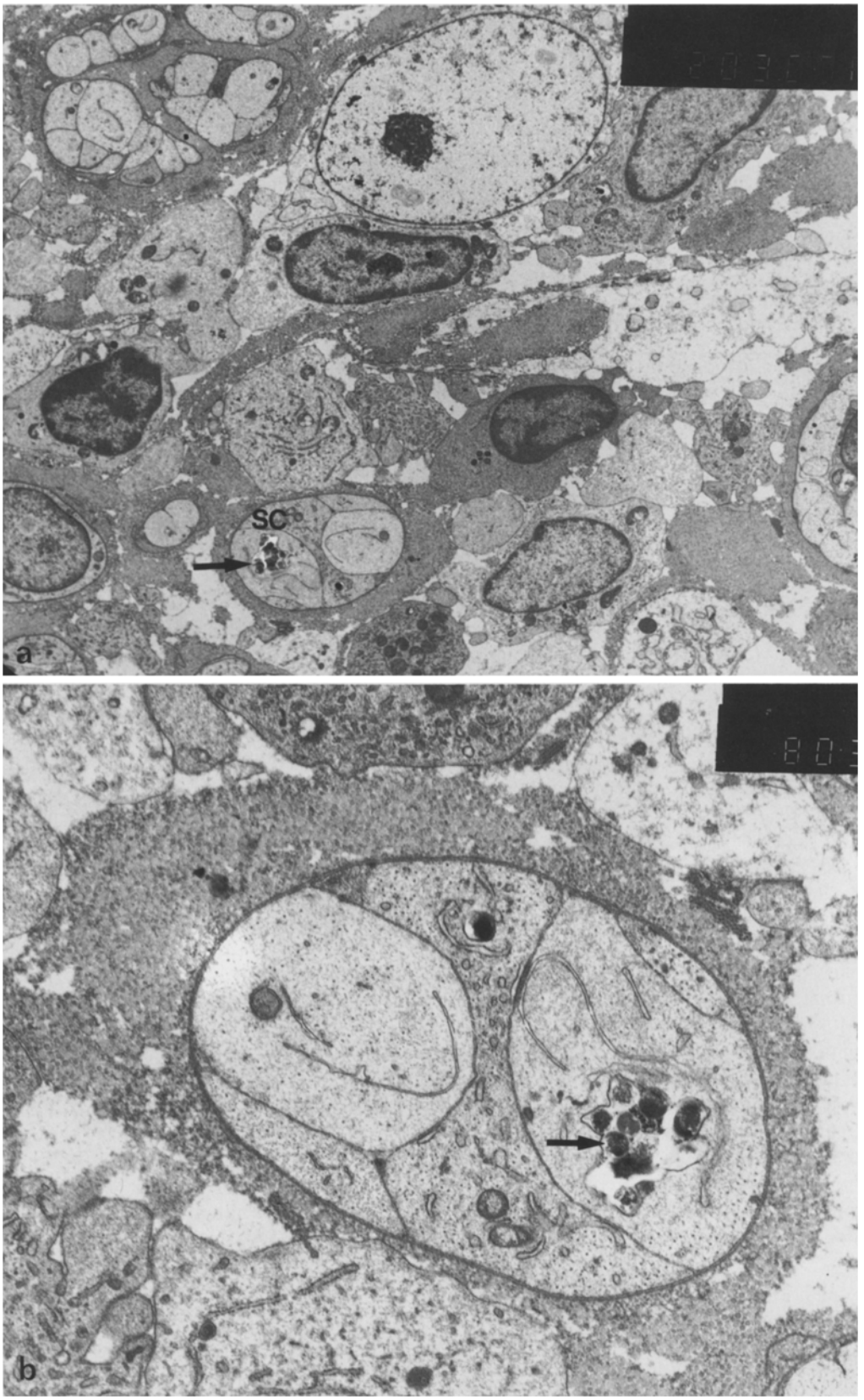

Fig. 1. a Ultrathin transverse section of an ulnar nerve funicle obtained from a tuberculoid leprosy patient (case 10) showing presence of bacilli (*arrow*) in a Schwann cell (*SC*) but absence in the surrounding infiltrating cells. × 3,600. **b** Higher manification of the above Schwann cell showing solid osmiophilic bacilli (*arrow*) in the cytoplasm. × 16,000

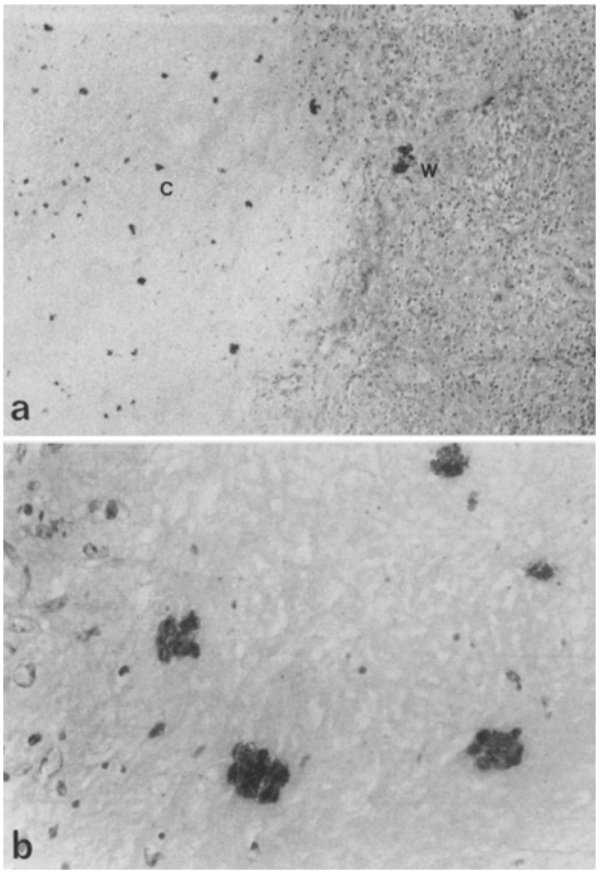

Fig. 2. a Transverse section of the caseous nerve abscess of case 6 showing distribution of mycobacterial antigen deposits in the wall (*W*) and cavity (*C*) of the abscess. The antigen is visualised by the use of anti-bacille Calmette-Guérin (BCG) using the peroxidase-antiperoxidase (PAP) technique. × 1,300. **b** Higher magnificationof **a** showing detail of antigen deposits. × 30,208

denervated Schwann bands (Fig. 1 a) (micrograph) but rarely in the macrophage-like cells of the surrounding granulomatous infiltrate (Fig. 1 b). The number of bacilli did not appear to correspond to the treatment status – the maximum number being seen in the case that was treated with diaminodiphenyl sulphone for 4 months, rather than the untreated. However, in patients treated for a relatively longer period, bacilli appeared irregularly electron dense in contrast to solid staining forms in the untreated or short-term-treated patients. Such variation in morphology may be attributed to treatment.

BCG staining. Despite the absence of AFB, this staining method detected varying degrees of antigen deposits. In some cases, few groups of antigen-bearing cells forming foci were dispersed throughout the section, whereas in others, the majority of the cells had stained positive. The number of foci varied in individual patients being maximum in patient 8. There appeared no specific confinement of antigen-bearing cells to a distinct area like the epineurium or perineurium.

Fig. 3. Longitudinal section of the ulnar nerve of case 6 showing mycobacterial antigen deposits as stained by PAP technique in endothelial cells (*arrow*). × 5,369

Qualitatively, the cytoplasmic staining demonstrated three patterns: (1) diffusion all over the cytoplasm with varying densities; (2) dark, dense cytoplasmic granules; and (3) a localised globular precipitate lying in the caseous necrotic cavity and its walls but without any cellular morphology (Fig. 2).

While the bacillary load, as seen under the EM showed no clear cut relation with treatment, immunoperoxidase staining was most marked in the untreated and showed a decrease following treatment, e.g. a 3-year-treated case showed only a marginal presence of anti-BCG.

Although lymphocytes constituted a large percentage of the infiltrating cells, they showed no presence of antigen. Infiltrating epithelioid cells were most commonly stained, but the proportion of antigen-bearing epithelioids varied in individual cases. Fifty percent of the cases studied showed BCG staining in Schwann cells. Endothelial and giant cells of a few cases were also seen to harbour antigen (Fig. 3).

An interesting observation was the staining of plasma-like cells with typical cart wheel nuclei amidst an unstained lymphocytic infiltrate in seven out of ten cases. However, the number of antigen-bearing plasma cells varied in individual cases (Fig. 4).

In addition to the above, there were two clinically involved nerves which showed a mild round cell inflammation but no AFB (cases 3 and 5). Although no bacilli were seen at the EM level, it was observed that these nerves had a considerable fibre drop-out. In one nerve (case 13), BCG staining showed significant amounts of antigen in both Schwann-like and epithelioid cells within the endoneurial area. Patient 5 had antigen in perineurial and Schwann cells but the epithelioid cells were devoid of staining (Fig. 5). This

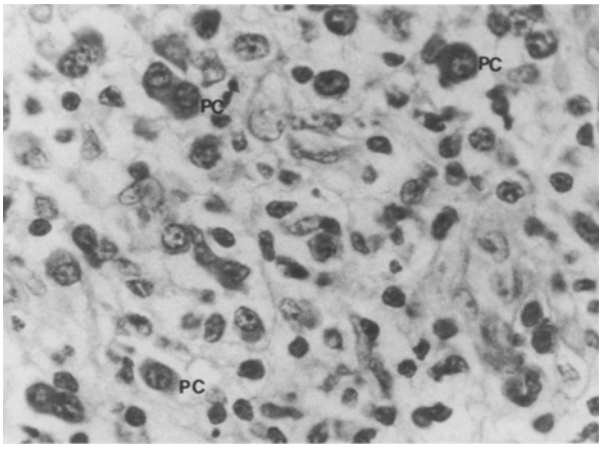

Fig. 4. Transverse section of greater auricular nerve of case 11 showing presence of mycobacterial antigen in plasma cells (*PC*) of the infiltrate. PAP technique, × 5,139

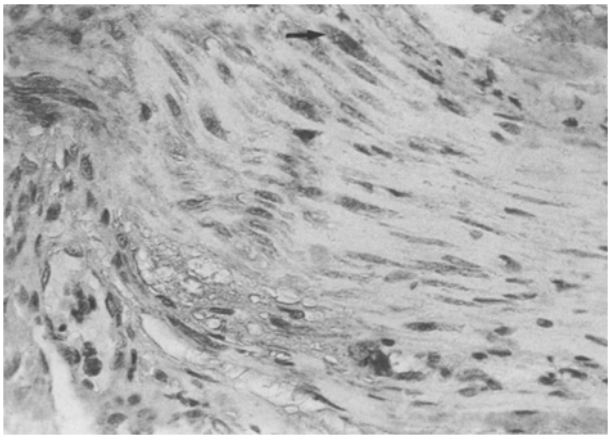

Fig. 5. Longitudinal section of ulnar nerve of case 5 showing mycobacterial antigen deposits in Schwann cells (*arrow*). PAP technique, × 3,835

case proved interesting in that antigen was located in more than half of the Schwann cells, yet ultrastructurally no bacilli were seen.

Clinically uninvolved nerves

Triff/Fite. All eight nerves appeared normal without any cellular infiltrate or AFB.

Electron microscopy. Significant ultrastructural changes were seen in these nerves suggesting both degenerative and regenerative activity among myelinated and unmyelinated fibers [10]. No nerves showed any bacilli.

BCG staining. In contrast to the involved nerves, these nerves did not take up any peroxidase staining indicating the absence of BCG-cross reacting antigen.

Discussion

The absence or paucity of *M. leprae* as detected by the standard carbol-fuchsin procedure makes it difficult to explain the cell-mediated immune damage in tuberculoid nerve lesions. Earlier findings [7] indicate that the inflammatory response towards intraneurally located *M. leprae* antigens may be necessary for nerve damage in tuberculoid leprosy. For the first time, the present study reports the concurrent presence of considerable amounts of *M. leprae* antigen, as well as of bacilli, under the TEM in clinically involved, heavily infiltrated tuberculoid nerves, which showed no acid-fast bacilli. The observation of bacilli solely at the ultrastructural level in six out of nine involved nerves suggests that osmium and carbol fuchsin may be staining two different components of *M. leprae*.

With the advent of the PAP method using anti-BCG, several workers [1, 6, 9] showed mycobacterial antigenic determinants in highly infiltrated tuberculoid skin lesions. Others used alternative approaches. Cells teased out and cultured in vitro, either from leprous skin lesions [3] or from caseous nerve abscesses of borderline tuberculoid patients [1], released antibody into the supernatant, directed largely against bacterial components and not against host tissue. Collectively, these observations imply that the specificity of the immune response in paucibacillary nerve lesions is probably against bacterial components.

We also searched for the presence of mycobacterial antigens in clinically normal, histologically uninfiltrated nerves of tuberculoid patients to explain our earlier findings of nerve damage occurring in the absence of infiltration [10]. The inability to demonstrate antigen in these nerves suggests an interplay of some other factors. One possibility could be that there is antigen which cannot be detected by anti-BCG. Secondly, as most of the ultrastructural changes are observed in the endoneurium rather than in the epineurium, it is possible that presence of *M. leprae* at a distal site acts as an adjuvant. This may lead to release of a soluble circulating factor directed against the nerve which can penetrate the blood-nerve barrier and cause damage to the nerve. Experimental evidence to this effect has been provided [11].

A possible cause of the ultrastructural damage could be Wallerian degeneration at distal sites, brought about by a proximal lesion. However, this can be ruled out because the nerves have been carefully examined both (1) clinically by using fine sensory modalities and (2) electrophysiologically, to exclude the presence of proximal lesions along the course of the nerve.

The interplay of differing specificities of the staining methods employed here poses an interesting question, i.e. in involved nerves, why are osmiophilic bacteria detected only in Schwann cells, and not in the granulomatous infiltrate?

It could be postulated that the *M. leprae*-harbouring Schwann cells, in the process of bacillary handling, may have stripped off the outer bacterial components responsible for acid-fast staining such as mycolic acids, whereas other components, which are osmiophilic, are retained and these permit the detection of bacilli under TEM. Alternatively, osmium staining may be depicting a non-acid-fast stage in the life cycle of *M. leprae* [2]. This question, in our opinion also indicates the differing antigen handling or processing capabilities of the Schwann cell and the inflammatory epithelioid cell.

In approximately 50% of the cases, positive cytoplasmic staining of antigen was noted in cells that classically fitted the description of plasma cells. Non-specific staining could be ruled out, since only a proportion of the plasma cells were seen to harbour antigen. This could imply a role for plasma cells in the processing and presentation [5] of *M. leprae* antigens.

The presence of antigen within and around the caseous mass despite extensive release of cellular enzymes that inevitably accompany such a process is interesting as well as indicative of the enzyme-resistant nature of the antigen. This persistent packet of antigen probably acts as a stable unit and may prove to be a stimulator for eliciting a subsequent cell-mediated immune response.

Thus, the presence of mycobacterial antigens in involved nerves, and the absence in quiescent nerve lesions of tuberculoid leprosy, suggests a dual role of specific immune response and auto-immune mechanisms in nerve damage in leprosy.

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