Appendix
Antineural Antibodies in Sera of Leprosy Patients

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A microtiter plate ELISA with semipurified human nerve sonicate antigen(s) (NA) was used to screen the sera of leprosy patients. High titers of IgG and low titers of IgM classes of antineural antibodies directed to peripheral nerve antigens were detected in LL, BL, BB, BT, and TT categories of leprosy. In the Western blot, leprosy sera recognized 50- to 55-, 85- and 108-kDa molecular weight protein bands of NA. The identity of these protein bands immunoreactive with leprosy sera was checked with a panel of commercially available antibodies to known neural proteins. The 50- to 55-kDa band reacted with anti-S100 and anti-glial fibrillary acidic protein antibodies while 85 and 108 kDa could not be identified. Whole immunoglobulins isolated from leprosy sera with high titers of antineural antibodies induced cytotoxicity of the cultured glial cell line in the presence of complement. © 1990 Academic Press, Inc.

INTRODUCTION

Leprosy is a chronic disease primarily involving peripheral nerves and skin. The etiological agent, *Mycobacterium leprae*, is an obligate intracellular parasite with high affinity for peripheral nerve cells, especially Schwann cells (1, 2). Invasion by *M. leprae* of the peripheral nerves leading to anesthesia and muscular atrophy is a key feature of leprosy (3). Clinically, nerve damage is seen not only throughout the spectrum but also in the early indeterminate stage of the disease.

Mechanisms underlying pathogenesis of leprous neuritis are not understood. Since *M. leprae* itself is nontoxic when a cell-mediated immune (CMI) response is not evoked, slow destruction of the nerve is thought to be due to (i) metabolic/biochemical alteration of the Schwann cell (4, 5) affecting the ability of these cells to maintain the myelin sheath and integrity of the axon, resulting in demyelination and degeneration of the nerve fibers, or (ii) circulating demyelinating factors (6). Such a mechanism may be implicated in the indeterminate and borderline lepromatous (LL-BL) categories of leprosy where no CMI against *M. leprae* antigen is evoked. In the tuberculoid and borderline tuberculoid (TT-BT) categories, the local CMI response is quite vigorous and is associated with the presence of mycobacterial antigens and severe nerve damage (3, 7, 8). Since the antigens involved have not yet been identified, it is speculated that here the immune response could be directed not only to mycobacterial antigens but also to self or nerve antigens. Such a contention is strengthened by recent evidence that Schwann cells can express MHC class I and class II antigens on their surface and present mycobacterial and/or autoantigens to T cells (9). The idea that autoimmune mechanisms may be involved in the pathogenesis of leprous neuritis is also supported by the observations that the nerve biopsies from patients with sensory loss show areas of breakdown of the myelin sheath and/or axons, and degenera-
tive changes in the Schwann cell cytoplasm, but no evidence of \textit{M. leprae} or invading inflammatory cells (2, 7, 8).

These findings call for a detailed investigation into autoimmune responses directed to the peripheral nervous system in leprosy. We examined the existence and the role, if any, of antineural antibodies. Earlier, Wright \textit{et al.} (10) reported antibodies directed to axons or the peripheral nerves in 40 and 20\% of lepromatous and tuberculoid leprosy patients, respectively. Eustis-Turf \textit{et al.} (11) first observed antibodies directed against the 45- to 55-kDa antigen in 38\% leprosy patients and on subsequent analysis to 42- and 35-kDa antigens (12). In both these studies, binding of leprosy sera to nerve sections in indirect immunofluorescence was taken as a measure of the presence of an antineural antibody. In the present study, antineural antibodies were measured in a microtiter plate ELISA using partially purified peripheral human nerve sonicate as antigen. High levels of antineural antibodies were detected in the LL, BL, BB, BT, and TT categories of leprosy. Initial results of the experiments relating to functional properties of these antibodies show that they may play a role in the complement-mediated lysis of the glial cells.

MATERIALS AND METHODS

\textit{Human nerve sonicate preparation.} Peripheral nerves were removed within 12 hr from autopsies of 20- to 50-year-old individuals, with no obvious history of neurological disorder. Under $\times 16$ magnification of a dissection microscope (Nikon, Japan), most of the nonneural tissues were removed and nerve funicles were isolated, minced, suspended in ice-cold phosphate-buffered saline (PBS), pH 7.4, and sonicated for 4 to 5 min using 40\% output of a Virtis Sonicator (Virsonic 300, New York) with a regular probe. Sonicate was centrifuged at 11,953g at 4°C for 30 min (Sorvall Instrument SS-34 rotor). The supernate was collected and its protein content was determined by the standard Lowry’s method (13).

\textit{Enzyme-linked immunosorbent assay (ELISA).} Ninety-six-well flat-bottom polystyrene Titerplates were coated with antigen at a volume of 50 $\mu$L per well in 0.2 M carbonate-bicarbonate buffer, pH 9.5. The plates were incubated overnight at 37°C, washed, and blocked with 150 $\mu$L of 3\% (w/v) bovine serum albumin (BSA)-fraction V (Spectrochem, India) for 1 hr at 37°C. Plates were then washed and human serum diluted in 1\% PBS–BSA was added and incubated for 1 hr at 37°C. After washing, the plates were reincubated for 1 hr at 37°C with rabbit anti-human immunoglobulins (Ig or IgG or IgM) conjugated to horseradish peroxidase (HRPO) (Dakopatts, Copenhagen, Denmark). Color was developed using substrate made up of 0.5 mg orthophenylene diamine/ml in 0.15 M citrate-phosphate buffer, pH 5.0, and 1 $\mu$L/ml of 30\% $\text{H}_2\text{O}_2$. Reaction was stopped with 5 $N$ $\text{H}_2\text{SO}_4$. The absorbance was read at 490 nm using a microplate autoreader (EL 309 Bio-tek Instruments). Antibody titers are expressed as the average optical density (OD) reading of duplicate wells subtracted from the mean OD of blanks consisting of OD of serum and conjugate alone.

In some experiments, the antigen-coated wells were treated with 100 $\mu$g/ml protease K (Sigma) for 2 hr at 37°C or with 0.05 M sodium periodate (SRL, India)
in 0.01 \( M \) sodium acetate buffer, pH 4.5, for 24 hr at 4°C (14). Plates were then washed and further steps of ELISA were followed as described above.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).** The proteins of the nerve sonicate were separated by SDS–PAGE according to the method described by Laemmli \textit{et al.} (15) using a 12.5% running gel and a 3% stacking gel. Proteins were solubilized in sample buffer containing 2% SDS, 10% glycerol, 0.05% mercaptoethanol, 0.0025% bromophenol blue, and 0.0625 \( M \) Tris–HCl, pH 6.8. These were vortexed and immersed in a boiling water bath for 5 min. Electrophoresis was carried out in a minislab gel apparatus (Hoefer Scientific Instruments, San Francisco, CA, Model SE250) for 1 hr at 20 mA per gel. Molecular weight standards (bovine serum albumin –66K, ovalbumin –45K, and carbonic anhydrase –29K, Sigma Laboratories) were run in parallel. The proteins were stained using Coomassie brilliant blue G-250 in 25% methanol and 10% acetic acid for 2 hr at room temperature and destained in 5% acetic acid and 5% ethanol.

**Immunoblot.** Proteins were transferred at 45 V for 2 hr at room temperature in 25 mM Tris, 0.2 mM glycine, and 20% methanol, pH 8.3, from the polyacrylamide gels to the nitrocellulose membrane (0.45 \( \mu \)m) in a Bio-Rad Trans-Blot apparatus. The membrane was cut into 4-mm strips, blocked with 5% PBS–BSA overnight at 4°C, and then probed with a 1:100 dilution of patients sera for 1 hr at room temperature with constant shaking. Alternatively, the strips were stained with mouse monoclonal antibodies such as vimentin and neurofilament and polyclonal antibodies were raised in rabbit against a panel of known nerve proteins like S100, myelin basic protein (MBP), glial fibrillary acidic protein (GFAP), and fibronectin (Dakopatts, Copenhagen, Denmark). The strips were then washed in PBS and then incubated with rabbit anti-human IgG/goat anti-mouse Ig/swine anti-rabbit Ig conjugated to HRPO at a 1:250 dilution for 1 hr at 37°C. The reaction product was visualized with the substrate consisting of 0.5 mg/ml of diaminobenzidine in PBS and 1 \( \mu \)l/ml of 30% \( H_2O_2 \).

**Sera.** Leprosy patients classified by the standard Ridley–Jopling criteria (16) were included in the study. Serum was separated from 10 ml of blood collected from each patient by standard procedures, aliquoted, preserved in 0.01% merthiolate, and stored at -70°C until use.

**Cytotoxicity assay.** The 33B Schwannoma cell line and the Vero cell line maintained by us routinely in the laboratory (5) were used for the cytotoxicity assay. The protocol described by Kull and Cuatrecasas (17) and Parish and Mullbacher (18) was followed with slight modifications. Briefly, 7 \( \times \) 10^4 cells per well were plated in a round-bottom 96-well plate (Linbro). After 3 hr the nonadherent cells were removed, and whole immunoglobulins isolated from leprosy or normal sera at 1:25, 1:50, and 1:100 dilutions were added and incubated for 1 hr at 37°C. The cells were washed and incubated with rabbit complement (Pel-Freeze) for 2 hr at 37°C. In some experiments the leprosy immunoglobulins were absorbed with whole nerve sonicate (NA), a protein fraction, and a lipid fraction of NA. To immunoglobulins diluted 1:100 in PBS were added protein (200 ng), lipid (1 \( \mu \)g), and NA (10 \( \mu \)g) and incubated for an hour at 37°C. These were centrifuged at 1912g and the supernate was used for the cytotoxicity assay. In parallel set of
experiments, the cytotoxicity assay was carried out with cultured Vero cells. Cells were again washed and then incubated with warm 0.036% neutral red in MEM for 30 min at 37°C. The dye taken up by the viable cells was released by lysing them with a mixture of 0.05 M acetic acid and 0.5% SDS. Optical density was measured by the ELISA reader at a 540-nm wavelength.

RESULTS

Antineural antibodies. Optimal concentrations of antigen and the sera dilution for the immunoassay were determined using standard titration procedures. A 5 μg/50 μl/well concentration of antigen and 1:400 sera dilutions found to be optimum were used for screening. Initially, 35 leprosy sera, 7 belonging to LL, BL, BB, BT, and TT leprosy and 7 normal sera, were screened for the presence of antineural antibodies. The antibody titers of the IgG type of antibody were high in the range of 1.4 to 2.7 OD in all the sera tested, while that for the IgM type of antibodies were in the range of 0.14 to 0.67 in 5/7 LL, 5/7 BL, 3/7 BB, 4/7 BT, and 5/7 TT sera. Therefore, in subsequent experiments, the IgG type of antineural antibodies were measured. Figure 1 gives the scattergram of titers of antineural antibodies in terms of the OD values measured in 258 sera of leprosy patients. In all the six categories of leprosy, measurable to high titers of antineural antibodies with OD values ranging between 0.6 and 2.0 were observed, as against the values of 0.05 and 0.1 of the apparently healthy normal individuals measured simultaneously. The high levels of antibodies were not reduced by absorption of test sera with mycobacterial antigens (1 mg/ml), while with nerve sonicate (1 mg/ml) the

![Graph showing serum antibody levels to human nerve antigen(s).](image-url)
OD value was reduced to 0.05-0.1. The specificity of the binding of antibodies was tested using peroxidase conjugated to the F(ab)_2 fraction of IgG. Figure 2 presents the data. It can be observed that IgG-F(ab)_2 bound to the sera to the same extent as whole IgG.

Characterization of the nerve antigen. The chemical nature of the antigens recognized by leprosy sera was investigated in the next series of experiments. The antigen–antibody reaction in ELISA was significantly reduced on prior treatment of the antigen with protease and marginally with sodium periodate, suggesting that the antigen(s) was primarily protein in nature (Fig. 3). Immunoblot with leprosy sera revealed that the antineural antibodies were directed to one to three antigenic determinants of 108-, 85-, and 50- to 55-kDa molecular weight nerve protein bands. No such binding was observed with normal sera tested (Figs. 4a and 4b). In the immunoblot, the 50- to 55-kDa antigen was recognized by anti-GFAP and anti-S-100 antibodies. The 85- and 108-kDa bands did not react to the following nerve antibodies such as fibronectin, GFAP, S-100, vimentin, neurofilament, and myelin basic protein.

Cytotoxicity. Table 1 presents the results of the neutral red uptake of 33B Schwannoma cells treated with whole Ig isolated from 10 pooled normal, LL, and TT sera in the presence of complement. Addition of leprosy Ig (both LL and TT) with complement reduced the neutral red uptake of cells significantly, indicating a reduction in the number of viable cells. Prior absorption of the leprosy immunoglobulins (Ig) with whole nerve sonicate, a protein fraction, and a lipid fraction significantly reduced the complement-mediated cytotoxic effect (Table 1). No such cytotoxic effect of leprosy Ig + complement was observed on Vero cells a nonglial cell line.

![Fig. 2. Mean OD values of three separate measurements of antineural antibodies in 10 pooled LL sera, using anti-human IgG conjugated to HRP (○-○) and anti-human IgG F(ab)_2 conjugated to HRP (○-○). The regression coefficient (r) was similar in both conditions (r being 0.73 and 0.70, respectively).](image-url)
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Before treatment

After treatment

FIG. 3. The effect of a prior treatment of the antigen with protease K and periodate. The results are a mean of three individual experiments. There was a marked decrease in the reactivity of the leprosy sera with the human nerve sonicate antigen following treatment with protease K. In treatment with periodate the reactivity was reduced by 20% only. The percentage decrease was calculated as \( \frac{(C - T)/C \times 100} \), where \( C \) = the OD value before treatment and \( T \) = the OD value after treatment with either protease K or periodate.

DISCUSSION

Peripheral nerve damage is not only the consistent feature but also the root cause for the classical limb deformities associated with leprosy. It is therefore vital to understand the mechanisms underlying the pathogenesis of leprous neuritis. The clinical and histopathological evidences suggest a main role for immune mechanisms, an exact role which remains to be defined. Recent observations show the capability of Schwann cells to express HLA class I and class II mole-

Fig. 4. (a) SDS-PAGE profile of the nerve sonicate antigen separated on a 12.5% gel. Lane 1 shows the profile of 20 \( \mu \)g of human nerve sonicate proteins. Lane 2 shows molecular weight markers of 29, 45, and 66 kDa, respectively. Note 15 clear bands. (b) Immunoblot of nerve sonicate proteins (a) electrobotted onto the nitrocellulose membrane. Note the prominent bands at the 50- to 55-, 85-, and 108-kDa regions. The results are an analysis of 10 individual sera. Strips 9 and 10 correspond to normal sera, while strips 1 to 8 correspond to eight leprosy sera, two each of LL, BL, and TT and one each of BB and BT, respectively.
<table>
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<th>Treatment</th>
<th>OD ± SD</th>
<th>% lysis</th>
<th>OD ± SD</th>
<th>% lysis</th>
<th>OD ± SD</th>
<th>% lysis</th>
<th>OD ± SD</th>
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<tr>
<td>Control (neutral red alone)</td>
<td>0.4786 ± 0.012</td>
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<td>Complement alone</td>
<td>0.478 ± 0.0113</td>
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<td>1:25</td>
<td>0.206 ± 0.032</td>
<td>57.5</td>
<td>ND</td>
<td>0.450 ± 0.007</td>
<td>5.62</td>
<td>0.4182 ± 0.0068</td>
<td>13.1</td>
<td>0.2706 ± 0.0047</td>
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<td>61</td>
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<td>ND</td>
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<td>60.7</td>
<td>0.450 ± 0.007</td>
<td>5.62</td>
<td>0.4182 ± 0.0068</td>
<td>13.1</td>
<td>0.2706 ± 0.0047</td>
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<td>1:25</td>
<td>0.2475 ± 0.056</td>
<td>48.8</td>
<td>ND</td>
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<td>34.46</td>
<td>0.2278 ± 0.006</td>
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<td>0.3295 ± 0.029</td>
<td>31.1</td>
<td>ND</td>
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<tr>
<td>1:50</td>
<td>0.347 ± 0.057</td>
<td>27.98</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>1:100</td>
<td>0.4030 ± 0.011</td>
<td>16.28</td>
<td>0.3680 ± 0.088</td>
<td>23.59</td>
<td>0.3638 ± 0.0063</td>
<td>24.47</td>
<td>0.3532 ± 0.01</td>
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Note. Results are expressed as the mean of five separate experiments, each carried out in triplicate. Ten sera with high antineural antibody titers were pooled in each group, viz. LL, TT, and 10 normal sera and their immunoglobulins isolated. Whole serum Ig was isolated from pooled LL, TT, and normal sera by precipitating serum with 50% saturated ammonium sulfate solution. The precipitate was dialyzed extensively against PBS and redissolved in PBS before use. Percentage lysis was calculated as \((C - T)/C \times 100\), where \(C\) = mean OD reading of cells treated with neutral red alone and complement alone and \(T\) = mean OD values of cells treated with Ig plus complement. Immunoglobulins absorbed with NA were assayed for cytotoxicity at a 1:100 dilution.
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Culcs, and present mycobacterial as well as autoantigens to T cells, indicating their participation in the local immune response (9) and the possible triggering of the autoimmune phenomenon. The present study was taken as a first step toward determining the possible role of antineural antibodies. ELISA and electroimmunoblot studies demonstrate a wide prevalence of antineural antibodies directed to one to three antigenic determinants of molecular weights ranging from 50- to 55-, 85, and 108 kDa in the sera of all categories of leprosy patients. These antigens are apparently different in molecular weight from those reported by other workers (10-12). These workers reported anti-Po, an intermediate filament present in less than 50% of leprosy patients (10-12). The high frequency and high titers of the antibodies observed in our study suggest that there is a good humoral immune response directed to peripheral nerves in this disease. Both IgG and IgM classes of antibodies were detected; the titer and frequency of IgG being higher than IgM. These antibodies specifically bound to epitopes present in the antigens coated on the plate and were not mediated nonspecifically by binding to the Fc region as is illustrated by immunoabsorption experiments with immunoabsorbed sera and in which the conjugate-linked F(ab)2 fractions were used.

The antigenic determinants recognized by these sera in the immunoblot have been partially characterized and will need further analysis. At this stage we can only say that these antigens are protein in nature as the reactivity was significantly reduced in ELISA by protease enzyme treatments. GFAP and S100 reacted to only one of the three bands, i.e., the 50- to 55-kDa protein. The 85- and 108-kDa proteins did not react to any of the six antineural antibodies used. The clinical significance and the pathogenic role of these antineural antibodies are currently being investigated in detail. Our preliminary observations with neutral red assay suggest that these antibodies may play a role in the complement-mediated lysis of the nerve cells. The role of antineural antibodies in human demyelinating diseases remains unclear. Antibodies directed against MBP, cerebrosides, gangliosides, and myelin-associated glycoprotein have been reported in other neuropathies (20-22). There are reports of a lack of antibodies to major proteins of myelin such as basic protein. Po, and galactocerebroside in human demyelinating disorders (23, 24). In leprosy, Eustis-Turf (11) reported antibodies to Po protein while Mshana et al. (25) could not detect antibodies to the related protein P2. It is difficult to find an explanation for these divergent results. Such a high frequency of antibodies associated with the varying pathology of nerves, ranging from nerves loaded with M. leprae in the LL to well-formed localized granulomas in TT observed in our study, also cannot be explained. Two possibilities can be speculated. Such a high frequency of antibodies may be due to some cross-reactivity between nerve proteins and M. leprae antigens. Recent work relating to the functional analysis of mycobacterial antigens suggests that many of these antigens may share a common structural sequence with human proteins, specifically the mycobacterial heat shock and stress proteins (26, 27). Therefore, the genesis of antineural antibodies may be triggered by the mycobacterial antigen-primed T cells recognizing the nerve proteins. Although this contention at the moment is speculative, it is persuasive. Recently, attention is being paid to the possible role of cytokines in tissue damage. More specifically, tumor necrosis factor has been suggested to play a role...
in demyelination or destruction of nervous tissue (28). Thus, in TT, cytokines may initiate the tissue damage leading to the release of otherwise sequestered nerve components into circulation and the production of antineural antibodies as a result.

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Diagnosis

Nerve antigen based serological tests for the diagnosis and prognosis of leprosy

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Humoral immune response to Mycobacterium leprae is significantly high in the lepromatous form (LL), but is low in the tuberculoid form (TT) of leprosy. Such an effect is thought to be due to varied antigenic load in these two forms of the disease. Hence most of the immunodiagnostic tests developed to detect the antigen or antibodies directed to these antigens are able to put into evidence only the LL form of the disease. An alternative approach could be to develop a test that would measure the ongoing destruction of the nerve as a result of M. leprae infection. It would have a dual advantage of being a tool for the diagnosis of the disease as well as be a measure for the ongoing nerve damage or its arrest. Such a contention is based on the observation that the peripheral nerve is the active site of pathological changes along the entire spectrum of the disease (Khanolkar, 1964). Symptoms of neural deficits such as loss of pain, touch and sweating are routinely used to discriminate leprous lesions from other dermatological disorders.

Nerve damage so far has been measured by estimating the degree of loss of sensory function or recording electrical activities. This study was initiated to find out if, a serological test could be developed for detection of nerve antigens/antibodies that would correlate with the symptoms of nerve damage in leprosy. Observations from in vitro tissue culture experiments and rhesus monkeys infected with M. leprae were pointers in this direction. Studies conducted in nerve tissue culture models reveal that the pathology may be initiated immediately after invasion by M. leprae in Schwann cells and changes in the neurons occur as a subsequent effect (Mukherjee et al., 1980a and b, Mukherjee and Antia, 1986). M. leprae primarily bind to Schwann cells in a specific manner (Mukherjee and Antia, 1986; Ity et al., 1986) and once internalized the organisms are metabolically active (Mistry et al., 1989) and multiply (Mukherjee and Antia, 1986) simultaneously causing inhibition of proliferation and migration of host Schwann cells (Mukherjee et al., 1989b). M. leprae infected Schwann cells are recognized by immune cells (Mehta et al., 1988). Schwann cells are capable of expressing MHC class I and class II molecules (Samuel et al., 1987) and present M. leprae autologous antigen to T-cells (Weckerle et al., 1987).

These experimental evidences suggest that invasion of peripheral nerves by M. leprae triggers an immune response not only to its constituents but also possibly to constituents of the peripheral nerves. Further it is established that there is no generalized autoimmune phenomenon in leprosy. Similarly, serum immunoglobulins have been found to rise only in LL but not TT type of leprosy (Rawlinson et al., 1987). The possibility of autoimmune response directed to nerve antigens have been previously explored using immunofluorescence and immunoblotting techniques (Wright et al., 1975 and Eustis Turf et al., 1986). Using these methods, antineural antibodies were detected in certain percentage of LL and TT patients. Such a result could be due to lack of production of antineural antibodies in those cases in which the immunofluorescence and immunoblot were negative or these two techniques are not sensitive enough to pick up the low titres of antibodies directed to nerve antigens.

Since nerve damage is the cardinal sign of leprosy and occurs throughout the spectrum of the disease, we speculated that if a sensitive immunoassay is developed it may be possible to measure presence, if any, of even low titres of antineural antibodies. Using human nerve sonicate antigens a microtitre plate ELISA system to measure the antineural antibodies was developed by us. Two hundred and fifty eight sera were screened of which 68 were LL, 35 BL, 34 BL, 43 BT, 10 TT and 8 neuritis. In this system, high titres of IgG class of antineural antibodies with OD values ranging from 1.4 to 2.7 were detected in the sera in all categories of leprosy. The antibodies were also elevated in some of the contacts and early indeterminate forms of leprosy. In further studies this assay was refined and made highly specific. It was sensitive enough to pick up 95% of the leprosy cases and was negative for tuberculosi,s dermatological disorders and other autoimmune disorders such as systemic lupus erythematosus, Guillain-Barré syndrome and diabetic neuropathy. The assay was negative for normal healthy individuals (70).

The contention that the ELISA based on nerve antigen could be quite sensitive to detect the early leprosy was also strengthened by our observations on rhesus monkeys inoculated with M. leprae. Infection triggered an early synthesis of antibodies to nerve in the sera of these animals. The antibody titres gradually increased and reached optimum within a year of infection before the overt signs of the disease appeared.
The high levels of antineural antibodies were associated with signs of nerve damage as evidenced by dryness, scaling, ulceration leading to resorption of phalanges and deformities mainly of forelimbs.

Attempts have been made to identify the antigens to which the antibodies are directed. Currently, steps are being taken to simplify the assay and routinise it.

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