CHAPTER 4

CIRCULATING ANTIBODIES TO PERIPHERAL NERVE COMPONENTS IN LEPROSY SERA
Introduction

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 (Mukherjee et al. 1980a; Mukherjee et al. 1985c) affecting the ability of these cells to maintain the myelin sheath and the integrity of the axon, resulting in demyelination and degeneration of the nerve fibers, or (ii) circulating demyelinating factors (Shetty et al. 1985). Such a mechanism may be implicated in the indeterminate and borderline lepromatous (LL-BL) categories of leprosy where very little CMI against *M. leprae* antigens is evoked. In the tuberculoid and borderline tuberculoid (TT-BT) categories, local CMI response is quite vigorous and is associated with presence of little mycobacterial antigens and severe nerve damage (Khanolkar, 1964; Dastur, 1977; Dastur et al. 1982). 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 evidences that Schwann cells can express MHC class I and class II antigens on their surface and present mycobacterial and/or autoantigens to T-cells (Wekerle et al. 1987). The idea that autoimmune mechanisms may be involved in the
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 degenerative changes in the Schwann cell cytoplasm, but no evidence of *M. leprae* or invading inflammatory cells (Job, 1971; Dastur, 1977; Dastur et al. 1982).

In this chapter we have measured the antibodies directed to peripheral nerves. The contention being that, as nerve damage is the cardinal sign of leprosy and occurs throughout the spectrum of the disease, and the nerve tissue is highly immunogenic, the immune response to peripheral nerve antigens must be generated in leprosy. Human peripheral nerve sonicate was used as antigen to measure the antineural antibody levels in a microtitre plate ELISA in the sera of leprosy patients. In the next series of experiments, we have studied the ontogenesis of the antineural antibodies in primate model following inoculation with *M. leprae*. In the third series, the basis for high frequency and titre of antineural antibodies in the sera of leprosy patients have been explored. Existence of cross-reactive antigenic determinants between mycobacterial and peripheral nerve proteins was found out.

**Methods**

**Preparation of Nerve antigens:** Human peripheral nerves were collected in semi-sterile conditions from cadavers within 6 to 12 hours of accidental death. These were transported in dry ice and stored at -70°C. For preparing the antigen(s),
the nerves were rapidly thawed and suspended in 10mM PBS, pH 7.2. The nerves were cleaned of the connective tissue under a dissection microscope (Nikon, Japan) at 16X magnification and each fascicle separated. Fascicles were pooled, cut into small pieces, minced and sonicated for 5-7 minutes in a Virtis Sonicator (Virsonic 300, New York) using a regular probe. Sonicate was centrifuged at 4000 rpm at 4°C for 30 minutes. The supernate was collected and used as antigen(s). Protein content of the preparation was estimated by Lowry's method (Lowry et al. 1951).

The monkey nerve antigen was prepared in a similar manner as described above. Peripheral nerves were collected from autopsies of normal monkeys immediately after death. The rest of the protocol has been described above.

ND-BSA was kindly made available by Dr. P.J. Brennan, Colorado State University, Colorado.

**Bacteria:** *M. avium, M. scrofulaceum, M. smegmatis, M. vaccae, M. w* and *M. tuberculosis* H37Rv were grown in Middlebrook 7H9 medium. The bacteria were harvested by centrifugation at 5000 rpm for 20 minutes. The bacterial pellet was washed twice with distilled water and finally suspended in 0.01M PBS, pH 7.2. *M. leprae* suspension was prepared from armadillo spleen. Briefly, spleen was homogenized in saline using a glass homogenizer. The suspension was centrifuged at low speed to remove tissue debris and the supernatant subjected to a high speed centrifugation (5000 rpm for 30 minutes) to obtain the bacillary pellet. The pellet was resuspended in known volume.
of saline and counted after staining with Ziehl Neelsen's carbol fuchsin.

M. tuberculosis M sonicate antigen was prepared as follows. Briefly, the mycobacteria cultured in Middlebrook 7H9 medium were harvested by centrifugation at 5000 rpm for 20 minutes. The bacterial pellet was washed twice with distilled water and finally suspended in 0.01M PBS, pH 7.2 containing 1mM EDTA as a chelating agent and 3mM PMSF as a proteolytic enzyme inhibitor. The bacterial suspension was sonicated for 30 minutes using a micro-tip Virtis sonicator. The sonicate was then centrifuged at 20,000 rpm for 60 minutes at 4°C. The supernatant was aspirated and dialysed against 0.01M PBS for 24 hours and then lyophilized to a minimum volume. The protein content of the sonicate was estimated by Lowry's method (Lowry et al. 1951) and the antigen stored in aliquots at -20°C.

M. tuberculosis H37Rv sonicate antigen was prepared in a similar manner as described above.

M. leprae sonicate was obtained from Dr. R.J.W. Rees, IMMLEP, M. leprae Bank, WHO.

Enzyme linked immunosorbent assay (ELISA): 96 well flat-bottom polyvinyl Titertek plates were coated with antigen at a volume of 50 ul per well in 0.05 M carbonate-bicarbonate buffer, pH 9.5. The plates were incubated overnight at 37°C, washed, blocked with 150 ul of 3% (w/v) bovine serum albumin (BSA)-fraction V (Spectrochem, India) for 1 hr at 37°C. Plates were then washed, human sera diluted in 1% PBS-BSA
was added and incubated for 1 hour at 37°C. After washing, the plates were re-incubated for 1 hr at 37°C with rabbit anti-human immunoglobulins (Ig or IgG or IgM) conjugated to horse radish peroxidase (HRPO) (Dakopatts, Copenhagen, Denmark). Color was developed using substrate made up of 0.5 mg orthophenylene diamine per ml in 0.15 M citrate phosphate buffer, pH 5.0 and 1 ul per ml of 30% H₂O₂. Reaction was stopped with 5N H₂SO₄. The absorbance was read at 490nm using a microplate autoreader (EL 309 Bio-tek Instruments). Antibody titres are expressed as the average OD reading of duplicate wells substracted from the mean OD of blanks consisting of OD of serum and conjugate alone.

EIA protocol followed for monkey nerve sonicate was similar to that described above, except the plates were coated with 100 ul of 50 ug/ml protein. Washing of the plates was done with PBS and revealing antibody used was HRP conjugated to anti-human IgG diluted 1:1000 in PBS-BSA.

To detect antibodies directed to ND-BSA, the synthetic phenolic glycolipid, ELISA was performed according to the method described by Cho et al. (1983). The protocol followed was as follows. Antigen was coated onto 96-well Titertek plates at a protein concentration of 20 ng per ml. The plates were incubated overnight at 37°C. Non-specific binding sites were saturated with 100 ul per well of 3% BSA in PBS (PBS-BSA) and washed. Sera diluted at 1:300 in 1% PBS-BSA were added to wells and incubated for 1 hour at 37°C. After washing with PBS containing Tween-20 (PBS-T), 100 ul of HRP
coupled to rabbit anti-human IgM diluted 1:1000 in PBS-BSA were added to each well and incubated for 1 hour at 37°C. The plate was washed thoroughly and color was developed by adding 100 ul/well of freshly prepared substrate (0.5 mg OPD/ml of 0.15M citrate phosphate buffer, pH 5.0 and 0.03% H₂O₂). The reaction was stopped by adding 50 ul/well of 5N H₂SO₄. Color intensity was read at 490 nm using EIA reader (EL 309, Biotek Instruments).

EIA to detect antibodies to M.w sonicate antigen was performed in a similar manner as above, except the antigen concentration used was 20 ug protein/ml of PBS and 1:400 sera dilution. The revealing antibody used was HRP coupled to rabbit anti-human Ig diluted 1:1000 in 1% PBS-BSA.

EIA protocol used for detecting cross-reactivity between M.tuberculosis H37Rv with MBP was similar to that of M.w sonicate antigen.

Selection of leprosy patients: Leprosy patients attending leprosy clinics in major hospitals of Delhi were the subjects of this study. They were classified according to Ridley-Jopling scale. The clinical diagnosis was confirmed by histopathological observations and bacteriological findings. Subjects apparently healthy and living in Delhi, a non endemic area served as controls.

Selection of Primates: Adult Rhesus monkeys of both sexes were used for this study. The monkeys were quarantined for 3 months to rule out existence of any infection, more
specifically tuberculosis by repeated negative finding in tuberculin tests and chest X-rays.

Inoculation with M. leprae: Each monkey was inoculated with $9.5 \times 10^7$ mycobacteria given intravenously in the forearm and $0.5 \times 10^7$ mycobacteria given subcutaneously at an adjacent site. These animals were also tested for lepromin reactivity. The inoculation protocol and the lepromin status of all the monkeys are shown in Table II.

Collection of sera: 10 ml of blood was collected from each monkey at an interval of 3 months. Blood was allowed to clot for 2 hours at $37^\circ$C following which it was centrifuged at 1800 rpm for 10 minutes. After centrifugation, serum was aliquoted into sterile vials containing 0.01% merthiolate and frozen at $-70^\circ$C till use.

During this period, the monkeys were examined for the appearance of lesions for any overt signs and symptoms of the disease. Slit-skin smears were taken from six different sites (ear lobes, eye brows and finger tips) and examined for the presence of mycobacteria.

Indirect immunofluorescence (IIF) assay: Bacterial smears were made on clean grease-free glass slides and allowed to air dry. The slides were placed in a humidified chamber and incubated with anti-neural antibodies diluted 1:50 in 0.01M PBS, pH 7.2, for 30 minutes at room temperature (RT). The slides were then washed in 0.01M PBS to remove unbound antibodies. After thorough washing, swine anti-rabbit Ig
### Table II Animal inoculation data

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Sex</th>
<th>Lepromin status*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMU-4</td>
<td>F</td>
<td>2 mm</td>
</tr>
<tr>
<td>MMU-5</td>
<td>F</td>
<td>6 mm</td>
</tr>
<tr>
<td>MMU-6</td>
<td>F</td>
<td>8 mm</td>
</tr>
<tr>
<td>MMU-7</td>
<td>F</td>
<td>5 mm</td>
</tr>
<tr>
<td>MMU-8</td>
<td>F</td>
<td>6 mm</td>
</tr>
<tr>
<td>MMU-12</td>
<td>F</td>
<td>8 mm</td>
</tr>
<tr>
<td>MMU-15</td>
<td>M</td>
<td>8 mm</td>
</tr>
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<td>MMU-18</td>
<td>M</td>
<td>5 mm</td>
</tr>
<tr>
<td>MMU-19</td>
<td>M</td>
<td>5 mm</td>
</tr>
</tbody>
</table>

Animals were inoculated with *M. leprae* isolated from armadillo spleen given $9.5 \times 10^7$ by i.v. route through anti-brachial vein of the forearm and $0.5 \times 10^7$ subcutaneously in the adjacent region. *Lepromin* status of the monkeys 6 months post-inoculation.
conjugated to FITC (fluorescein isothiocyanate) diluted 1:40 were added and the slides further incubated for 30 minutes at RT. The conjugate was then washed off and the smears were fixed in 4% formal-saline for 10 minutes at 4°C. The slides were again washed in PBS and then mounted using clean coverslips with glycerol-PBS containing 10⁻⁴ M paraphenylene diamine and sealed with nail varnish. The slides were viewed under x100 objective of Nikon microscope equipped with epifluorescence.

Results

Enzyme immunoassay (EIA): Using the human nerve sonicate antigen we screened leprosy sera belonging to all categories of the disease. To determine the optimal antigen concentration and sera dilution to be used, checker board titrations were carried out. For this standard EIA protocol as described in Methods was followed.

Sera of 10 lepromatous leprosy (LL) patients and 10 normal healthy individuals were mixed to generate two pools of sera, one of LL and the other of normal controls. For determining optimum antigen concentration, reactivity of pooled LL and normal sera at a dilution of 1:400 was studied with different concentrations of nerve antigen. The results are given in Fig. 2a. Whole Ig coupled to HRP was used as revealing antibody. The reactivity was best evident at antigen concentration of 5μg/well. At this concentration of the antigen there was minimal reactivity with the normal serum.
Fig. 2. Presents checker board determination of optimum concentration of nerve sonicate antigen and leprosy sera carried out in a microtitre plate ELISA.

a. Reactivity of pooled LL and normal sera was checked with different antigen concentrations. An antigen concentration of 5 ug/50ul/well gave optimum reactivity with pooled LL sera and minimal reactivity with the pooled normal sera.

b. Using standard antigen concentration of 5 ug/well, varying dilutions of pooled leprosy sera were tested. 1:400 sera dilution was found to give optimum reactivity with the antigen.
To determine the sera dilution to be used, varying dilutions of pooled LL and normal sera were used at an antigen concentration of 5 ug/well. The results are presented in Fig. 2b. Optimum sera dilution was found to be 1:400, since at this dilution there was minimal reactivity with the normal serum.

Using antigen concentration of 5ug/well and sera dilution of 1:400, leprosy sera belonging to all categories were screened, with normal sera as controls. Results are presented in Fig. 3 which gives the scattergram of antineural antibodies in terms of the O.D. values in 258 sera of leprosy patients. Out of these 68 were LL, 35 BL, 34 BB, 43 BT, 10 TT and 8 neuritic leprosy respectively. In all these six categories the O.D. values of antineural antibodies ranged between 0.6 and 2.0, as against the O.D. values of 0.05 and 0.1 of the apparently healthy normal individuals. Note the clustering of antibody titres around the O.D. values of 0.78 to 1.0 and 1.8 to 2.0. The dotted line in the figure denotes Mean ± 2 S.D. of the normals. These results were seen using anti-human Ig coupled to HRP as the revealing antibody. The results are expressed as the average of duplicate wells after subtracting the difference from the mean of blanks consisting of wells coated with serum and conjugate alone. Thus 5ug/50 ul/well concentration of antigen and 1:400 sera dilutions was found to be optimum and used in the subsequent assays. Thirty-five leprosy sera, seven each belonging to LL, BL, BB, BT and TT and seven
Fig. 3. Presents the serum antibody levels to the human nerve sonicate antigen(s). The O.D. values are plotted on the Y axis, while the X axis gives the category of leprosy. The number on top indicates the number of patient sera in each category of leprosy. Each dot represents the antibody titre of an individual patient. Note that the antibody levels in leprosy are significantly higher than those in normal individuals. The dotted line represents the mean + 2 S.D. antibody levels of normal individuals.

N = neuritic leprosy.
normal sera were screened for antineural antibodies with peroxidase conjugated to the whole anti-human immunoglobulins (Ig), IgG or IgM, in order to identify the class of immunoglobulins. Fig. 4 presents the data. The anti-IgG antibody titres were high in the range of 1.4 to 2.7 (O.D. values) in all the 35 leprosy sera tested as against 0.1 to 0.14 O.D. values for the normal sera. IgM type of antibodies were detected in low titres (O.D. values 0.14 to 0.67) in 5 LL, 5BL, 3BB, 4BT and 5TT from a total of 35. Since the titres of IgG class of antibodies were significantly high the specificity of the binding of antibodies was tested using peroxidase conjugated to whole IgG and IgG-F(ab)₂. Fig. 5 presents the results on ten pooled LL sera. It can be observed that binding of IgG-F(ab)₂ to the antineural antibody was comparable to IgG. These antibodies were specifically binding to epitopes present on the antigen(s) and were not mediated non-specifically by binding to the Fc region.

Ontogenesis of antineural and antimycobacterial antibodies: The genesis of antibodies directed to neural antigens and mycobacterial antigens at different time points following inoculation with M. leprae upto a period of 15 months was done. Table III presents the status of the rhesus monkeys at different time points when blood was withdrawn to measure antibody levels. It can be observed that the 9 monkeys in the study had not yet developed experimental leprosy, but showed
Fig. 4. Presents the levels of antineural antibodies when measured as belonging to whole immunoglobulins (Ig), IgG class and IgM class.
Fig. 5 Presents the mean O.D. values of three sets of experiments. Antineural antibodies were measured in a pool of 10 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 = 0.73 and 0.70 respectively).
Δ O.D. at 490 nm

Sera dilutions

100 200 300 800 1600 3200 6400
Table III: Signs and symptoms of nerve damage in monkeys fifteen months after infection with M. leprae

<table>
<thead>
<tr>
<th></th>
<th>Dryness</th>
<th>Scaling</th>
<th>Resorption* of terminal phalanges</th>
<th>Deformities**</th>
<th>Ulceration***</th>
</tr>
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<tbody>
<tr>
<td>MMU-4</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
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</tr>
<tr>
<td>MMU-5</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MMU-6</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
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<td>MMU-7</td>
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<td>+++</td>
<td>-</td>
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<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MMU-12</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>MMU-15</td>
<td>++</td>
<td>++</td>
<td>++(wrist drop)</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>MMU-18</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-</td>
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<tr>
<td>MMU-19</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
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</table>

* Resorption of terminal phalanges of upper limbs seen in eight monkeys and that of lower limbs in three monkeys.

** Deformities in the upper limb more severe than lower limb.

*** Ulceration was more frequently seen within 3-6 months of infection.
early signs of peripheral nerve damage. The skin smears were still bacillary negative at the end of one year.

Fig. 6 presents the comparative levels of antibodies, directed to the three antigens tested. Panel 1 presents the antibody levels directed to monkey nerve (MN) sonicate. The antibody titres were increased in 4 of the 9 monkeys within 3 months. Levels increased further in these animals after 6 months of inoculation, while after 9 months high levels of antibodies were recorded in all the 9 monkeys. The O.D values ranged between 0.7 and 1.0. This level fluctuated a little on the 12th month, but was still significantly high. Levels remained high on the 15th month post inoculation in all the monkeys.

The antibodies directed to ND-BSA (natural disaccharide of M. leprae conjugated to BSA) was detected in 4 monkeys throughout the period. In the other 5 monkeys low anti-PGL antibodies were detected, the titres of antibodies as compared to that for monkey nerve sonicate being lower. It still showed the trend of gradual increase.

Antibodies to M.w cross-reactive mycobacterial antigen was detected within 3 months and the level was significant in 6 out of 9 monkeys after 6 months, 8 out of 9 monkeys after 9 months and all 9 monkeys within one year. On 15 months, the antibody titres decreased in 7 monkeys and in the other two continued to increase.

Cross-reactivity: The basis for the generation of antibodies to M. leprae in leprosy was tested by examining the possible
Fig. 6. Ontogenesis of antineural and antimycobacterial antibodies in rhesus monkeys, following inoculation with \textit{M. leprae}. The antineural antibodies showed a steady increase all through the 15 months, while antibodies to ND-BSA and \textit{M.w} antigens varied with each of the monkeys. MN-monkey nerve sonicate; ND-BSA - Natural disaccharide antigen of \textit{M. leprae} conjugated to BSA; \textit{M.w} - cross-reactive mycobacterial antigen.
existence of cross-reactivity between mycobacterial and neural antigens. Using IIF assay, a panel of seven mycobacteria viz. *M. avium*, *M. scrofulaceum*, *M. vaccae*, *M. smegmatis*, *M. w*, *M. tuberculosis* H37Rv and *M. leprae* were stained using antibodies to known neural proteins. The antineural antibodies used were anti-MBP, anti-S100 and anti-GFAP (polyclonal) and anti-neurofilament, anti-vimentin (monoclonal). Table IV presents the data. All the mycobacteria stained positive with MBP and S100 antibodies and were negative with the remaining antibodies. Amongst the mycobacteria, *M. leprae* and H37Rv were strongly positive showing bright green fluorescence under UV light. Fig.7 illustrates bright fluorescence of H37Rv stained with anti-MBP antibodies.

In the next series of experiments, the reactivity between neural antibodies and mycobacterial antigens was quantitated in a microtitre plate ELISA system. In an ELISA using H37Rv sonicate and anti-MBP antibodies, a checkerboard assay was carried out to determine the optimum antigen and antibody dilutions. Fig.8 a & b gives the results of the titrations. An antigen concentration of 250ng and antibody dilution of 1:200 was found to be optimum. Using these antigen concentration and antibody dilution, absorption studies were carried out, to determine the specificity of the cross-reactive antigen-antibody reaction. Fig. 9 gives the results. Absorption experiments were carried out using H37Rv sonicate and anti-MBP antibodies. Absorption was done using 300 ng of H37Rv sonicate antigen with 1:500 of anti-MBP antibody raised.
**Table IV**: IIF assay with various mycobacterial strains and antineural antibodies

<table>
<thead>
<tr>
<th></th>
<th>MBP</th>
<th>S 100</th>
<th>GFAP</th>
<th>Neurofilament</th>
<th>Vimentin</th>
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<tbody>
<tr>
<td><em>M. avium</em></td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td><em>M. scrofulaceum</em></td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>M. Vaccae</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. tuberculosis H$_{37}$Rv</em></td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>M. leprae</em></td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>M. w.</em></td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>M. smegmatis</em></td>
<td>++</td>
<td>++</td>
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</table>

*Results are mean of 4 separate experiments*
Fig. 7. Indirect immunofluorescence study using anti-MBP antibody on a smear of \textit{M. tuberculosis} H37Rv. Note bacilli fluorescing bright green.
Fig. 8. Presents the checkerboard determination of optimum concentration of *M. tuberculosis* H37Rv sonicate antigen and anti-MBP antibodies (polyclonal sera) carried out in a microtitre plate ELISA.

a. Reactivity of anti-MBP antibodies was checked with different H37Rv antigen concentrations. Note the steady increase of the reactivity with increasing antigen concentration.

b. Using an antigen concentration of 250ng varying anti-MBP antibody dilutions were tested. 1:200 antibody dilution was found to be the optimum.
Fig. 9. Percent neutralization of the reactivity of anti-MBP antibodies (polyclonal sera) (1:500) with H37Rv sonicate (300 ng) checked with varying concentration of H37Rv sonicate antigen.
Onti-MBP (H1:500) tar 37Rv (300ng)

% Decrease

(ng/ml)

0 10 20 30 40 50 60 70

1000 500 250 125 62.5
in rabbit. The absorption was carried out for 2 hrs at 37°C, following which the antigen-antibody mixture was centrifuged at 4000 rpm for 5 minutes. The supernatant was then used to check reactivity with various concentrations of H37Rv sonicate antigen. The reactivity was considerably reduced, as can be observed from the values following absorption with H37Rv and anti-MBP suggesting that the cross-reactivity observed both by IIF and EIA was specific.

We also obtained recombinant mycobacterial proteins from WHO viz. M.tuberculosis (38 kDa); M.leprae (18 kDa; 65 kDa). In an ELISA using anti-MBP monoclonal antibodies we could observe moderate reactivity with M.tuberculosis-38K while there was very low reactivity seen with the M.leprae proteins. Table V presents the data.

Discussion

Nerve damage is the cardinal sign of leprosy, and is also the basis of the classical limb deformities associated with this disease. It is therefore vital to understand the mechanisms responsible for the pathogenesis of lepromatous neuritis. The clinical and histopathological evidences suggest the role of multiple mechanisms and an early interaction between the immune cells and the nerve cells may be playing a role. However, the exact role of the immune system remains to be defined. Recently there have been reports that the myelin-synthesizing cells, Schwann cells, express HLA Class I and Class II molecules and are capable of
**TABLE V:** ELISA of recombinant mycobacterial proteins with anti-MBP monoclonal antibody

<table>
<thead>
<tr>
<th></th>
<th>M. tuberculosis</th>
<th>M. leprae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>38 kDa</td>
<td>65 kDa</td>
</tr>
<tr>
<td></td>
<td>18 kDa</td>
<td></td>
</tr>
<tr>
<td>% Inhibition</td>
<td>0.634 ± 0.113*</td>
<td>0.301 ± 0.010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.121 ± 0.0</td>
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</table>

* Mean of 5 sets of experiments

+ anti-MBP monoclonal antibody used at 20μg/ml concentration
presenting mycobacterial as well as auto antigens (Wekerle et al. 1987).

Earlier studies have reported the presence of antineural antibodies in less than 50% of leprosy patients (Wright et al. 1975; Eustis-Turf et al. 1986; Benjamins et al. 1989). The techniques used in these studies and our study differ. We have used a sensitive microplate ELISA method using semi-purified nerve antigen, while in the other studies, the sera were probed mostly with the sections of the spinal cord, a component of the central nervous system, in an indirect immunofluorescence system and sections of the peripheral sensory nerves. Nerve damage in leprosy is limited to the peripheral nerves and does not proceed beyond the dorsal root ganglion. It is quite possible that all antigenic determinants present in the sensory nerve are not present in the spinal cord.

The high frequency and high titres of the antibodies observed in our study suggest that there is a good humoral immune response directed to peripheral nerves in this disease. The isotype of these antibodies were mainly of the IgG class. IgM type of antibodies were detected in lesser percentage of patients as compared to IgG type. These antibodies were specifically binding to epitopes present in the antigens coated on the plate and were not mediated non-specifically by binding to the Fc region as is illustrated by the experiments in which the conjugates linked to whole IgG and F(ab)_2 fractions were compared. The antibody titres were significantly high as compared to the levels in apparently
normal healthy individuals. We could not see any measurable levels of antibodies in a total of 60 normal healthy sera tested by us, as compared to the earlier reports (Wright et al. 1975; Eustis-Turf et al. 1986; Benjamins et al. 1989). The difference in these studies could be attributed to the sera dilutions. We used sera at dilutions of 1:400 in our study, while in the other studies, dilutions of 1:10 and 1:20 have been employed.

There are sufficient studies reporting that monkeys—African-green, mangabey and rhesus, develop leprosy on inoculation with \textit{M.leprae}. Further, some of these monkeys developed signs and symptoms of nerve damage (Waters et al. 1978; Wolf et al. 1983; Samuel et al. 1984; Martin et al. 1984). Subsequently there were reports that African green monkeys inoculated intravenously and intracutaneously with \textit{M.leprae} developed active leprosy infection in peripheral nerve with extensive inflammation and with polyneuritic features (Baskin et al. 1987).

This study monitored the kinetics of antibodies developed against an antigen unique to \textit{M.leprae} (synthetic PGL-I), and a cross-reactive mycobacterial strain \textit{M.w} (Mustafa, 1988; Ganju et al. 1990). It also monitored the ontogenesis of antibodies directed to peripheral nerves. The results show that antibodies to nerve antigens and cross-reactive mycobacterial antigen appeared early and lasted much longer than the antibodies directed to synthetic PGL-I. This pattern of antibody response in the animals could be due to
the humoral immune response to inoculated *M. leprae* and clinical manifestation of the disease. These monkeys would have to be monitored for longer periods to see if any of them would eventually develop experimental leprosy, and if so the type of the disease they develop i.e. whether LL, BL, BB, BT or TT. However, what was interesting was the earliest clinical sign of nerve changes seen in the monkeys. The lack of high titre of antibodies to anti-PGL can be explained, as several workers have reported that the antibody titres correlate with the bacteriological index (BI) status. Significant levels of antibodies are observed only in subjects with high BI, while the antibody titres to cross-reactive antigen, *M. w*, does not show any such correlation (Moudgil et al. 1989). The basis for the generation of antineural autoantibodies directed to peripheral nerves will have to be studied and this model provides a good system for undertaking such a study.

The antineural antibodies observed in our study could be quite specific to the peripheral nerve pathogenesis and could not be a consequence of generalized autoimmune response. Several studies have been carried out measuring the immunoglobulins in leprosy. The findings of the various investigators have been summarized by Rawlinson et al. (1987). Hyperglobulinemia is reported to be present in certain percentage of the lepromatous type but not the tuberculoid type of the disease (Malaviya et al. 1972). There have also been reports of raised levels of antibodies to rheumatoid factor, thyroglobulin and nuclear material.
The frequency of these antibodies too are low. Clinical significance and pathogenic role, if any, of these antineural antibodies remains to be determined. Antibodies directed against MBP, cerebrosides, gangliosides and myelin associated glycoprotein has been reported in some other neuropathies such as encephalomyelitis and polyneuritis (Ruutiainen et al. 1982; Wajgt and Gorny, 1983). Moreover, very few leprosy patients clinically manifest any other symptoms of autoimmune disorder excepting nerve damage, while nerve damage of varying degree and extent are present in all the forms of leprosy. Such high frequency of antibodies associated with varying pathology of nerves, loaded with \textit{M. leprae} in the LL to well formed localized granulomas in TT cannot explain the basis. One can speculate that such high frequency of antibodies may be due to some cross-reactivity between nerve antigens and \textit{M. leprae} antigens. Recent work relating to mycobacterial antigens suggests that many of these antigens may share a common structural sequence with human antigens, specifically the heat shock and other stress proteins (Young, 1988; Young et al. 1988). From our present study we could infer that such a phenomenon could be taking place. In preliminary experiments using IIF and ELISA, we could detect presence of cross-reactive antigenic determinants between the mycobacteria and neural proteins.