CHAPTER 6

FUNCTIONAL CHARACTERIZATION OF ANTINEURAL ANTIBODIES
Introduction

There have been reports of high levels of immunoglobulins and circulating autoantibodies in leprosy (Malaviya et al. 1972; Shwe, 1972; Rawlinson et al. 1987). IgG, IgA and IgM isotypes of immunoglobulins have been reported to increase in lepromatous form, but much less frequently in tuberculoid form of leprosy. There have also been reports of raised levels of anti-neural antibodies in leprosy sera (Wright et al. 1975; Eustis-Turf et al. 1986). The functional role of these circulating antineural antibodies have yet to be determined.

The peripheral nervous system, although often referred to as an immunologically privileged site, can be the target of immune-mediated tissue injury. Systemic immune sensitization with myelin antigens in humans can cause acute disseminated encephalomyelitis, in animals it can result in acute or chronic relapsing form of experimental allergic encephalomyelitis (Saida et al. 1972).

Leprosy, a disease caused by Mycobacterium leprae, involves mainly the peripheral sensory nerves. The diffuse destruction of the nerves is not always associated with accumulation of mononuclear cells, and if and when associated, the disease is not limited to the area of infiltration (Weddell et al. 1964). These observations suggest the role of circulating factors such as antibodies. This study suggests a role for antibodies, more specifically, antibodies directed against the peripheral nerve components.
Methods

Schwann cell culture: Sciatic and brachial nerves from 3-4 day old inbred BALB/c mice were used to prepare dissociated Schwann cell cultures. The nerves were dissected in sterile conditions under a dissection microscope (Nikon, Japan). Funicles of nerves were collected into a petridish containing DMEM, chopped into fine pieces and treated briefly with 0.25% trypsin (Difco, U.S.A). The enzyme treatment was stopped using foetal calf serum (FCS), and minced pieces were then mechanically disrupted by passing them through 21G and 23G hypodermic needles twice each, to obtain a single cell suspension. The cells were plated out on collagen coated coverslips. Collagen was prepared by the method described by Bornstein, 1958. The cultures were fed with 10% feeding medium (DMEM containing 10% FCS) on alternate days and incubated at 37°C in presence of 5% CO₂ and 100% humidity.

Sera collection: The protocol followed was similar to that described in Chapter 4.

Precipitation of whole immunoglobulins: Pooled sera from 10 LL, TT and 10 normal subjects were precipitated with 50% saturated ammonium sulphate solution in order to enrich the immunoglobulins (Ig). The precipitate was dissolved in PBS and dialysed extensively against PBS.

Indirect immunofluorescence assay: 5 to 7 day old Schwann cell cultures were used for the assay. The coverslips were
rinsed in DMEM containing 5% FCS (DMEM-FCS), and then incubated with normal rabbit serum diluted 1:10 for 30 minutes at 37°C, to block the non-specific sites. The cultures were then washed by immersing the coverslips in DMEM-FCS. Appropriately diluted leprosy/normal sera were then added on to the coverslips and cultures were further incubated for 30 minutes at 37°C in a humidified chamber. After thorough washing in DMEM-FCS, rabbit anti-human Ig conjugated to FITC diluted 1:40 was added to the cultures and the coverslips were incubated for 30 minutes at 37°C. The conjugate was then washed off and coverslips fixed in 4% formal saline for 10 minutes at 4°C. The coverslips were then washed and mounted with glycerol-PBS containing 10^{-4}M paraphenylenediamine and sealed with nail varnish. The cultures were viewed under X100 objective of Nikon microscope equipped with epifluorescence.

**Cell Lines:** The 33B Schwannoma cell line and Vero cell lines maintained routinely in the laboratory were used.

**Cytotoxicity assay:** To study the functional role of the antineural antibodies cytotoxicity assays were set up. Five to seven day old Schwann cell cultures or 33B Schwannoma cells were used for the assay. Schwann cell cultures were incubated with varying dilutions of leprosy/normal sera, and incubated for 1 hour at 37°C. The cultures were then washed with PBS, and then incubated with rabbit complement (Pel-Freeze, USA) for 2 hours at 37°C.
After this the cultures were again washed thoroughly with PBS and processed either for light microscopy (LM) or SEM. For LM a drop of 5% Eosin was added and the cultures observed under the inverted microscope (Nikon, Japan) for cytotoxicity. Appropriate controls, such as cultures with sera alone, complement alone or Eosin alone were included. For SEM, the cultures were processed according to the protocol described as follows.

Scanning Electron Microscopy (SEM): Schwann cell cultures after incubation with leprosy/normal sera and then complement were washed with PBS and fixed for 2 hours in 2.5% glutaraldehyde diluted in 0.1M Sodium cacodylate, pH 7.4. The coverslips were washed with cacodylate buffer and left in the buffer at 4°C overnight. Cultures were then dehydrated through ethanol grades and were immersed in 100% Amylacetate for 15 minutes. The coverslips were then dried in a critical point drier, coated with gold palladium and scanned under the JEOL 35 CF scanning electron microscope.

For quantitative estimation of cytotoxicity, the protocol described by Kull and Cuatrecasas (1981) and Parish and Mullbacher (1983) was followed with slight modifications. Briefly, $7 \times 10^4$ cells (33B/Vero) per well, were plated in a round bottom 96 well plate. After 3 hours the non-adherent cells were removed, whole immunoglobulins isolated from leprosy or normal sera were added at 1:25, 1:50 and 1:100 dilution and incubated for 1 hour at 37°C. The cells were washed and incubated with rabbit complement for 2 hours at
37°C. Cells were again washed and then incubated with warm 0.036% neutral red in MEM for 30 minutes at 37°C. The dye taken up by the viable cells was released by lysing them with a mixture of 0.05M acetic acid and 0.5% SDS. Optical density was measured by the ELISA reader (EL309, Bio-tek instruments) at 540 nm. In some experiments, the leprosy immunoglobulins were absorbed with whole nerve sonicate (NA), protein fraction and lipid fraction of NA. Immunoglobulins diluted 1:100 in PBS were absorbed with either NA (10 ug) or protein (200 ng) or lipid (1 ug), and incubated for an hour at 37°C. These were centrifuged at 4000 rpm and the supernatant was used for the cytotoxicity assay. In parallel set of experiments, the cytotoxicity assay was carried out with Vero cell cultures.

Results
Murine Schwann cells in culture appears as bipolar spindle shaped cells. These cells were recognised in culture using the morphological criteria (Bunge et al.1967; Brockes et al. 1979) and their ability to bind to S100 protein. Five to seven days old cultures (when they attained semi-confluence) were used to investigate the binding of leprosy sera. Fig. 12 illustrate the observations made in one such experiment. The sera bound to the Schwann cell surface and the Schwann cells exhibited bright green fluorescence. Binding experiments were carried out with pooled sera of ten patients in each category. Subsequently individual sera of
Fig. 12. Shows an indirect immunofluorescence of murine Schwann cells reacting to TT sera. Schwann cells fluoresce bright green showing positive reaction following addition of sera followed by anti-human Ig conjugated to FITC.
these patients were also tried. All the sera tested were positive in the assay. At 1:50 dilutions the normal sera did not bind to the Schwann cells and there was no evidence of autofluorescence. The binding pattern of leprosy sera varied depending upon the point in the spectrum. The pattern of fluorescence in LL was punctuated and formed a border or outline while in BT/TT the whole cell fluoresced (Fig. 12). Similar experiments were carried out on teased nerve fibres. One centimeter long nerve pieces were teased out on glass slides, and indirect immunofluorescence assay was carried out as described earlier. The sera bound to the inner lip of myelin membrane, node of Ranvier and to the nerve fibres. In the next series of experiments, after the treatment of sera, cells were treated with rabbit complement to study the antibody dependent complement mediated cytotoxicity. Addition of complement in presence of leprosy sera induced cytotoxicity. Cells were observed under the light microscope and also under the Scanning Electron microscope to check for cytotoxicity which was evidenced by detachment of cells.

Under the light microscope, cytotoxicity was evidenced by rounding of the bipolar Schwann cells. TT sera was found to be most toxic to the cells as compared to LL sera. The assay was repeated four times and degree of cytotoxicity was determined by quantitating the number of rounded cells under the x100 oil immersion objective. A total of 200 cells were counted and the number of rounded cells on addition of TT sera was 189.25 ± 6.5; BT- 179 ± 14.6; BB- 193.25 ± 3.94; BL-
146.25 ± 12.5 and LL- 133 ± 4.76. No such effect was seen with sera alone or complement alone. Normal sera did not produce any cytotoxicity following addition of complement. Under SEM, the cytotoxic cells appeared punched out and rounded, while normal cells looked healthy, had a smooth surface and were bipolar (Fig. 13 a & b). Table X presents the results of neutral red uptake of 33B Schwannoma cells treated with whole Ig isolated from 10 pooled normal, LL and TT sera in presence of complement. Addition of leprosy Ig (both LL and TT) with complement reduced the neutral red uptake of cells significantly, indicating reduction in the number of viable cells. Prior absorption of the leprosy immunoglobulins (Ig) with whole nerve sonicate, protein fraction and lipid fraction significantly reduced complement mediated cytotoxic effect. No such cytotoxic effect of leprosy Ig + complement were observed on Vero cells, a non glial cell line.

Discussion
The raised levels of antineural antibody could be of functional significance as is indicated in the present study. It is well established that the Schwann cells are the target cell of M. leprae. In the lepromatous end of the spectrum, Schwann cells are found loaded with M. leprae while in the tuberculoid end of the spectrum there is infiltration with mononuclear cells (Weddell et al. 1964; Antia 1982). Ultrastructural studies have revealed reduction in the number
Fig. 13 a&b: SEM micrographs of Schwann cells following antibody dependent complement mediated cytotoxicity assay.  
a) Normal Schwann cell, smooth surface and processes.  
b) Cells after treatment with TT sera and complement, cells have rounded off and have totally detached themselves from the substratum.  
Note the punched out appearance of the cell (arrow).  

### Table X

Lysis of Schwannoma cells induced by immunoglobulins from leprosy patients in presence of complement

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein fraction</th>
<th>Absorption with lipid fraction</th>
<th>whole NA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O.D ± S.D % lysis</td>
<td>O.D ± S.D % lysis</td>
<td>O.D ± S.D % lysis</td>
</tr>
<tr>
<td>Control (Neutral red alone)</td>
<td>0.4786 ± 0.012</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Complement alone</td>
<td>0.478 ± 0.0113</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TT-Ig+Compl.</td>
<td>1:25</td>
<td>0.206±0.0.32 57.5%</td>
<td>0.482±0.0068 13.1% 0.2706±0.0047 43.94%</td>
</tr>
<tr>
<td></td>
<td>1:50</td>
<td>0.189±0.012 61%</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>0.190±0.0059 60.7%</td>
<td>0.450±0.007 5.62%</td>
</tr>
<tr>
<td>LL-Ig+Compl.</td>
<td>1:25</td>
<td>0.247±0.056 48.8%</td>
<td>0.316±0.007 34.46% 0.2278±0.006 52.89%</td>
</tr>
<tr>
<td></td>
<td>1:50</td>
<td>0.247±0.07 48.87%</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>0.186±0.01 61.49%</td>
<td>0.317±0.005 34.17%</td>
</tr>
<tr>
<td>Nor-Ig+Compl.</td>
<td>1:25</td>
<td>0.329±0.029 31.1%</td>
<td>0.316±0.007 34.46% 0.2278±0.006 52.89%</td>
</tr>
<tr>
<td></td>
<td>1:50</td>
<td>0.347±0.057 27.98%</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>0.403±0.011 16.28%</td>
<td>0.368±0.088 23.59% 0.3638±0.0063 24.47% 0.3532±0.01 26.69%</td>
</tr>
</tbody>
</table>

Viability in terms of neutral red uptake

Results are expressed as mean of 5 separate experiments, each carried out in triplicates.

10 sera with high antineural antibody titres 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 sulphate solution. The precipitate was dialysed extensively against PBS and redissolved in PBS before use.

% lysis was calculated as C-T x 100% where C=mean O.D reading of cells treated with neutral red alone and complement alone.

T= mean O.D values of cells treated with Ig plus complement.

Immunoglobulins absorbed with NA were assayed for cytotoxicity at 1:100 dilution.
of Schwann cells and small unmyelinated fibres (Dastur, 1972). It may be postulated that the loss in these fibres as well as Schwann cells could be due to circulating antibodies. In the present study we observed binding of leprosy sera to the various components of the mouse peripheral nerves. The antibodies bound to the node of Ranvier, inner lip of myelin membrane and to the axons. The antibodies were also cytotoxic in nature as evidenced by our above studies. The cytotoxicity was antibody dependent and complement mediated. Sera alone had no cytotoxic effect to the cultured nerve cells. The antibodies bound and caused cytotoxicity specifically to the peripheral nerve cells, no such effect was seen with a non-gliaal cell line such as Vero cells. Our observations with neutral red uptake assay suggest that these antibodies may play a role in the complement-mediated lysis of the nerve cells.