Detection of Antibodies in Sera of Leprosy Patients and Contacts by Enzyme Linked Immunoabsorbent Assay (ELISA)

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Introduction

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*. Infection in the host induces a complex immune response involving both cellular immune reactions and antibody production. Immunological aspects of the disease have been studied extensively and in the low resistant form polar lepromatous leprosy the cell mediated immunity is lacking with presence of circulating antibodies against antigens shared by *M. leprae* and other mycobacterial species. In the high resistant form the tuberculoid type of leprosy is characterized by the development of adequate cell mediated immunity with infrequent occurrence of antibodies. When sensitive techniques are employed, antibodies could be demonstrated throughout the spectrum of leprosy(1). There is strong evidence to show that antibodies formed appear to have an adverse effect and are considered to be responsible for erythema nodosum leprosum(2,3) (Wemambu 1968, Moran, 1972).

Why are the antibodies of interest in leprosy? The study of antibodies in leprosy may help to understand the period between infection with *M. leprae* and the actual appearance of clinical disease.

This communication is concerned with use of enzyme-linked immunoabsorbent assay (ELISA) system for detection of (i) antibodies throughout the disease spectrum of leprosy and from groups of controls, i.e. normals, contacts, staff including tuberculosis patients(4); (ii) if so, whether specific antibodies could be detected with prior absorption with BCG; (iii) class specific antibodies such as IgG and IgM could be detected; and (iv) to compare the ELISA findings with FLA-ABS test(5).

Materials and methods

Serum samples: Serum samples of leprosy patients were obtained from patients attending Anandaban Leprosy Hospital. All patients were clinically and histologically classified according to Ridley-Jopling scale. Serum samples of tuberculosis patients were obtained from Kathmandu clinic. These were cases verified by specific culture and X-ray. Control sera were included from normal healthy Caucasian tourists. All samples were stored at $-20^\circ$C.
Antigens Source Dilution for use
1 M. leprae Armadillo derived, provided by Dr. R. J. W. Rees (AB22 1 mg/ml) 1 : 800
2 B. C. G. Suspensions (5% in PBS) Dr. M. Abe (Japan)
3 M. Vaccae II Dr. J. L. Stanford (2 mg/ml) 1 : 800

Enzyme Conjugates
Enzyme : Alkaline phosphatase sigma (Type VII)
Rabbit Anti-human
1 Immunoglobulin Dako 1 : 2000
tfractions (poly-specific)
2 IgG, specific for γ-chains II 1 : 4000
3 IgM, specific for μ-chains II 1 : 100

Note: The antisera had been raised without the use of complete freunds adjuvant, therefore, no anti-mycobacterial antibodies were present.

**Elisa assay**: The method used was that of Samuel et al(6). A diagramatic outline of this type of assay is given in Figure 1. The optimum concentration of the antigen to be used was obtained by means of chequer-board ELISA titration of antigen dilution against a reference positive and negative serum. A reference positive serum was prepared as a pool of sera from 20 lepromatous leprosy patients. A reference negative serum was obtained from a Caucasian who had never had tuberculosis. The highest dilution of antigen which gave the maximal difference between the reference sera was used, i.e. 1 : 800. After obtaining the optimum dilutions of antigen and serum to be used, the microplate ELISA was carried out as follows: (1) The wells of microtitre plates (Cooke M29AR) were passively sensitized for 3 hours at 4°C with 0.2 ml of M. leprae antigen diluted in carbonate/bicarbonate buffer (1.59 g Na$_2$ CO$_3$, 2.93 g NaHCO$_3$/litre, pH 9.6). (2) Plates were washed; this was done by emptying plates then flooding with phosphate buffered saline pH 7.4 0.15M+0.05% Tween 20. This procedure was repeated three times and finally plates were shaken dry. (3) 0.2 ml. of test serum, diluted in PBS Tween was added to separate wells of the plate and incubated for 2 hours at room temperature in a moist chamber. (4) Washing procedure was repeated as in (2). (5) 0.2 ml. of enzyme-labelled antibody freshly diluted in PBS Tween was added to each well and incubated overnight at 4°C. (6) Washing as in (2). (7) 0.2 ml. of enzyme substrate-p-nitrophenyl phosphate substrate (Sigma 104) solution was added to each well. (5 mg. tablet of substrate was dissolved in 5 ml. of 10% diethanolamine buffer). (8) The enzyme reaction was stopped by adding to each well 50 ml 3 M NaOH when reference positive reached an absorbance value of more than one. (9) The absorbance of the contents of each well was determined by reading absorbance at 400 nm in a spectrophotometer (Vitatron UPM).

**Enzyme-linked immunoabsorbent assay after absorption with BCG**: The assay for M. leprae specific antibodies after absorption with BCG sonicate was made as follows: Test serum was diluted 1 : 800, mixed with BCG sonicate 50 ml. and incubated for 10 minutes at 37°C.
The tubes were spun at 2000 rpm for 20 minutes and the supernatant of 200 ml was added to each well.

**Enzyme-linked immunoabsorbent assay-antibody class response to leprosy antigen:** One problem with IgM tests is that they may detect a rheumatoid factor IgM, which reacts with IgG bound to the solid phase, giving a false positive reaction. Thus all sera for study of IgM were filtered through a sephedex G200 column.

**Results:**
(1) A positive/negative value was determined as follows (Wolters, 1976):
\[
P/N = \frac{E_{\text{sample}} - E_{\text{blank}}}{E_{\text{negative}} - E_{\text{blank}}} 
\]

Fig. 1 The principle of the enzyme-labelled antiglobulin method for measuring antibody (indirect method)

A serum sample with P/N value of 2.1 was considered as significant. All values are given as mean values of double tests.

(2) Results of specific assays are expressed as percentage of antibody left after absorption.

**FLA-ABS test**: FLA-ABS test was carried out as described by Abe et al(5). The reagents were supplied by Dr. Abe.

**Results**

Figure 2 shows considerable antibody detected in the lepromatous leprosy patients. The antibody activity showed a striking variation from very high to low values. The antibody activity in BT/TT group of patients was less pronounced than that in the LL patients. The borderline lepromatous had higher antibody activity than the BT/TT patients. The control group had strikingly low antibody activity against *M. leprae*. Tuberculosis patients mean value was higher than that of BT/TT patients. The antibody activity among normal population of India, Bhutan and Nepal where leprosy is endemic is variable.

Figure 3 shows the striking difference of antibody levels between pulmonary and extra pul-
monary patients. The mean value of leprosy patients was lower than that of Tb patients. Therefore, it was concluded that the normal subjects from non-endemic countries had negligible antibody to *M. leprae* and *M. tuberculosis* antigens and that each antigen detects higher activity in patients with leprosy and tuberculosis respectively.

Figure 4 shows that the absorbed sera with BCG when tested against *M. tuberculosis* antigen gives values lower than the lower limit set for a positive reaction.

Figure 5 shows the percentage of antibody remaining after absorption with BCG sonicate in patients and control subjects. Except for two, all Nepaleese had no antibody activity to *M. leprae*. These were later skin tested with *Leprosin A*, and the test was negative at 48 hours. Similarly, the six staff members and three contacts of multibacillary patients showed high antibody activity to *M. leprae*. These were skin tested with *Leprosin A* and the result was negative. Additional clinical examination in all eleven subjects did not reveal any clinical signs of leprosy.

Figure 6 shows the striking findings of high IgM values in the lepromatous leprosy patients, and as one moves towards the BT/TT side of the spectrum the values decrease considerably. It is evident that high IgM values are noted in contacts of leprosy patients.
Fig. 4 To show leprosy sera contained specific antibody; after absorption with BCG. Sera were then tested against *M. leprae* and *M. tuberculosis*. Each point indicates an individual serum. The broken line shows the lower limit set for a positive reaction.

Fig. 5 Percentage of antibody activity remaining after absorption with BCG. Each point indicates an individual serum. The horizontal broken line shows the lower limit set for a positive reaction.

In Table 1, the results in both tests among indeterminate type of leprosy patients is equivocal. However among contacts the FLA-ABS test seems to pick up those negative in ELISA assay.

**Table 1** To Compare the Results of ELISA and FLA-ABS Test.

<table>
<thead>
<tr>
<th>Total Number of cases</th>
<th>Type</th>
<th>FIA-ABS</th>
<th>ELISA</th>
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<tbody>
<tr>
<td>94</td>
<td>Contacts</td>
<td>32/52 (61%)</td>
<td>6/20 (30%)</td>
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<tr>
<td></td>
<td>Indeterminate</td>
<td>20/42 (47%)</td>
<td>4/10 (40%)</td>
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**Discussion**

ELISA has proved to be a sensitive technique to be applied in the study of humoral responses of the diseases caused by Mycobacteria. The advantage that offers over other techniques is the sensitivity as well as the speed with which large numbers of samples could be analysed, a
feature applicable in epidemiological studies. The present study showed that with ELISA specific antibodies could be detected in sera of patients and in particular contacts of patients. This might be used as an epidemiological tool in the diagnosis of subclinical leprosy and in identifying persons probably incubating *M. leprae*.

The combined use of ELISA and FLA-ABS tests might help to delineate subjects on a priority basis for regular follow up as a "high risk" group in leprosy control programmes. This "high risk" group of individuals may be candidates for immunisation. Such immunological epidemiological studies are under progress.

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**References**