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Original Research Article

Leprosy: a brief study of pediatric leprosy patient in Gwalior region, India

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A B S T R A C T

Leprosy is one of the most ancient diseases known to man, leprosy may be defined as a chronic, infectious, granulomatous disease caused by acid-fast bacilli, *Mycobacterium leprae*. It is classified under order of actinomycetalis and family *Mycobacteriacae*. There are several characteristic antigens present in *Mycobacterium leprae.* About 20 antigens of *M. leprae* has been recognized by antibodies in the sera from leprosy patients. *Mycobacterium leprae* is the only species of *Mycobacteria* to infect peripheral nerves and specifically Schwann-cells. In the present study only 18% of leprosy children were found to be positive for this antibody where as only one healthy child was positive. In our study, the antibody response against 35KDa antigen in MB (multi bacillary) patients was found to be significantly more as compared to the PB (paucibacillary) patients and healthy controls.

Introduction

Mycobacterium leprae is a straight, rod shaped and about 1–8 µm long and 0.3 µm in diameter. It is non-spore forming bacillus with pointed, rounded club shaped ends. *M. leprae* divides by binary fission, it is gram positive and less strongly acid fast with carbol fuchsin. The acid-fastness of *M. leprae* is weaker than any other *Mycobacteria*. This acid fastness is due to presence of mycolic acid in the cell wall Barksdale and Kim (1977). When bacilli are numerous as LL (Lepromatous) cases, it is generally arranged in clumps, rounded masses (globi) or in group of bacilli side by side, its structure consists of a capsule, double layered cell wall, membrane and cytoplasm.

M. leprae has not been cultivated in any acceptable *in-vitro* culture media so far, identification depends on clinical characterization of disease and criteria other than those used routinely for cultivable *Mycobacteria*. *M. leprae* multiplies extensively in tissues of nine banded armadillo (*Dasypus noveminctus)* led the use of this animal model to produce large quantities of bacilli, which can be extracted from host, liver for purification and study (Draper, 1982). Cho *et al.*, (1983) described the stable phenolic glycolipid (PGL) and showed its specificity of *M. lepare. M. lepare* is the only species of *Mycobacteria* to infect peripheral nerves and specifically Schwann-cells (Hasting,

1994). Leprosy transmitted too many ways by contact, inhalation, ingest of Breast milk, through insects. So keeping in views the present studies were carried out in the following objectives:

1) Analysis of antibody response in paediatric group of leprosy patients.
2) Antibody response (IgG) against *M.leprae* soluble antigens in sera (MLSA).
3) Evaluation of IgG sub-classes (IgG1, IgG2, IgG3 and IgG4) against MLSA.
4) Evaluation of specific antibody response against 35 KDa antigens.
5) IgM response against Natural disaccharide-octyle-Bovine Serum Albumin antigen (ND-O-BSA)

Materials and Methods

Serum sample: Sera samples of paediatric leprosy patients stored in department of Immunology, GR Medical College were used. These sera samples had been collected from leprosy patients classified according to the classification laid by Indian Association of Leprologist (1982). Patients having less than five lesions were included in PB (paucibacillary) group and five or more than five lesions were included in MB (multi bacillary) group. A total of 40 patients sera were used, out of which 9 Tuberculoid (TT), 2 Borderline Tuberculoid (BT), 15 Borderline (BB), 11 Borderline Lepromatous (BL) and 3 are Lepromatous (LL) patients. 19 sera samples of healthy children were included; all these sera samples were from children below 14 years of age. 62 samples of adult patients were also used out of which 13 BT, 10 BTR, 21LL, 3BB, 4BL and 11 were healthy control.

Antigens

(1) *Mycobacterium leprae* soluble antigen (MLSA):- It was obtained from Dr. P.J. Brennan (Colorado State University, Fort Collins, Colorado USA; NIH contract no.I-AI-55262).

(2) Phenolic Glycolipid-1 (PGL-1) (*M. leprae* sonicated):- P.J. Brennan (Colorado State University, Fort Collins, Colorado, USA; NIH contract no. I-AI-55262).

ND-O-BSA (Natural di-saccharide-octyle-Bovine serum albumin), a synthetic sugar of PGL-1 *M. leprae*, was provided by Dr. Dlphi Chatterjee, Colorado State University, Fort Collins, Colorado, USA (contract no. I-AI-55262).

(3) 35 KD antigen (Welcome laboratory, UK)

Chemicals/Reagents

(1) For ELISA

- Carbonate bicarbonate buffer, pH 9.6 [Anhydrous sodium carbonate (Na_2CO_3). (AnalaR* Glaxo), Sodium bicarbonate ($NaHO_3$) (QUALIGENS)].
- Phosphate buffered saline (PBS) pH 7.4. [Monobasic sodium phosphate (NaH_2Po_4). (SISCO RESEARCH LABORATORIES) Dibasic sodium phosphate (Na_2HPO_4) (MERCK), Sodium Chloride (NaCl). (QUALIKEMS).].
- Bovine serum albumin (BSA), (SIGMA, USA)
- Tween-20, 0.05% (MERCK, INDIA)
- Antihuman IgG conjugated to Horse radish peroxidase (HRPO)
- Ortho-phenylene diamine dihydrochloride tablet (5 mg/10 ml distilled water). (SIGMA USA)

- 30% hydrogen peroxide (H_2O_2) (MERCK)
- 7% H_2SO_4 (QUALIGENS)
- Sodium chloride (Qualichem)

(2) For IgG sub classes against MLSA

Mouse Monaclonal against antihuman IgGI, IgG2, IgG3, IgG4. (SIGMA)

Equipments

- Centrifuge (COSTAR.).
- ELISA washer. (Tri Continent).
- ELISA reader (Titer-tek Multiscan plus^R UK)

Plasticwares

- Eppendorf tubes.
- Polystyrene micro titer plate. (Nunc-Denmark).
- Pipette of 1000 µl, 200 µl, 100 µl, 10-40 µl, 0.5-10 µl.

Method

Preparation of buffer and solution for ELISA procedure:-

(1) **Carbonate bicarbonate buffer (antigen diluting buffer)**

Solution (A): 0.2 molar anhydrous sodium carbonate (Na_2Co_3).

Solution (B): 0.2 molar sodium bicarbonate ($NaHCo_3$).

16 ml of solution (A) and 34 ml of solution (B) were mixed well and pH was adjusted to 9.6, volume was made up to 200-ml. the molarity of the solution becomes 0.05 M.

(2) **Phosphate buffered saline**

Solution (A): 0.15 molar solution of monobasic sodium phosphate (NaH_2Po_4).

Solution (B): 015 molar solution of dibasic sodium phosphate (NaH_2Po_4) in 500 ml of D/W.

0.015M 1x PBS buffer was prepared by dilution of the above solution for 10 times and 8.7 gm Sodium Chloride (NaCl) was added. (Final volume 1000 ml).

(3) **Blocking buffer: -** 1% BSA in 1 X PBS buffer.

(4) **Washing buffer: - 0.05**% tween-20 in 1X PBS buffer (PBS-T).

(5) **Antibody (serum) diluting buffer:-** 1 % BSA in 1X PBS buffer.

(6) **Preparation of conjugate: -** Antihuman IgG, anti mouse IgG conjugated to horse radish peroxidase (HRPO) and anti human IgM conjugate with HRPO enzymes were diluted 10,000 fold with PBS-T-BSA.

(7) **Preparation of substrate:** 1 OPD tablet (5 mg) was dissolved in 10 ml D/W then 30 µl of H_2O_2 (hydrogen peroxide) was added to it.

(8) **Stop solution:** 7% H_2SO_4.

ELISA procedure

ELISA using MLSA antigen for the detection of IgG

(a) Antigen coating: MLSA antigen (3 µg/ ml) was dissolved in carbonate bicarbonate buffer and mixed properly. ELISA plate was taken and 100 µl of diluted MLSA antigen was added to each well with the help of pipette. After that ELISA plate was sealed by plate sealer and incubated at 37°C for 4.5 hours. After incubation it was preserved at 4°C over night.

(b) Washing: Next day plate was washed with PBS only for 3 times at 2 min. interval through ELISA washer. After washing, the plate was dried using folds of filter paper or tissue pad.

(c) Blocking: The plate was blocked by each well with 200 µl of 3% PBS-BSA. Then it was sealed and incubated for 1 hour at 37°C.

(d) Serum dilution: Hundred fold dilution was added in each well. Only diluents buffer was added to the first column which served as blank, it was sealed and incubated at 37°C for 1.5 hours.

(e) Washing: Plate was washed with PBS with Tween for 3 times at 2 min interval through ELISA washer. After washing the plate was dried using folds of filter paper or tissue pad.

(f) Conjugate: Anti human IgG conjugated with HRPO was diluted in PBS-t-BSA (1:1000 dilution). Then 100 µl was added to each well. Plate was sealed by sealer and incubated at 37°C for 1 hour.

(g) Washing: Plate was washed with PBS with tween for 3 times at 2 min interval through ELISA washer. After washing the plate was dried using folds of filter paper or tissue pad.

(h) Substrate: One tablet of OPD was dissolved in 10 ml of D/W and 30 µl H_2O_2 (30%) was added in to it, 100 µl substrate was added in each well and the plate was kept in dark for 20–30 min at room temperature. The whole process was done in dark. It can be stopped before the color developed in blank column.

(i) Stop reaction: 25 µl of H_2SO_4 (7%) was added to each well of the plate.

(j) Measurement of colour: After 20 min optical density (OD) was read on ELISA reader at 492 nm.

ELISA for IgG subclasses against MLSA antigen:

(i) Antigen coating: 3 µg /ml of MLSA antigen was used. ELISA plate was taken and 100 µl antigens were added in each well. The plate was sealed by sealer and incubated at 37°C for 4.5 hours. After incubation it was preserved at +4°C for overnight.

(ii) Washing: Next day plate was washed with PBS only for 3 times at 2 min interval through ELISA washer. After washing the plate was dried using folds of filter paper or tissue pad.

(iii) Blocking: The plate was blocked by 200 µl each well with 3% PBS-BSA. Then it was sealed and incubated for 1 hour at 37°C.

(iv) Serum dilution: Hundred fold dilution was performed for each sample. It was diluted in PBS-t-BSA. 100 µL diluted sample was added in each well. Only diluent buffer was added to the first column which served as blank, it was sealed and incubated at 37°C for 1 hour.

(v) Washing: Plate was washed with PBS only for 3 times at 2 min interval through ELISA washer. After washing the plate was dried using folds of filter paper or tissue pad.

(vi) Anti human IgG subclasses: Anti human IgG1 monoclonal antibody was used after diluting it to 2000 fold in PBS-t-BSA.

Anti human IgG2, IgG3 and IgG4 monoclonal antibodies were used after diluting them to 5000 fold in PBS-t-BSA. Then plate was sealed and incubated at 37°C for 1 hour.

(vii) Washing: Plate was washed with PBS only for 3 times at 2 min interval through ELISA washer. After washing the plate was dried using folds of filter paper or tissue pad.

(viii) Substrate: One tablet of OPD was dissolved in 10 ml of D/W and 30 µl H_2O_2 (30%) was added in to it, 100 µl substrate was added in each well and the plate was kept in dark for 20–30 min at room temperature. The whole process was done in dark. It was stopped before the color developed in blank column.

(ix) Stop reaction: 25 µl of H_2SO_4 (7%) was added to each well of the plate.

(x) Measurement of colour: After 20 min OD was read on ELISA reader at 492 nm.

ELISA for the detection of antibody against ND-o-BSA (PGL-1 ELISA):

(i) Antigen coating: Antigen was diluted to 4 µg/ml in carbonate bicarbonate buffer. ELISA plate was taken and 100 µl prepared antigen was added in A-D rows and in E-H rows only bicarbonate buffer was added (100 µl/wells). The plate was sealed by sealer and incubated at 37°C for 18 hours. After incubation, it was preserved at 4°C.

(ii) Washing: Next day plate was washed with PBS only for 3 times at 2 min interval through ELISA washer. After washing the plate was dried using folds of filter paper or tissue pad.

(iii) Blocking: The plate was blocked by 200 µl each well of 1% PBS-BSA. Then it was sealed and incubated for 1 hour at 37°C.

(iv) Serum dilution: Three hundred fold diluted sera were added in quadruplicate wells, two wells with antigen coating and the other two with buffer coating. Then plate was incubated at 37°C for 2 hours.

(v) Washing: Plate was washed with PBS-T only for 3 times at 2 min interval through ELISA washer. After washing the plate was dried using folds of filter paper or tissue pad.

(vi) Conjugate: Anti-human IgM conjugated to HRPO was diluted 10,000 folds in PBS-BSA. Then 100 µl was added in each well. Plate was incubated at 37°C for 2 hours.

(vii) Washing: Plate was washed with PBS-T only for 3 times at 2 min interval through ELISA washer. After washing the plate was dried using folds of filter paper or tissue pad.

(viii) Substrate: One tablet of OPD was dissolved in 10 ml of D/W and 30 µl H_2O_2 (30%) was added in to it, 100 µl substrate was added in each well and the plate was kept in dark for 2030 min at room temperature. The whole process was done in dark. It was stopped before the color developed in blank column.

(ix) Stop reaction: 25 µl of H_2SO_4 (7%) was added to each well of the plate.

(x) Measurement of colors: After 20 min OD was read on ELISA reader at 492 nm.

Indirect ELISA against 35KD antigen:

(i) Antigen coating: 35 KD antigen of *M. lepare* was diluted in 0.125 µg/ml bicarbonate buffer. It was coated 50 µl in each well then the plate was sealed and incubated at 4°C in humid chamber over night.

(ii) Washing: Next day plate was washed with PBS-T only for 3 times at 2 min interval through ELISA washer. After washing, the plate was dried using folds of filter paper or tissue pad.

(iii) Blocking: The plate was blocked by 1% BSA which was dissolved in PBS. In each well 100 µl of PBS-BSA was added. The plate was sealed by sealer and incubated at room temperature for 2 hours. After incubation the BSA was removed and the plate was dried using filter paper. Washing was not done.

(iv) Serum dilution: Serum was serially diluted up to 1:000 and 1:200 in PBS-BSA. 50 µl of diluted serum was added in each well. Plate was incubated at 37°C for 1.5 hours.

(v) Washing: Plate was washed with PBS only for 3 times at 2 min interval through ELISA washer. After washing, the plate was dried using folds of filter paper or tissue pad.

(vi) Conjugate: Anti human IgG-HRPO was diluted 5000 times in PBS-BSA. Then 50 µl conjugate was added in each well and incubated at 37°C for 2 hours.

(vii) Washing: Plate was washed with PBS only for 3 times at 2 min interval through ELISA washer. After washing, the plate was dried using folds of filter paper or tissue pad.

(viii) Substrate: One tablet of OPD was dissolved in 10 ml of D/W and 30 µl H_2O_2 (30%) was added in to it, 100 µl substrate was added in each well and the plate was kept in dark for 20–30 min at room temperature. The whole process was done in dark. It can be stopped before the color developed in blank column.

(ix) Stop reaction: 25 µl of H_2SO_4 (7%) was added to each well of the plate.

(x) Measurement of colors: After 20 min OD was read on ELISA reader at 492 nm.

Results and Discussion

In this study 40 sera samples of paediatric leprosy patients and 15 samples of healthy children were analyzed. All these samples were from children below the age of 14 yrs. For comparison, 51 sera samples from adult leprosy patients and 11 samples from healthy adults were taken.

Various classes and sub-classes of IgG antibodies were analysed in sera of these individuals by serological assay (ELISA).

Total IgG antibodies against MLSA were analysed in sera of paediatric samples and healthy controls. Mean level of antibodies is shown in Table 1.

The mean IgG level in MB patients was found to be significantly higher when compared to healthy children ($P < 0.05$) or in PB and MB patients.

Sero-positivity for this antibody is depicted in Table 2.

Cut- off point was calculated by taking the mean OD of healthy children +2SD. Cut-off point=0.498. The percentage of seropositivity for this antibody in healthy children is 5.2, which is less than MB children (18.7) and PB children (18.1). The total IgG antibody against MLSA antigen was analyzed in sera of adult samples. Mean level of antibodies is shown in Table 3 and seropositivity for this antibody is depicted in Table 4.

Mean IgG antibody level against MLSA was found to be higher in MB patients and PB patients with reaction compared to healthy individuals and this difference was statistically significant (P <0.01). However, there was no difference in antibody level between PB and MB patients.

Cut off point was calculated by taking mean OD of health individual +2SD. Cut off point = 0.335.

Only one healthy children was positive for this antibody (9%). 30% cases are positive in BT, 80% cases are positive in BTR and 75% cases are positive in MB for this antibody.

The IgG1, IgG2, IgG3 and IgG4 antibody against MLSA antigen was analyzed in sera of paediatric samples. Mean level of antibodies is shown in Table 5.

Among four IgG subclasses IgG2 level was found to be higher in MB patients but it was not significant statistically. Moreover, there was no difference in mean antibody level among healthy individuals, PB and MB individuals.

The total IgM antibody against ND-O-BSA antigens was analyzed in sera of paediatric samples. Mean level of antibody is shown in Table 6.

There was no difference in mean level of IgM antibody among healthy children and paediatric leprosy patients (P >0.05). But a significant difference was there in between MB children and PB children (P <0.01).

Seropositivity for this antibody is depicted in Table 7.

Cut-off value was calculated by taking mean OD of healthy individual +2SD. Cut-off value = 0.456. There are two samples of healthy individuals which were found positive, (10.5%).

The IgG antibody against 35 KD antigen was analyzed in sera of paediatric samples. Mean level of antibody is shown in Table 8.

The antibody level in PB patients was not found to be significantly different as compared to healthy individuals (P >0. 10). But in MB patients, antibody response were found to be significantly more as compared to healthy individuals (P=0.05). In PB and MB patients there was no difference in antibody level against 35KDa antigen (P >0. 10). Seropositivity for this antibody was shown in Table 9.

Only one healthy individual is positive for this antibody. In paucibacillary two samples are positive, but in multibacillary three samples are positive for this antibody.

In present study 55 subjects were included, out of which 40 were leprosy cases and rest 15 were healthy children. All these samples were from children below 14 years of age. For comparison, 51 sera samples of adult leprosy patients and 11 samples from healthy adults were taken. In paediatric patients, antibodies titer in sera was estimated against MLSA, ND-o-BSA and 35KD antigens. But in all adult leprosy cases and healthy controls, antibodies titer in sera was estimated only against *M. lepare* soluble antigen (MLSA).

In our study, the mean level of IgG against MLSA antigen in paediatric leprosy patients (MB) was found to be significantly more as compared with healthy children, but in PB patients, there was no significant difference. Only 18% of leprosy children were found to be positive for this antibody whereas only one healthy child was positive. A significant proportion of adult leprosy patients were positive for this antibody compared to healthy controls. We have also observed that higher percentage of MB patients were positive as compared to PB patients. Beuria *et al.* (1999) have also reported a similar finding of MB patients having high antibody level compared to PB patients. This finding suggests that paediatric leprosy patients show a similar tendency of antibody response as those of adult leprosy patients.

Table.1 Mean level of IgG against MLSA antigen in paediatric leprosy patients

Type of disease	Mean OD±SD
Healthy control N=15	0.130 ± 0.181
Paucibacillary N=10	0.265±0.320
Multibacillary N=30	0.344±0.417

Statistical analysis was done by student 't' test

Table.2 Sero-positivity against MLSA antigen in leprosy children

Type of disease	No. of individuals	No. of positivity	Percentage
Healthy control	15	1	5.2
Paucibacillary	10	2	18.1
Multibacillary	30	6	18.7

Table.3 Mean level of antibody against MLSA antigen in adult leprosy patients

Type of disease	Mean OD±SD
Healthy control N=19	0.090 ± 0.101
BT N=13	0.409±0.502
BTR (PB with reaction) N=10	0.722±0.561
Multibacillary N=28	0.661±0.503

Statistical analysis was done by student 't-test'

Table.4 Antibody response against MLSA antigen

Type of disease	No. of individuals	No. of positivity	Percentage
Healthy control	11	1	9
Paucibacillary	13	4	30
BTR	10	8	80
Multibacillary	28	21	75

Table.5 Level of IgG subclasses against MLSA antigen in paediatric leprosy patients

	IgG1	IgG2	IgG3	IgG4
Types of disease	MeanOD±SD	MeanOD±SD	MeanOD±SD	MeanOD±SD
Healthy control N=15	0.06 ± 0.13	0.04 ± 0.06	0.007 ± 0.01	0.02 ± 0.07
Paucibacillary N=10	0.085 ± 0.206	0.035 ± 0.037	0.01 ± 0.015	0.005 ± 0.005
Multibacillary N=30	0.083 ± 0.154	0.117 ± 0.252	0.026 ± 0.057	0.025 ± 0.037

Statitistical analysis was done by student‘t’ test:-

Table.6 Mean level of IgM antibody against ND-O-BSA antigens in sera of paediatric leprosy patients

Types of disease	Mean OD ± SD
Healthy control n = 15	0.140±0.156
Paucibacillary n = 10	0.272±0.217
Multibacillary n = 30	0.587±0.488

Table.7 Seropositivity against ND-O-BSA antigens

Type of disease	No. of individuals	No. of positivity	Percentage
Healthy control	15	2	10.5
Paucibacillary	10	2	18.1
Multibacillary	30	16	50

Table.8 Mean level of IgG against 35 KD antigens in paediatrics leprosy patients

Type of disease	Mean OD ± SD
Healthy control N=15	0.140±0.084
Paucibacillary N=10	0.190±0.215
Multibacillary N=30	0.196±0.084

Table.9 Antibody response against 35KD antigen

Type of disease	No. of individuals	No. of positivity	Percentage
Healthy control	15	1	5.2
Paucibacillary	10	2	18.1
Multibacillary	30	3	9.3

Cut-off value =0.308.

In our study on IgG subclasses, in paediatric patients, among four subclasses IgG2 was found to be in higher level in MB patients, but it was not significantly different from other. Earlier, it was reported that the mean level and seropositivity value for IgG1, IgG2 and IgG3 were significantly higher in BL/LL patients as compared to BT/TT patients. IgG3 antibody level was found to be low as compared to the level of IgG1 in LL patients (Beuria *et al.,* 1999).

There was no difference in mean level of IgM between healthy children and paediatric leprosy patients (P >0.05). But there was significant difference between PB and MB children (P <0.01). In our study, 50% of MB children were found to be positive. In previous study by Joshi *et al.* (2007), 70% of active LL patients were positive for IgM antibody to ND-o-BSA antigen.

In our study, the antibody response against 35KDa antigen in MB patients was found to be significantly more as compared to the PB patients and healthy controls. (P= 0.05). Joshi *et al.* (2007) have also reported a similar finding in MB patients.

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