Indian J Lepr 2009, 81 : 63-68 http://www.ijl.org.in

Original Article

Study of cross-reactivity of *Mycobacterium leprae* reactive salivary IgA with other environmental mycobacteria

RR Kamble[1], VS Shinde[2], SP Madhale[3], RS Jadhav[1]

Majority of the endemic population is exposed to *Mycobacterium leprae* but very few develop disease. Humoral mucosal immune response mediated through *M. leprae* reactive salivary antibodies has been suggested to be quite important in the protective immunity. As the endemic population is also exposed to many environmental mycobacteria, we tested saliva from 121 subjects for the cross-reactivity of the *M. leprae* reactive salivary antibodies to mycobacteria like *M. smegmatis* and *M. phlei.* Saliva samples were treated with these two mycobacteria prior to testing *M. leprae* reactive antibodies by ELISA. In 59 subjects (48.76%), original and cross-reacted saliva showed same absorbance values suggesting no cross-reactivity. 26 subjects (21.49%) showed less than 25% drop in the OD values whereas 21 subjects (17.4%) showed 25% to 50% drop after reacting saliva with the mycobacteria. 15 subjects (12.4%) showed more that 50% drop in OD The data suggest that though in half of subjects antibodies did not cross-react with mycobacteria tested, there were subjects where antibodies showed cross-reactivity to mycobacteria suggesting that positive salivary *M. leprae* reactive IgA response could be to some extent due to exposure to environmental mycobacteria and it could also be protective against *M. leprae.*

Key words : Salivary antibodies, Mucosal immunity, Leprosy

Introduction

Leprosy is a chronic granulomatous disease caused by *Mycobacterium leprae*. The deformities caused by the disease are responsible for the stigma attached to it. The present MDT regimen used for treating leprosy was introduced in the early 1980's, since then the prevalence of the disease has dropped down significantly but the new case detection has not shown proportionate drop. The global leprosy situation at the beginning of 2007 was estimated to be around 2, 31,361 registered cases and new cases detected in 2006 were 2,65,661 (WHO 2007). The transmission of leprosy has been studied in great detail and various routes of transmission have been suggested. Though, there is a long held belief that the skin was the exclusive portal of entry (Satapathy et al 2005), the respiratory route of entry is being considered as major mode of transmission as evidence in its favor is on increase as it has been shown that transmission of *M.leprae* infection was possible in T/900R mice by *M.leprae* infected aerosol exposure (Rees and McDougall 1977). Lepromatous leprosy cases harbor in their body enormous number of leprosy bacilli and discharge them freely from the nose (Davey and Rees 1974). Hence, coughing and

[1]Stanley Browne Laboratory, TLM Community Hospital, Nand Nagari, Delhi-110093, India
[2]Blue Peter Research Centre, Cherlapally, Hyderabad, India
[3]Richardson Leprosy Hospital, Sangli-Miraj Road, Miraj - 416 410, Dist-Sangli, Maharashtra, India
Correspondence to : RS Jadhav **Email :** rupenjadhav@yahoo.com

sneezing can give rise to droplets and droplet nuclei which suggest the respiratory system to be the likely primary site of initial infection.

Most people within leprosy-endemic populations have been exposed to *M. leprae* (Izumi et al 1998) but few develop disease and it seems likely that the majority of the population develops protective immunity. If the site of initial infection is in the nose, dissemination of bacilli around the body to skin and nerve implies that the initial infection is bacilliferous. Presence of *M. leprae* on the nasal mucosa has been studied by the polymerase chain reaction (Gillis and Williams 1991) which suggests wide spread sub-clinical infection in the endemic population (Jadhav et al 2001).

Though PGL-1 antigen is *M. leprae* specific antigen and can detect *M. leprae* specific responses in sera of leprosy patients and their contacts (Foss et al 1993), salivary anti-PGL1 IgA antibody responses in leprosy patients were weak (Nagao-Dias et al 2007). The use of whole *M. leprae* cells as an antigen for the detection of mucosal immune response is more useful as IgA antibodies detected are against the exposed antigens that provoke mucosal immune response which plays an important role in neutralizing the bacteria before it could establish itself on the nasal mucosa. It is possible that the *M. leprae* which enters the nose through inhalation and settles on the nasal mucosa may trigger production of antibodies that could play an important role in elimination of these *M. leprae* (Cree and Smith 1998).

The salivary IgA response to *M. leprae* (sML-IgA) is estimated by an ELISA based assays. The protocol uses γ-irradiated whole *M. leprae* cells as an antigen. But since, *M. leprae* shares antigenic similarities with other environmental mycobacteria (Ivanyi et al 1983, Kingston et al 1986), it is important to establish the specificity of the sML-IgA antibodies and rule out any cross reactivity against other environmental mycobacteria. As in our earlier studies, we had seen *M. leprae* reactive antibodies in saliva (Smith et al 2004) of normal healthy population; we tested cross-reactivity of the salivary antibodies against environmental mycobacteria using competitive ELISA assay.

Materials and Methods

Collection of samples

Approximately 10 ml of saliva samples were collected from 121 individuals from an area in Miraj (leprosy prevalence at the time of collection 1.3 per 10,000 population), Maharashtra, India in sterile containers. The samples were cooled in ice buckets with coolant packs (0°C) and transported to the lab where it was processed by centrifugation at 2,000 x g for 10 minutes to remove food particles. 10µl of preservatives (Thiomerosal and EDTA) per one ml of saliva was added and stored at -20°C until further use. 1:4 dilution of saliva in phosphate buffered saline (PBS) containing 0.3% gelatin and 0.1% bovine serum albumin (BSA) was used in the assay.

Preparation of cells

The γ-irradiated *M. leprae* cells were kindly provided by Prof Patrick Brennan from Colorado State University, US. *M. smegmatis* and *M. phlei* cultures were obtained from National Chemical Laboratory, Pune, India. The cultures were maintained on Lowenstein-Jensen's medium. For use in the assay, the cultures were grown in Middlebrook's Broth and after overnight incubation, they were centrifuged and washed with PBS containing 0.05% Tween 20. The cells were finally re-suspended in 1 ml of normal saline (sterile 0.85% w/v NaCl solution in water). The cells were counted by using Neubauer Chamber.

Pre-treatment of saliva to test cross-reactivity

For competition ELISA, 0.2 ml of saliva was treated with $1X10^8$ cells each of *M. phlei* and *M. smegmatis* in 0.6 ml of PBS. Optimisation for incubation periods for the complete adsorption of antibodies to *M. phlei* and *M. smegmatis* was done prior to testing of cross-reactivity of antibodies. 0.2ml of saliva was treated with $1X10^8$

cells each of *M. phlei* and *M. smegmatis* in 0.6 ml of PBS for various time periods and then tested by ELISA to check any unadsorbed antibody. After 2 hrs incubation period, we could not detect any antibodies reactive to either *M. phlei* and *M. smegmatis*; we used this incubation period for our further studies. For competition ELISA, saliva was treated with the mycobacteria for 2 hrs at 37°C and centrifuged at 8,400 x g for 10 minutes. The supernatant was collected and used for ELISA.

ELISA for the detection of IgA antibodies

To detect the presence of IgA antibodies against *M. leprae*, *M. smegmatis* and *M. phlei*, the 96 wells microtiter plate (Thermo Scientific 6382 Immulon 2 HB) was coated with $1X10^8$ cells/ml of *M. leprae*, *M. smegmatis, M. phlei* respectively. The protocol used was as described earlier by Ramaprasad et al (1997). 0.1% gelatin (Sigma-G2500) was dissolved in distilled water at 60°C for 1 hour in water bath. The microtiter plates were immersed vertically in gelatine twice and tapped on bench to remove bubbles. The solution was flicked off and the plates tapped on towel to remove excess of gelatin. The plates were dried for 2 hrs at 60°C in a dry oven. Mycobacterial cells were re-suspended in volatile ammonium acetate-carbonate buffer pH 8.3. The plate was marked for test and control wells, the test wells were coated with 50ml of *M. leprae* cells/well and incubated at 37°C for 16-18 hrs. The plate was washed with wash buffer (4.3 mM Na_2HPO_4, 137mM NaCl, 2.7mM KCl, 1.4mM KH_2PO_4 pH 7.4; 0.05% Tween 20, 0.1% bovine serum albumin, 0.3% gelatin). The plate was then blocked with 250ml/well of blocking solution (3% bovine serum albumin in wash buffer) by incubation under humid conditions at 37°C for 2 hrs. The plate was washed with wash buffer for 4 times over 15 min. Untreated saliva was diluted 1:4 using wash buffer whereas saliva treated with mycobacteria was used directly as it was already diluted 1:4 during treatment with mycobacteria. 100µl of the saliva was used to carry out ELISA. Following saliva addition to the wells, the plate was incubated in humid chamber at 37°C for 2 hrs. After washing the plate, 100 µl of anti-IgA antibody conjugated to HRPO (1:40000 diluted) was added to all wells and the plates were incubated in humid chamber at 37°C for 90 min. Plates were then washed with PBS and 100µl/well of o-Phenylene diamine dihydrochloride substrate (1mg/ml in 10 ml phosphate citrate buffer containing 10µl 0.3% H_2O_2) was added and the plate was incubated in dark at 37°C for 15 min. The reaction was terminated by adding 50µl/well 3N HCl. The optical density (OD) was measured using ELISA Reader (Dynatech MR 500) at 490nm as test filter and 630nm as reference filter.

Results

Majority of the subjects showed antibodies reactive to *M. leprae, M. phlei* and *M. smegmatis* (Figures 1, 2) in their saliva. Saliva from 11 subjects each did not show any detectable antibodies reactive to either *M. phlei* or *M. smegmatis*. We observed that only three subjects did not show *M. leprae* reactive antibodies. Out of these three individuals, one showed low levels of antibodies reactive to both the mycobacteria tested. Of the remaining two, one individual each did not show detectable antibodies reactive to either *M. phlei* or *M. smegmatis*.

As described in the methods, saliva was pretreated with *M. smegmatis* and *M. phlei* so that any of the antibodies that will react with these cells will then be not available for subsequent ELISA used for the detection of *M. leprae* reactive antibodies. As shown in Figure 3, mixed results were seen in different individuals; almost half of the subjects did not show any drop in OD values after pre-treatment of saliva.

As shown in Table 1, out of 121 subjects; saliva from 59 (48.76%) subjects did not show any drop in optical density (OD) values for *M. leprae* reactive salivary antibodies even after pre-treatment with *M. smegmatis* and *M. phlei.* Saliva from 26 subjects (21.5%) showed less than 25% drop in OD values. Saliva from 21 subjects (17.4%)

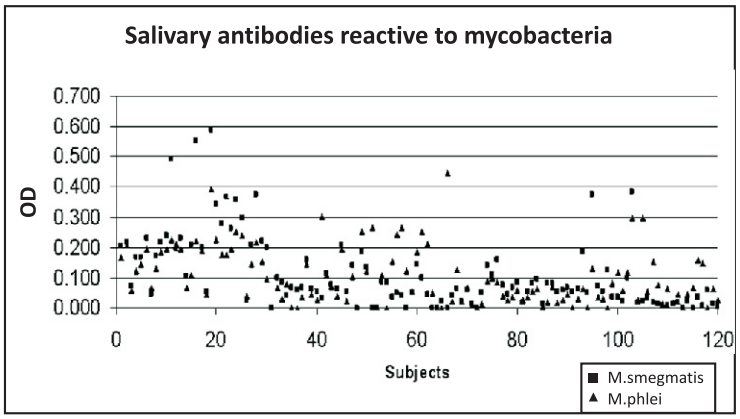

Figure 1 : Scatter plot showing optical density values of IgA antibodies reactive to *M. phlei* and *M. smegmatis* in saliva. Antibodies in saliva were tested by ELISA using microtitre plates coated with either *M. phlei* or *M. smegmatis*.

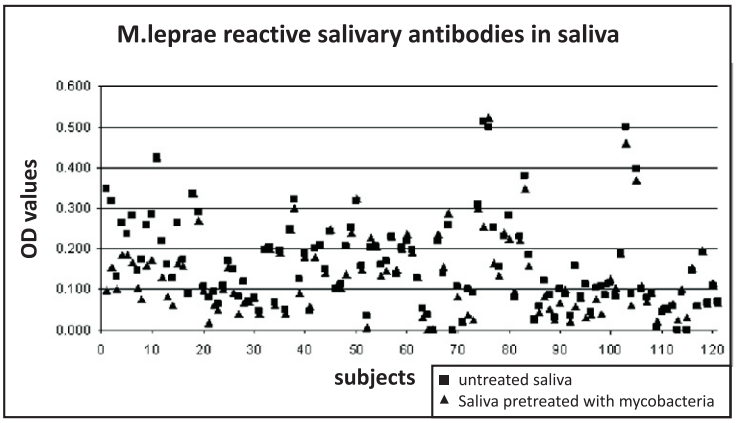

Figure 2 : Scatter plot showing optical density values of IgA antibodies reactive to *M. leprae* in saliva. Antibodies in saliva were tested before and after pretreatment with mycobacteria (*M. phlei* and *M. smegmatis*) by ELISA using microtitre plates coated with *M. leprae.*

Table 1: Cross-reactivity of the *M. leprae* reactive salivary antibodies (sML-IgA) to mycobacteria tested

Cross-reactivity	Number of subjects (%)
No cross-reactivity	59 (48.7)
Less than 25% drop in OD	26 (21.5)
25-50% drop in OD	21 (17.4)
More than 50% drop in OD	15 (12.4)

showed 25-50% drop in OD values. It was observed that saliva from only 15 subjects (12.4%) showed more than 50% drop in OD values.

Discussion

As nasal mucosa is considered as major portal of entry for *M. leprae*, nasal swabs were tested by different research groups using polymerase chain reaction for the detection of *M. leprae* and have reported that significantly large population from leprosy endemic countries show direct evidence

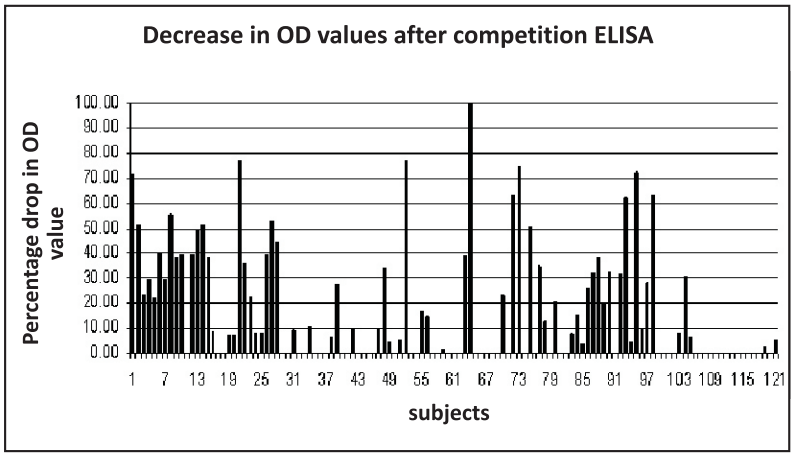

Figure 3 : Decrease in optical density for the IgA antibodies *reactive to M. leprae* in saliva pretreated with mycobacteria. Antibodies in saliva reactive to *M. leprae* prior to pretreatment and after pretreatment with *M. phlei* and *M. smegmatis* were tested using ELISA

of *M. leprae* in the nasal cavity (Klatser et al 1993, Beyene et al 2003, Smith et al 2004). But, so far the significance of detection of *M. leprae* on the nasal mucosa in relation to the actual out come of the disease is not yet established. If we look at the actual outcome of the diseases, it is quite evident that majority of the population in the endemic countries is immune to the disease. It has been shown that in leprosy endemic areas 60-80% of the population has *M. leprae* reactive IgA antibodies in the saliva which could be an indirect evidence for the exposure to *M. leprae* (Smith et al 2004).

In the earlier studies reported on *M. leprae* reactive salivary IgA (ML-IgA), whole *M. leprae* cells were used in the ELISA assay (Ramprasad et al 1997, Smith et al 2004). It was necessary to see whether the *M. leprae* reactive IgA antibodies cross-react with environmental mycobacteria sharing antigenic similarities. What is detected as humoral mucosal immune response against *M. leprae* in such assays could be just a cross-reactivity of the antibodies produced against environmental mycobacteria to which the subjects are exposed. Fine et al (2001) have earlier reported widespread sensitivity to antigens of environmental mycobacteria in the population of rural Malawi. They observed cross protection from natural exposure to certain environmental mycobacteria which may explain geographic distributions of mycobacterial disease.

In this study, IgA from saliva was allowed to react with *M. phlei* and *M. smegmatis*. If at all the IgA antibodies in the saliva cross-reacted with the environmental bacteria, they would not be free for the subsequent binding during ELISA testing. The supernatant with the free IgA that had not reacted with the mycobacteria used were detected by the *M. leprae* ELISA assay. As saliva from almost half of the tested subjects had antibodies that did not show any cross-reactivity with the other mycobacteria tested (no drop in the OD values), these antibodies could be either *M. leprae* specific or may have cross-reactivity with the other untested mycobacteria. Saliva from rest other subjects did show different levels of cross-reactive antibodies to mycobacteria tested. The IgA antibody responses against environmental mycobacteria could be affected due to other defense mechanism of nasal mucosa which eliminates the mycobacteria before it could be exposed to the immune system. Most of the microorganisms come in contact with a range of

soluble mediators present in the mucus, such as lysozyme, lactoferrin, collectin and defensins, produced by cells of the respiratory tract and the production of these molecules can lead directly to lysis of pathogens (Boyton and Openshaw 2002).

The mixed *M. leprae* reactive IgA responses in the subjects studied indicate that possible mucosal immunity could be either by direct exposure to *M. leprae* or by exposure to other mycobacteria which share common surface antigens with *M. leprae*, involved in eliciting mucosal humoral immune response. Nevertheless, as the antibodies react with *M. leprae*; they may play important role in protection against it irrespective of what elicited the response. This has important implications for the mechanisms and measurement of protection against mycobacterial diseases and evaluation of immune responses against vaccines as reported earlier (Fine et al 2001).

Acknowledgements

We wish to acknowledge support of The Leprosy Mission, India to carry out this work. We also acknowledge Dr Jeyakumar Daniel, Director, TLM India, Dr PSS Sundar Rao, Research Coordinator, TLM, India and Prof Cairns Smith from University of Aberdeen, UK for their guidance and Mrs. Mallika Lavania for her help in the preparation of this manuscript.

References

1. Beyene D, Aseffa A, Harboe M et al (2003). Nasal carriage of *Mycobacterium leprae* DNA in healthy individuals in Lega Robi village, Ethiopia. *Epidemiol Infect.* **131**: 841-848.
2. Boyton RJ and Openshaw PJ (2002). Pulmonary defences to acute respiratory infection. *Br Med Bull*. **61:** 1-12.
3. Cree IA and Smith WC (1998). Leprosy transmission and mucosal immunity: towards eradication? *Lepr Rev.* **69**: 112-121.
4. Davey TF and Rees RJW (1974). The nasal discharge in leprosy: clinical and bacteriological aspects. *Lepr Rev.* **45**: 121-134.
5. Fine PE, Floyd S, Stanford JL et al (2001). Environmental mycobacteria in northern Malawi: implications for the epidemiology of tuberculosis and leprosy. *Epidemiol Infect*. **126:** 379-387.
6. Foss NT, Callera F and Alberto FL (1993). Anti-PGL1 levels in leprosy patients and their contacts. *Braz J Med Biol Res*. **26**: 43-51.
7. Gillis TP and Williams DL (1991). Polymerase chain reaction and leprosy. *Int J Lepr Other Mycobact Dis.* **59**: 311-316.
8. Ivanyi J, Sinha S, Aston R et al (1983). Definition of species specific and cross-reactive antigenic determinants of *Mycobacterium leprae* using monoclonal antibodies. *Clin Exp Immunol.* **52**: 528-536.
9. Izumi S, Budiawan T, Matsuoka M et al (1998). Present situation of leprosy in highly endemic area of tropical Asia–a seroepidemiological study of *Mycobacterium leprae* infection in general inhabitants. *Jpn J Lepr*. **67**: 401-408.
10. Jadhav RS, Macdonald M, Bjune G et al (2001). Simplified PCR detection method for nasal *Mycobacterium leprae*. *Int J Lepr Other Mycobact Dis.* **69**: 299-307.
11. Kingston AE, Stagg AJ and Colston MJ (1986). Investigation of antigen cross-reactivity of *Mycobacterium leprae*-reactive murine T-cell lines and clones. *Immunology.* **58***:* 217-223.
12. Klatser PR, van Beers S, Madjid B, Day R, de Wit MYL (1993). Detection of *Mycobacterium leprae* nasal carriers in population for which leprosy is endemic. *J Clin Microbiol.* **31**: 2947-2951.
13. Nagao-Dias AT, Almeida TL, Oliveira Mde F et al (2007). Salivary anti-PGL IgM and IgA titers and serum antibody IgG titers and avidities in leprosy patients and their correlation with time of infection and antigen exposure. *Braz J Infect Dis.* **11**: 215-219.
14. Ramaprasad P, Fernando A, Madhale S et al (1997). Transmission and protection in leprosy: indications of the role of mucosal immunity. *Lepr Rev.* **68**: 301-315.
15. Rees RJW and McDougall AC (1977). Airborne infection with *Mycobacterium leprae* in mice. *J Med Microbiol.* **10**: 63-68.
16. Satapathy J, Kar BR and Job CK (2005). Presence of *Mycobacterium leprae* in epidermal cells of lepromatous skin and its significance. *Indian J Dermatol Venereol Leprol.* **71:** 267-269.
17. Smith WC, Smith CM, Cree IA et al (2004). An approach to understanding the transmission of *Mycobacterium leprae* using molecular and immunological methods: results from the MILEP2 study. *Int J Lepr Other Mycobact Dis*. **72:** 269-277.
18. World Health Organization (2007). Weekly epidemiological record, no. 44, 2 November http://www.who.int/wer/2007/pdf. 2007.