Symposium on the IMMUNOLOGY OF LEPROSY, Oslo, Norway, 1986
Lepr Rev (1986) 57, Suppl 2, 213-223

Anti-mycobacterial antibodies in saliva

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Summary
Modified techniques for the fluorescent leprosy antibody absorption (FLA-ABS) test and the enzyme-linked immunosorbent assay (ELISA) using phenolic glycolipid-I (PG) antigen of *M. leprae* were employed for detecting anti-*M. leprae* antibodies in sera and saliva from the patients with leprosy, household contacts and inhabitants in leprosy endemic areas. The FLA-ABS test with saliva was positive in 20 (58.8%) of the 34 household contacts, 92 (39 %) of the 236 schoolchildren and 37 (38.1 %) of the 97 adults who had social contacts with leprosy, but negative in the 23 patients with pulmonary tuberculosis. Serological specificity of salivary anti-*M. leprae* antibodies detected in the specimens of saliva from the schoolchildren was checked by cross-reactions and additional absorption tests. Only 7 out of the 55 specimens of saliva showing a positive FLA-ABS test were cross-reactive with some species of mycobacteria. Positive reactions against *M. leprae* were not influenced at all by additional absorptions with cross-reacting mycobacteria, but became negative after an additional absorption with *M. leprae*. Therefore, the modified technique of FLA-ABS test was found specific to *M. leprae* and useful for detecting subclinical leprosy infection. The PG-ELISA activity was most frequently found in the serum and salivary IgA from the patients with leprosy, the percentage of positive reactions in saliva being comparable to that of FLA-ABS test. The PG-ELISA using anti-IgA-enzyme conjugate was negative in the specimens of saliva from the patients with tuberculosis and the schoolchildren who had no suspicious symptom nor FLA-ABS reactivity, but was positive in 3 (11.1 %) of the 27 specimens of saliva from the schoolchildren who had suspicious symptoms and/or FLA-ABS reactivity and in 17 (17.5 %) of the 97 specimens of saliva from the adults who had social contacts with leprosy. Therefore, the PG-ELISA with saliva was also found specific to *M. leprae* and useful for detecting the secretory type of anti-PG atibodies. A significant difference in the percentage of positive reactions between FLA-ABS and PG-ELISA was discussed from a point of view concerning specific epitopes of *M. leprae*.

Introduction
A possible transmission of leprosy through mucous membrane has been discussed by several investigators [1-5]. One of the evidences supporting this possibility would be the detection of secretory type antibodies against *Mycobacterium (M) leprae*. The authors [6] found such antibodies in the nasal and oral secretions of leprosy patients using the fluorescent leprosy antibody absorption (FLA–ABS) test. Since this test has been used for detecting subclinical leprosy infection in household contacts [7, 8] and inhabitants in an endemic area [9], it was anticipated that the production of salivary IgA antibodies might be induced by a minute amount

of *M. leprae,* an amount which might not be capable of inducing the production of an appreciable amount of circulating antibodies.

An enzyme linked immunosorbent assay (ELISA) using a phenolic glycolipid-I (PG) antigen of *M. leprae* has recently been introduced by some investigators [10-14] for detecting *M. leprae*-specific antibodies in leprosy patients and their contacts. If this antigen plays any role in the development of mucosal immunity, a secretory type of anti-PG antibody may also be found by using the ELISA with saliva.

Materials and methods

The subjects of this study consisted of the 61 leprosy patients whose sera and saliva were used in the previous study [6], 34 household contacts, 236 schoolchildren in Miyako Islands, Okinawa, 97 residents and staffs in a home for aged people among whom a new case with lepromatous leprosy has recently been found, and 23 patients with pulmonary tuberculosis hospitalized in a non-endemic area. All the specimens of saliva were collected after administration of an oral tablet containing 100 or 200 mg of ascorbic acid. The salivas of non-leprosy cases were used for serological tests without concentration.

The technique of FLA-ABS test was modified slightly so as to adapt to these specimens: 0.5 ml of saliva was mixed with 0.1 ml each of 5 % (w/v) BCG and 10 % (v/v) *M. vaccae* suspensions, incubated at 37°C for 30 min and centrifuged twice at 2,000 rpm for 15 min and at 10,000 rpm for 5 min to remove the bacterial cells completely. The supernatant was diluted to 1:4 of original volume with 0.1 % (w/v) BSA in PBS and further diluted with the same medium in serial 4-fold dilutions. A suspension of *M. leprae* purified from an infected armadillo's liver (W73) according to an IMMLEP protocol 1/79 was used as the antigen throughout the study. A commercial product of FITC-conjugated anti-human alpha-chain specific goat antibody (Miles-Yeda, Ltd, Tokyo) was used as the secondary antibody. The other techniques and those for the test with serum were the same as described previously [6, 7].

A specimen of PG was prepared by one of the authors (YY) from an infected armadillo's liver residue according to the method described by Hunter et al [15], and its serological activity was confirmed to be almost equal to that of another specimen of PG offered from Dr. Rees through IMMLEPSC. According to his suggestion, the authors used ELISA conditions modified from those described by Cho et al [10]. Only the conditions modified were described below: PG (100 μg/ml) was suspended in an ammonium acetate-carbonate buffer (pH 8.2) by direct sonication until a milky stable suspension was obtained. The suspension was diluted to a required concentration (usually 2 μg/ml) with the same buffer; 50 μl was added to the wells of polystyrene microtiter plates (U-bottom, «Immulon» 1, Dynatec laboratories, Inc. Alexandria, Virginia, USA) which were incubated at 37°C for 14 to 16 hr in a moist chamber. A 5 % BSA (Armour Pharamaceutical Co, Kankakee, Illinois, USA) solution in PBS was used for blocking the wells and diluting the test serum or saliva. A goat anti-human IgG- or IgM- or IgA-peroxidase conjugated reagent (Cappel Laboratories Inc. Malvern, Pennsylvania, USA) was used as a secondary antibody at an optimum dilution which had been determined by a boxtitration. Reactions were terminated with 2.5N sulfuric acid after a 30 min incubation with a substrate-dye reagent, and the absorbance was read at 490 nm by using a Micro ELISA autoreader MR580 (Dynatec Laboratories, Inc).

Results and discussion

FLA-ABS TEST WITH THE SALIVA OF HOUSEHOLD CONTACTS

The results of FLA-ABS test with 34 specimens of saliva collected from household contacts are shown in Table 1. The percentage of positive reaction was 58.8 in total cases. This value was significantly smaller than that of the sera examined by the previous study [7]. The difference of sex or blood relationship did not cause any significant difference in the percentage. However, the contacts with lepromatous patients showed a higher percentage of positive reaction than those with tuberculoid patients. The percentage in the former was comparable to that of the sera in the previous study. Providing that the salivary antibodies of household contacts are also induced by subclinical infection with *M. leprae,* the above-metioned difference in percentage may be explained by different eexposure to the bacilli discharged from index cases.

Table 1.
Salivary IgA antibody activity of household contacts.

Contacts	No. of cases	FLA-ABS test Positive	%	X^2
Total	34	20	58.8	
Male	18	12	66.7	0.97
Female	16	8	50.0	
Child & grandchild	26	17	65.4	0.98
Spouse	8	3	37.5	
Lepromatous	23	18	78.3	8.75
Tuberculoid	11	2	18.2	

FLA-ABS TEST IN SCHOOLCHILDREN

During the annual survey conducted in 1984, 236 specimens of saliva were collected from the schoolchildren in 3 districts of Miyako Islands, Okinawa, where the highest incidence of leprosy in Japan had ever been reported [16]. The results of FLA-ABS test are shown in Table 2. The test was positive in 92 (39 %) children. This percentage was significantly lower than that in the household contacts, but was comparable to that of the sera in previous report [9]. The difference of sex and the presence or absence of neural symptoms, such as the enlargement of peripheral nerve without sensory loss, gave a significant difference in the percentage of positive reaction, as indicated by chi-square (X^2) values. Since only 10 out of the 54 children with neural symptoms were girls, the difference in percentage by sex may be due to neural symptoms. Saliva and sera were collected simultaneously from 234 schoolchildren. The results of FLA-ABS test with these specimens are shown in Table 3. Concordant positive and negative reactions were seen in 73.5 % children. It is noteworthy that 34 children showed salivary antibody-positive but circulating antibody-negative responses. Immunological significance of this discrepancy will be discussed later.

Table 2.
Salivary IgA antibody activity of schoolchildren in Miyako Islands.

Children	No. of cases	FLA-ABS test Positive	%	X^2
Total	236	92	39.0	
Boy	148	67	45.2	6.6
Girl	88	25	28.4	
Neural symptoms	+ 54	32	59.3	11.1
	− 182	60	32.6	

Table 3.
FLA-ABS test with serum and saliva of schoolchildren in Miyako Islands.

Test with serum	Test with saliva		Total
	Positive	Negative	
Positive	58	28	86
(%)	(24.8)	(12.0)	(36.8)
Negative	34	114	148
(%)	(14.5)	(48.7)	(63.2)
Total	92	142	234
(%)	(39.3)	(60.7)	(100)

Serological specificity of salivary FLA-ABS test in the schoolchildren was checked by the cross-reactions with 8 species of mycobacteria and by the additional absorption [9] with the cross-reacting mycobacteria. The results are shown in Tables 4 and 5. Only 7 out of the 55 specimens of saliva showig positive FLA-ABS reaction were cross-reactive with some species of mycobacteria other than *M. leprae*. Cross-reactions with BCG and *M. vaccae* were still observed in some specimens of saliva, though these mycobacteria had been used for the absorption of crossreacting antibodies. Then, additional absorption test was conducted with 6 specimens of saliva except that of HT. The antibody titers against *M. smegmatis* or *M. tuberculosis* became negative after the additional absorption, while the titers against *M. leprae* were not influenced at all by the absorption. However, the latter became negative after the additional absorption with *M. leprae*. These results indicate that both *M. leprae*-specific and cross-reacting IgA antibodies were present in these specimens of saliva.

Table 4.
Cross-reactivity of salivary IgA antibodies.

Mycobacterium	Saliva of TS	AS	HT	TM	YU	KT	HS
leprae	+	+	+	+	+	+	+
tuberculosis	–	–	+	–	+	–	+
kansasii	–	–	+	–	–	–	–
marinum	–	–	+	–	–	–	–
smegmatis	+	+	+	+	–	–	–
phlei	–	–	+	–	–	–	+
avium	–	–	+	–	–	–	–
BCG	+	+	+	–	–	+	+
vaccae	–	–	+	–	–	–	+

Table 5.
Additional absorption of saliva with cross-reacting mycobacteria.

Antibody titer of	Antigen for absorption	Antigen in smear M. l.	M. s.	M. t.	BCG
TS	None	16	64	/	16
	M. s.	16	0	/	16
	M. l.	0	/	/	/
AS	None	16	256	/	64
	M. s.	16	0	/	0
	M. l.	0	/	/	/
TM	None	16	256	/	/
	M. s.	16	0	/	/
	M. l.	0	/	/	/
YU	None	16	/	16	/
	M. t.	16	/	0	/
	M. l.	0	/	/	/
KT	None	16	/	/	4
	M. t.	16	/	/	0
	M. l.	0	/	/	/
HS	None	16	/	4	4
	M. t.	16	/	0	0
	M. l.	0	/	/	/

Abbreviations: M. l.: *M. leprae*, M. s.: *M. smegmatis*, M. t.: *M. tuberculosis*.

COMPARISON BETWEEN PG-ELISA AND FLA-ABS TESTS

Figure 1 shows the absorbance at 490 nm (A490) of PG-ELISA in 61 pairs of sera and saliva from the patients with leprosy, of which 20 were lepromatous (L), 21 borderline (B) and 20 tuberculoid (T). The sera were diluted to 1:300 and the salivas were diluted to 1:3 for IgG and IgM or to 1:10 for IgA. Average absorbance in each group is indicated by a short hori-

zontal line in the figure. The average of IgM antibody activity in the sera was highest in the L, middle in the B and lowest in the T, while those of serum IgG and IgA antibody activities showed no such correlation with the spectrum of leprosy. The average of salivary IgA antibody activity was somewhat higher than that of salivary IgG and IgM irrespective of the spectrum.

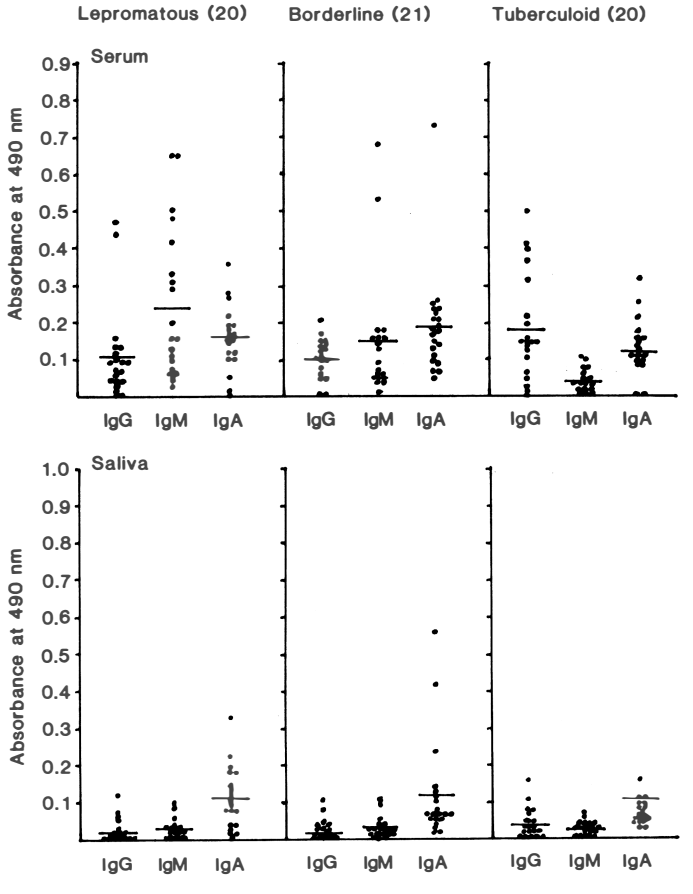

Figure 1.
PG-ELISA activity in sera and saliva from leprosy patients.

The results of PG-ELISA in schoolchildren, adults in a home for aged people and the patients with pulmonary tuberculosis are shown in Figure 2. Dilutions of the specimens were the same as those used for the patients with leprosy. Sixty-nine schoolchildren were selected as a control group, because they had no suspicious symptoms nor FLA-ABS reactivity. The 99 % rejection limits of mean absorbance in this group were used for determinig a cut-off point of positive reaction with each antibody-enzyme conjugate, and they are shown by broken lines in the figure. The upper limit was 0.088 for IgG, 0.097 for IgM and 0.096 for IgA. In the saliva, however, the upper limit was 0.034 for IgG, 0.032 for IgM, whereas that for

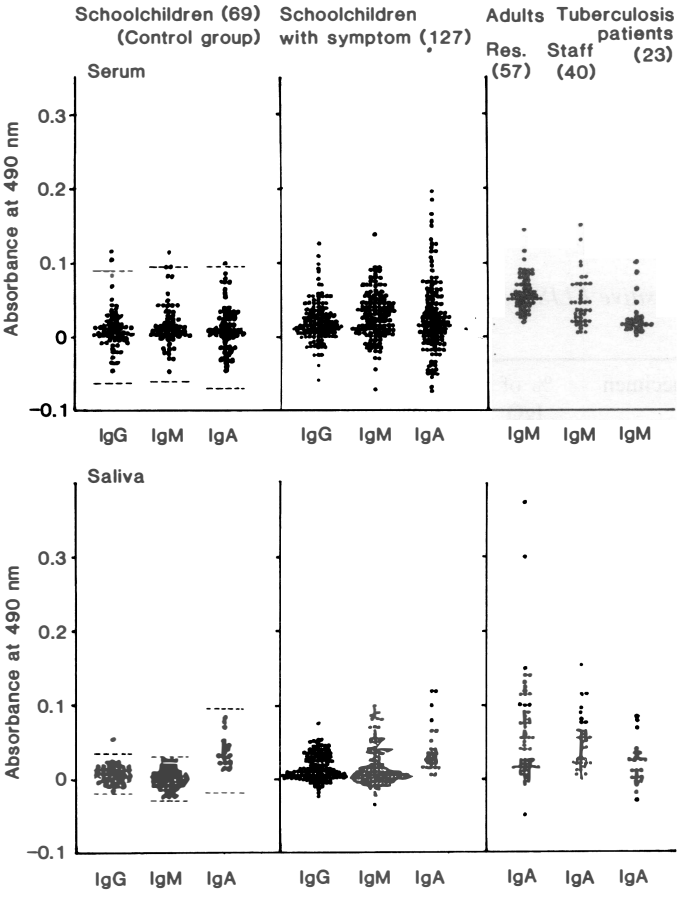

Figure 2.
PG-ELISA activity in sera and saliva from schoolchildren, adults and the patients with pulmonary tuberculosis.

IgA was 0.097 which was comparable to that in the serum. As the values for salivary IgG and IgM were included in a range of variations due to technical error, these values were substituted by those for serum IgG and IgM. The percentage of positive ELISA reactions in each group was calculated by using these cut-off points and compared with that of positive FLA-ABS tests in the same group. The results are shown in Tables 6 and 7. IgA-ELISA in the serum showed higher percentages than those of IgA-FLA-ABS test, irrespective of the classification of leprosy. In the saliva, however, the percentage of positive IgA-ELISA activity was highest in the L, middle in the B and lowest in the T, the order being a reverse of what was seen in the IgA-FLA-ABS test. IgA-ELISA in saliva was found to be specific for leprosy, because the specimens of saliva from patients with pulmonary tuberculosis and schoolchildren in the control group showed no reactivity against PG antigen of *M. leprae*. On the other hand, IgA-ELISA was positive in 15 (11.0 %) out of the 127 specimens of serum from schoolchildren having suspicious symptoms and/or FLA-ABS reactivity and in 3 (11.1 %) out of the 27 specimens of saliva from the same group. The percentages of positive

ELISA with IgG and IgM were far lower than the above. In the adults who had social contacts with leprosy, the percentage of positive ELI-SA activity in their sera was highest in IgG, middle in IgA and lowest in IgM, the order being similar to that of tuberculoid patients. Moreover, positive IgA-ELISA activity was found in 17 (17.5 %) out of the 97 specimens of saliva from these adults. However, these percentages of positive reactions were significantly lower than those obtained by the FLA-ABS tests with sera and saliva from the two groups of children and adults.

Table 6.
Percentages of positive ELISA and FLA-ABS tests in sera and saliva from the patients with leprosy.

Patients (Number)	Specimen	% of positive ELISA with IgG	IgM	IgA	% of positive FLA-ABS with IgG	IgM	IgA	Igs*
Lepromatous	Serum	50	70	85	95	90	30	95
(20)	Saliva	5	0	50	5	0	15	10
Borderline	Serum	61.9	47.6	81.0	85.7	90.5	28.6	85.7
(21)	Saliva	4.8	9.5	33.3	4.8	9.5	47.6	9.5
Tuberculoid	Serum	75	10	70	60	70	15	75
(20)	Saliva	10	0	20	0	0	50	35
Total (61)	Serum	62.3	42.6	78.7	80.3	83.6	24.6	85.2
	Saliva	6.6	3.3	34.4	3.3	3.3	37.7	18.0

* A mixture of IgG, IgM and IgA.

Table 7.
Percentages of positive ELISA and FLA-ABS tests in sera and saliva from schoolchildren, adults and the patients with pulmonary tuberculosis.

Group (Number)	Specimen	% of positive ELISA with IgG	IgM	IgA	% of positive FLA-ABS with IgA	Igs*
Schoolchildren	Serum	2.9	1.4	1.4	–	0
(control) (69)	Saliva	0	0	0	0	–
Schoolchildren	Serum	3.9	0.8	11.0	–	59.1
with symptoms (127)	Saliva	0	0.8	11.1**	66.1	–
Adults with social	Serum	38.1	6.2	25.7	–	54.6
contacts (97)	Saliva	–	–	17.5	38.1	–
patients with	Serum	–	4.3	–	–	4.3
tuberculosis (23)	Saliva	–	–	0	0	–

* A mixture of IgG, IgM and IgA.
** 27 specimens at 1:10 dilution.

Since anti-PG antibody activity was frequently found in IgA of both serum and saliva, concordance and discordance of IgA-ELISA activity between the pair of specimens was examined by using the results in the 3 groups as shown in Table 8. Concordant positive reaction was most frequently found in the L and nearly 50 % of the total patients were positive in the serum but negative in the saliva. The reverse discordant activity was seen in 3 cases each of the patients with leprosy and the schoolchildren with symptoms and in 11 adults having social contacts with leprosy. Table 9 shows concordance and discordance of FLA-ABS test between serum and saliva from the adults. Concordant positive and negative reactions were seen in 67 % of the adults. Eight persons showed positive reactions in the saliva but negative in the serum. Similar cases were found in 34 schoolchildren as shown in Table 3. Based on these findings, it may be concluded that salivary IgA antibodies against *M. leprae* can be found by both FLA-ABS and PG-ELISA in a significant number of individuals who were negative in these tests with their sera. Such a local mucosal immune response might have been induced by a minute amount of *M. leprae* infected in the oral mucosa.

Table 8.
Concordance and discordance of IgA-ELISA between serum and saliva.

IgA-ELISA		Percentage of responders in					
activity in serum	saliva	Leprosy patients L	B	T	Total	School-children	Adults
Positive	Positive	45	23.8	20	29.5	0	6.2
Positive	Negative	40	57.1	50	49.2	14.8	19.6
Negative	Positive	5	9.5	0	4.9	11.1	11.3
Negative	Negative	10	9.5	30	16.4	74.1	62.9
Number of cases		20	21	20	61	27	97

Table 9.
Concordance and discordance of FLA-ABS test between serum and saliva from the adults having social contacts with leprosy.

FLA-ABS test with serum	FLA-ABS test with saliva Positive	Negative	Total
Positive	29	24	53
(%)	(29.9)	/24.7)	(54.6)
Negative	8	36	44
(%)	(8.2)	(37.1)	(45.4)
Total	37	60	97
(%)	(38.1)	(61.9)	(100)

The percentages of positive FLA-ABS test with saliva from the group of schoolchildren and adults were significantly higher than those of IgA-ELISA activity in the same specimens, as shown in Table 7. This difference may not be due to the difference in sensitivity be-

tween the 2 tests, because the percentage of positive IgA-ELISA was significantly higher than that of FLA-ABS test with the saliva of lepromatous patients. A most probable explanation may be the difference in antigenic substance(s) participating in the reaction. Since whole cells of *M. leprae* are used in the FLA-ABS test, antibodies other than anti-PG might also cause positive reactions. In fact, some sera from the patients with lepromatous leprosy inhibited the immunofluorescent staining of *M. leprae* with 2 specimens of monoclonal antibody, one of which (MC2404) recognized proteins and another (MC2817) recognized PG, as described in a TDR report of WHO [16]. In other words, at least 2 kinds of antigens, i.e. protein and PG, can participate in the FLA-ABS test. The production and secretion in saliva of the antibodies against these antigens seem to be not necessarily parallel in each individual. Table 10 shows concordance and discordance between the 2 tests with saliva. Anti-PG antibodies were more frequently found in the saliva from the patients with multi-bacillary forms of leprosy than those from the other groups. Another kind of antibodies, probably anti-protein, were more frequently found in the saliva of schoolchildren and adults than those from the patients with multibacillary forms of leprosy. It is therefore anticipated that the use of *M. leprae*-specific protein antigen in any serological test may be more favorable than the use of PG or related antigen for the detection of subclinical leprosy infection.

Table 10.
Concordance and discordance between IgA-ELISA and FLA-ABS activities in saliva.

IgA-ELISA	FLA-ABS	Percentage of responders in					
		Leprosy patients				School-children	Adults
		L	B	T	Total		
Positive	Positive	10	0	5	13.1	3.7	9.3
Positive	Negative	40	33.3	15	21.3	7.4	8.2
Negative	Positive	0	9.5	30	24.6	66.7	28.9
Negative	Negative	50	57.1	50	41.0	37.0	53.6
Number of cases		20	21	20	61	27	97

Acknowledgements
This investigation received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and from the Japanese Leprosy Panel of the US-Japan Cooperative Medical Science Program. The authors express sincere thanks to Dr. Sanada for collecting the specimens from tuberculosis patients and to all participants in the survey held by the Division of Prophylaxis, Department of Environment and Health, Okinawa Prefecture.

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