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Salivary Immunoglobulins and Antibody Activities in Leprosy[1]

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The biologic function of the secretory immune system has been described in various diseases ([17]). Although leprosy of the upper respiratory tract and oral mucosa was described by Hansen and Looft ([8]) in the last century and reviewed recently by Yoshie ([19]), little information regarding the immunologic defense mechanism of the mucosa in leprosy is available in the literature, except for three reports ([5, 14, 15]) in which the levels of secretory immunoglobulins in leprosy are described. However, the existence of secretory antibodies reacting with *Mycobacterium leprae* has not yet been shown by these investigators. In 1974, the authors ([3]) reported the levels of immunoglobulins and *M. leprae*-specific antibodies in the nasal secretions of leprosy patients and described part of the results separately ([1, 2]). The number of patients examined in this study was not large enough to lead to a definite conclusion because it was very difficult to collect the nasal secretions from a large number of patients, especially those with tuberculoid leprosy. The collection of saliva is far easier and less troublesome than nasal washing. Moreover, if saliva is available for a serologic test instead of serum, this test will be acceptable to many persons who regard bleeding as traditionally taboo.

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MATERIALS AND METHODS

Patients and controls. A total of 110 patients with known classifications of leprosy were examined in this study. They were classified as: lepromatous leprosy (L) = 50, borderline (B, including BL, BB, and BT) = 24, and tuberculoid (T) = 36. Twenty-one cases were outpatients at a skin clinic in Naha, Okinawa; 69 cases were inpatients at the National Leprosarium Airaku-en, Okinawa, and the remaining 20 cases were inpatients at the National Leprosarium Tama Zensho-en, Tokyo, Japan. Their ages ranged from 19 to 81, and 42 cases were females. All of the patients were under treatment and 78 of them were inactive at the time of this study. Erythema nodosum leprosum (ENL) was simultaneously found in one BL and nine L cases. The controls were 10 healthy individuals, 6 males and 4 females aged 16 to 62. Only salivas were collected from this group.

Collection and analysis of specimens. Blood was taken from a vein and saliva was collected after administration of an oral tablet containing 200 mg of ascorbic acid. The serum was separated from the clotted blood on the next day and kept in a freezer (−20°C) together with the saliva collected from the same patient. These materials were collected at Okinawa and brought by plane to the authors' laboratory. The saliva was concentrated by ultrafiltration to approximately $\frac{1}{10}$–$\frac{1}{20}$ of the original volume. Protein concentration of the serum was determined by an Atago refractometer for sugar and protein determination; whereas that of the saliva was determined colorimetrically by the method of Lowry, *et al.* ([11]). IgG, IgM, and IgA levels were determined by the Hyland Immunoplates III GAM Kit. Low-level human serum was used for the determination of immunoglobulins in the concentrated sa-

liva, and the levels of IgG, IgM, and IgA were expressed as mg/dl of the original saliva.

Fluorescent leprosy antibody absorption (FLA-ABS) test. *M. leprae*-specific antibody activity of IgG, IgM, and IgA in the serum and saliva was examined by using the FITC-conjugated goat antibodies monospecific for the respective immunoglobulins (Miles-Yeda Co., Ltd., Tokyo, Japan) as the second antibodies in the FLA-ABS test ([4]). These fluorescent antibodies were previously absorbed with an equal volume of 5% w/v BCG suspension and diluted to an optimal dilution which had been determined by a box-titration against a known positive serum. A double volume of saliva (0.1 ml) and the usual volume of serum (0.05 ml) were used in the FLA-ABS test and diluted to 1:20 and 1:40, respectively, after the absorption. Subsequent serial, fourfold dilutions were conducted in the same manner. Since almost all of the saliva specimens did not give a distinct reaction at the dilutions of 1:80 or higher, the test was considered to be positive if 2+ or more intense fluorescence was observed at the 1:20 dilution of saliva.

Statistical analysis was performed by Student's *t* test and the chi-square test.

RESULTS

Total protein and immunoglobulin levels in the serum and saliva of three groups of leprosy patients and those in the saliva of healthy individuals are summarized in Table 1. Serum protein and immunoglobulin levels in the leprosy patients were not significantly different among the L, B, and T groups. Salivary IgM was detected in only two L cases and three T cases. Salivary total protein, IgG, and IgA levels showed no significant difference according to the classification of leprosy.

Salivary IgG and IgA levels in the control group were also not significantly different from those in the leprosy patients, although the total protein level was significantly higher in the control group. Since the levels of these immunoglobulins in the saliva of the leprosy patients ranged widely, from a trace to the maximum of 9.75 mg/dl in the case of IgG, the percentages of the concentrations in each specimen were calculated. Results are shown in Figure 1. The numbers under the zero line indicate those cases in which the immunodiffusion test was negative. The average percentage of IgA concentrations seems to increase from L to T, but the difference was not statistically significant. Ratios of serum IgG to salivary IgG in each patient and of serum IgA to salivary IgA in the same patients are shown in Figure 2. The distribution of these ratios and their averages did not show any significant difference among the L, B, and T groups. Figure 3 shows a ratio of salivary IgA/IgG to serum IgA/IgG. This ratio was used for indicating a function of the mucous membrane secreting IgA ([9]). Its distribution and the average showed no significant difference among the L, B, and T groups. These find-

TABLE 1. *Total protein and immunoglobulin levels in serum and saliva of leprosy patients and normal subjects.*

Specimen	Level	Leprosy patients			Normal subjects (10)
		L (50)[a]	B (24)	T (36)	
Serum	Total protein (g/dl)	8.16 ± 0.83	8.60 ± 1.21	7.99 ± 0.57	ND[b]
	IgG (mg/dl)	1293 ± 378	1143 ± 311	1176 ± 308	ND
	IgM (mg/dl)	151 ± 105	133 ± 67	132 ± 97	ND
	IgA (mg/dl)	371 ± 150	311 ± 142	333 ± 134	ND
Saliva	Total protein (mg/ml)	1.35 ± 0.68	1.32 ± 0.77	1.34 ± 0.61	2.20 ± 1.54
	IgG (mg/dl)	1.13 ± 0.79 (43)	0.73 ± 0.33 (18)	0.93 ± 0.39 (27)	1.18 ± 0.79 (5)
	IgM (mg/dl)	0.63 ± 0.30 (2)	Undetected	0.82 ± 0.45 (3)	ND
	IgA (mg/dl)	1.50 ± 0.99 (49)	1.16 ± 0.59 (23)	1.37 ± 0.82 (36)	0.90 ± 0.61 (9)

[a] Number in parentheses indicates the number of cases when a mean ± S.D. is calculated. Undetected cases are omitted from this calculation.

[b] Not done.

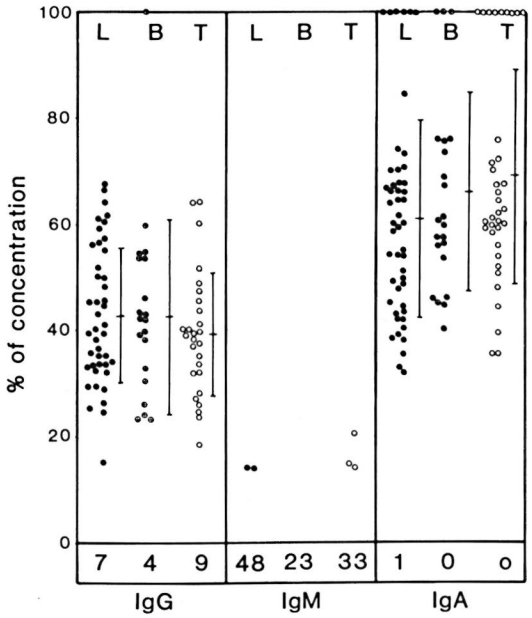

FIG. 1. Percent of immunoglobulins in saliva.

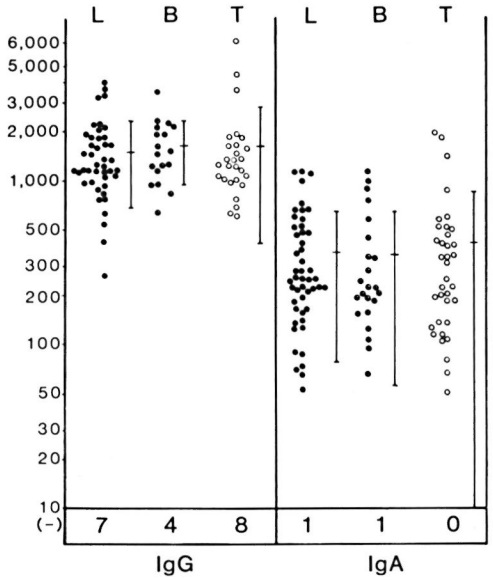

FIG. 2. Ratio of serum IgG to salivary IgG and serum IgA to salivary IgA.

ings suggest that leprosy patients secrete salivary immunoglobulins in a fashion which is not significantly different according to the classification of leprosy nor different from that of healthy individuals.

The results of the FLA-ABS test are shown in Table 2. The numbers and the percentages of positive reactions in the test used as a routine are shown in the columns under Igs (a mixture of IgG, IgM, and IgA). The percentage in the test with serum was highest in the L, middle in the B, and lowest in the T cases. The difference between L and T is statistically significant ($p < 0.01$). A similar result was also obtained by using anti-IgG fluorescent antibody in the test with serum. The difference between 87.5% in B and 58.3% in T is also significant statistically ($p < 0.05$). Therefore, it was found that the antibody activity in serum IgG was higher in L and B cases than in T cases. The percentages of positive reactions in the serum by means of anti-IgM and anti-IgA antibodies did not show any significant difference according to the classification of leprosy, although the percentages with anti-IgA are clearly lower than those with the other two antibodies. Based on these findings, it is concluded that *M. leprae*-specific antibodies in the serum of leprosy patients are found mainly in IgG and IgM and less frequently in IgA.

On the other hand, these tests with saliva of the same patients showed quite a different result. Appreciable numbers of positive reactions were not obtained by the test with anti-IgG and anti-IgM antibodies. The percentage in the column under Igs was lowest

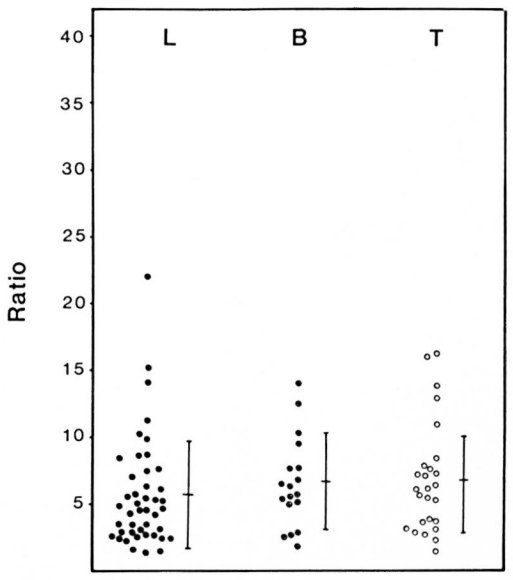

FIG. 3. Ratio of salivary IgA/IgG to serum IgA/IgG.

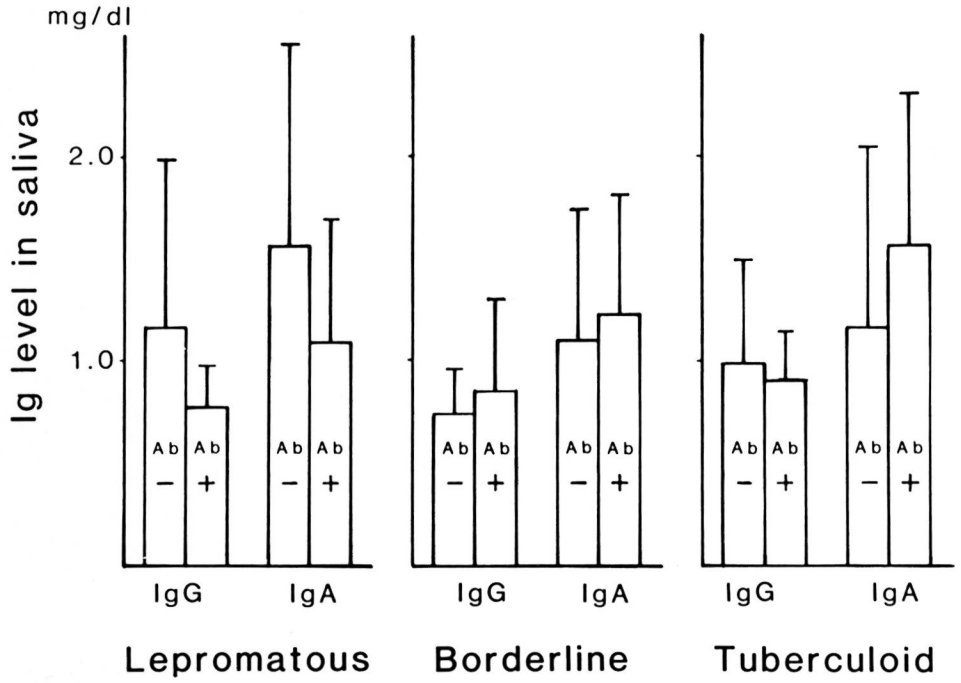

FIG. 4. Salivary Ig level and antibody (Ab).

in the L, middle in the B, and highest in the T cases, a reverse order as compared with that in the serum. The difference between 6.0% in L and 27.8% in T is statistically significant ($p < 0.02$). A cause of such a difference is clearly shown in the result of testing with anti-IgA fluorescent antibody. This antibody gave significant numbers of positive reactions in the saliva of every group of patients, but the percentage of positive reactions was far lower in L cases than in B and T cases ($p < 0.01$). The B and T percentages were also higher than those in the sera of the same patients. These findings indicate that *M. leprae*-specific IgA antibodies are found more frequently in the saliva than in the sera of patients with borderline or tuberculoid leprosy, and the antibodies are found less frequently in the saliva of patients with lepromatous leprosy.

A relationship between the serum and the salivary antibodies is expressed by the four possible combinations of results as shown in Table 3. The number of patients giving

TABLE 2. *FLA-ABS test with serum and saliva of leprosy patients.*

Type of leprosy	No. cases	Test with	Positive reaction with FITC-antibody to							
			Igs[a]		IgG		IgM		IgA	
			No.	%	No.	%	No.	%	No.	%
Lepromatous	50	Serum	49	98.0	47	94.0	45	90.0	9	18.0
		Saliva	3	6.0	3	6.0	1	2.0	6	12.0
Borderline	24	Serum	21	87.5	21	87.5	22	91.7	6	25.0
		Saliva	5	20.8	1	4.2	2	8.3	12	50.0
Tuberculoid	36	Serum	27	75.0	21	58.3	27	75.0	5	13.9
		Saliva	10	27.8	0	0	1	2.8	16	44.4
Total	110	Serum	97	88.2	89	80.9	94	85.5	20	18.2
		Saliva	18	16.4	4	3.6	4	3.6	34	30.9

[a] Mixture of IgG, IgM and IgA.

TABLE 3. *Serum and salivary antibodies to* M. leprae *in leprosy patients.*

Ig antibody class	FLA-ABS test with		Type of leprosy		
	Serum	Saliva	L (50)[a]	B (24)	T (36)
Igs	+	+	3	5	8
	+	−	46	16	19
	−	+	0	0	2
	−	−	1	3	7
IgG	+	+	3	1	0
	+	−	44	20	21
	−	+	0	0	0
	−	−	3	3	15
IgM	+	+	1	2	1
	+	−	44	20	26
	−	+	0	0	0
	−	−	5	2	9
IgA	+	+	1	2	3
	+	−	8	4	2
	−	+	5	10	13
	−	−	36	8	18

[a] Numbers in parentheses indicate the number of cases in each classification.

each of these results is shown in the table. The patterns of distribution are not significantly different in the tests with Igs or IgG or IgM. On the other hand, tests with IgA showed a different pattern in that this class of antibodies was positive in the saliva but negative in the serum in a significant number of patients. The numbers among the B and T cases were two or more times larger than those in the L cases. Salivary IgG and IgA levels in the group of patients who were positive for salivary IgA antibodies were compared with those in the group of patients who were negative for salivary IgA antibodies to ascertain whether or not the low frequency of these antibodies in lepromatous leprosy is due to a low level of salivary IgA. As shown in Figure 4, no significant difference in the average level of IgG or IgA was found in the two groups of patients, irrespective of the classification of leprosy. This may be the result of a wide range of values in the specimens of saliva, but it is unlikely that an average level of IgA in the antibody-negative patients is lower than that in the antibody-positive patients.

The percentage of salivary IgA antibody-positive cases was compared among the subgroups defined by clinical findings, and the results are shown in Table 4. Sex, age,

TABLE 4. *Salivary IgA antibody and clinical findings.*

Clinical findings	No. cases	IgA antibody positive cases	
		No.	%
Male	68	19	27.9
Female	42	15	35.7
39 yrs. old or less	30	7	23.3
40–59	31	10	32.3
60 yrs. or more	49	17	34.7
Active	32	5	15.6
Inactive	78	29	37.2[a]
Less than 5 yrs. treatment	66	28	42.4
5 and more yrs. treatment	44	6	13.6[b]
ENL present	10	2	20.0
ENL absent	100	32	32.0

[a] Significantly more than active group, $p < 0.05$, χ^2.
[b] Significantly less than the less than 5 year treatment group, $p < 0.01$, χ^2.

and the presence or absence of ENL at the time of this study did not give significant differences in the percentages. Salivary IgA antibodies were found more frequently in inactive cases and in those who had been treated for less than five years. The difference may be due to the fact that the lepromatous cases included 25 of the 32 active cases and 34 of the 44 cases which had been treated for five and more years. The cross-reactivity of the IgA antibodies in 22 saliva specimens collected from 4 L cases, 9 B cases, and 9 T cases were examined by using six species of mycobacteria which had also been used in the previous study ([4]). Results are shown in Table 5. Several specimens of unabsorbed saliva crossreacted with these

TABLE 5. *Crossreactivity of salivary IgA antibodies.*

Mycobacterial species	No. of positive reactions with	
	Unabsorbed saliva	Absorbed saliva
M. leprae	22[a]	22
M. tuberculosis	4	0
M. kansasii	2	0
M. marinum	3	1
M. smegmatis	4	0
M. phlei	2	0
M. avium	3	0

[a] Cases = 4 lepromatous, 9 borderline, 9 tuberculoid.

mycobacteria, but only one B case showed a crossreaction with *M. marinum* after the absorption. Therefore, the salivary IgA antibodies detected by the FLA-ABS test were found to be specific for *M. leprae.*

DISCUSSION

Total protein and immunoglobulin levels in the serum and the saliva of the leprosy patients shown in Table 1 were somewhat different from those reported by other authors ([5, 14]). The average levels in both serum and saliva showed no significant differences according to the classification of leprosy. This might be due to the inactive cases included in the respective groups of patients in this study. The total protein and immunoglobulin levels in the saliva shown in Table 1 are generally lower than those reported by other authors ([5, 14]). This may be explained by a different method for collecting the saliva and the use of ascorbic acid which stimulates salivary secretion. Such a stimulant should not be used for the determination of physiological levels of salivary proteins. However, the purpose of this study was fulfilled by knowing the relative difference in protein levels according to the classification of leprosy as well as the background of salivary antibody activity. Moreover, the use of ascorbic acid is convenient for collecting saliva within a short period of time and it helps in preventing microbial growth by reducing the pH (the minimum pH was 3.4). The pH was always controlled by the buffered medium at the time of the immunodiffusion and FLA-ABS tests, and the effect of ascorbic acid itself on these tests was found to be negligible.

The percentage of immunoglobulins in the saliva and the ratios of IgG and IgA in the serum vs saliva as shown in Figures 2 and 3 were not significantly different among the L, B, and T groups. As shown in Figure 4, salivary IgG and IgA levels were also not significantly different between the positive and negative FLA-ABS test groups, irrespective of the classification of leprosy. Accordingly, a deficiency of salivary IgA antibody against *M. leprae* in lepromatous leprosy cannot be explained by the level of immunoglobulins in the saliva. In the previous study ([3]), *M. leprae*-specific IgA antibody was scarcely found in the nasal washings of the patients with lepromatous leprosy; whereas it was positive in those patients with tuberculoid leprosy. Abnormal secretion of IgA was not found in these patients. Therefore, it is conceivable that secretory IgA antibody against *M. leprae* is deficient in lepromatous leprosy. The production of secretory antibody is considered to be an expression of local immunity ([17]). According to a review by Lamm ([10]), the secretion of IgA is thymus dependent. Therefore, the deficiency of cell-mediated immunity against *M. leprae* in lepromatous leprosy may be a cause of the deficient secretion of IgA antibody from the nasal and oral mucosa. The low frequency of serum IgA antibodies against *M. leprae* may also be associated with this mechanism.

On the other hand, nasal excretion of *M. leprae* has been reported by many investigators ([6, 7, 13, 16]), and the possibility of nasal transmission in leprosy has been discussed ([12]). Therefore, an alternative explanation of our findings could be that, even if local production of secretory IgA antibody was not impaired in lepromatous leprosy, almost all of the excreted antibodies might be neutralized by combining with *M. leprae* or its antigenic component discharged from the local lepromatous lesion. If all of *M. leprae* or its antigens are combined with the antibodies in the external secretion, the antigen-antibody complex may inhibit the adhesion of *M. leprae* to the mucous membrane and result in protection against reinfection or it may aid in the disposal of antigen in the same way as with other bacterial infections ([18]).

The percentage of positive FLA-ABS tests in saliva is not sufficiently high so that the test could be useful for the diagnosis of leprosy. However, saliva as a substitute for serum might be useful for the detection of subclinical infection in leprosy. It is anticipated that the production and secretion of salivary IgA antibodies may be induced by a local immune response against infection by a minute amount of *M. leprae,* an amount which might not be able to induce the production of an appreciable amount of circulating antibodies. This problem must be solved by future study.

SUMMARY

The technics of immunodiffusion and the fluorescent leprosy antibody absorption

(FLA-ABS) test were used to determine the levels of immunoglobulins and their antibody activities against *Mycobacterium leprae* in the serum and the saliva collected from a total of 110 patients with leprosy (50 lepromatous, 24 borderline, and 36 tuberculoid). The average levels of serum IgG, IgM, and IgA were not significantly different among these patients. In saliva, however, IgM was detected in only two cases with lepromatous leprosy and three tuberculoid cases. Salivary IgG and IgA levels and their ratios to those in the sera were not significantly different according to the classification of leprosy.

The percentages of positive FLA-ABS tests in the sera and saliva were compared by using fluorescent antibodies specific for IgG, IgM, and IgA, respectively. The results indicated that *M. leprae*-specific antibodies in the serum were mainly found in IgG and IgM and, less frequently, in IgA. IgG antibodies were found more frequently in lepromatous and borderline patients than in tuberculoid cases. On the other hand, salivary IgA antibodies against *M. leprae* were found in a significant number of specimens; whereas IgG and IgM antibodies were scarcely found. However, the percentage of positive FLA-ABS tests caused by salivary IgA antibodies was higher in the patients with tuberculoid or borderline leprosy than in those with lepromatous leprosy. A significant number of patients with tuberculoid or borderline leprosy secreted *M. leprae*-specific IgA antibodies into saliva without detection of circulating IgA antibodies. A deficiency in salivary IgA antibodies in lepromatous leprosy was not due to the lowered secretion of IgA in saliva, because the average levels of IgG and IgA did not show significant differences between the IgA antibody-positive and -negative patient groups, irrespective of the classification of leprosy. These findings coincide with the results of our previous study on nasal secretions collected from leprosy patients and suggest that secretory antibodies play an important role in the local defense mechanism of the mucous membrane against *M. leprae.*

RESUMEN

Se usaron las técnicas de la inmunodifusión y de la absorción del anticuerpo fluorescente (FLA-ABS) para medir los niveles de inmunoglobulinas y su actividad de anticuerpo contra *M. leprae,* en el suero y en la saliva de 110 pacientes con lepra (50 lepromatosos, 24 intermedios y 36 tuberculoides). Los niveles promedio de IgG, IgM e IgA en el suero, no difirieron significativamente entre los grupos de pacientes. Sin embargo, en saliva, la IgM sólo se encontró en dos casos de lepra lepromatosa y en 3 casos tuberculoides. Los niveles en saliva de IgG e IgA y su relación con los niveles séricos, tampoco difirieron significativamente entre los distintos tipos de lepra.

Comparando el porcentaje de pruebas FLA-ABS positivas en los sueros y salivas, con la clase (IgG, IgM e IgA) de anticuerpos específicos para *M. leprae,* se encontró que éstos fueron más frecuentemente de las clases IgG e IgM, y menos frecuentemente de la clase IgA. Los anticuerpos IgG fueron más frecuentes entre los pacientes lepromatosos e intermedios que entre los tuberculoides. En saliva, los anticuerpos anti-*M. leprae* de la clase IgA fueron más frecuentes que los de las clases IgG o IgM. Sin embargo, el porcentaje de pruebas FLA-ABS positivas debidas a anticuerpos salivales IgA fue más elevado entre los pacientes con lepra tuberculoide o intermedia, que entre los lepromatosos. Algunos pacientes con lepra tuberculoide o intermedia secretaron en su saliva anticuerpos IgA contra *M. leprae* sin tenerlos en su sangre. La deficiencia en los anticuerpos IgA salivales de los casos lepromatosos no se debió a una disminuída secreción de IgA porque, independientemente de la clasificación de la lepra, los niveles promedio de IgG e IgA entre los pacientes con anticuerpos IgA positivos y los negativos no mostraron diferencias significativas. Estos hallazgos coinciden con los resultados de un estudio previo sobre secreciones nasales colectadas de pacientes con lepra y sugieren que los anticuerpos secretorios juegan un papel importante en los mecanismos defensivos de las membranas mucosas contra el *M. leprae.*

RÉSUMÉ

On a eu recours aux techniques d'immunodiffusion et à l'épreuve d'absorption des anticorps lépreux fluorescents (FLA-ABS) pour déterminer les niveaux d'immunoglobulines et leur activité d' anticorps contre *Mycobacterium leprae,* dans du serum et dans de la salive recueillis chez 110 malades atteints de lèpre, dont 50 lépromateux, 24 dimorphes et 36 tuberculoïdes. Les taux sériques moyens d'IgG, d'IgM, et d'IgA ne différaient pas significativement chez ces malades. Toutefois, dans la salive, on a détecté des IgM chez deux cas seulement parmi les malades atteints de lèpre lépromateuse, et chez trois cas souffrant de lèpre tuberculoïde. Les taux salivaires d'IgG et d'IgA, de même que leur ratio par rapport aux taux observés dans le serum, ne présentaient pas de différence significative selon le type de la maladie.

Le pourcentage d'épreuves positives FLA-ABS dans le serum et dans la salive a été comparé en utilisant les anticorps fluorescents spécifiques pour, respectivement, IgG, IgM, et IgA. Les résultats ont montré que

dans le serum, les anticorps spécifiques pour *M. leprae* étaient principalement trouvés parmi les IgG et le IgM; on les trouvait également, moins fréquemment, parmi les IgA. Les anticorps IgG étaient détectés plus fréquemment chez les lépromateux et les malades dimorphes que chez les cas tuberculoïdes. Par ailleurs, des anticorps salivaires IgA contre *M. leprae* ont été relevés dans un nombre significatif d'échantillons, alors qu'il était rare d'observer des anticorps IgG et IgM. Néanmoins, le pourcentage d'épreuves positives FLA-ABS dû à des anticorps salivaires IgA était plus élevé chez les malades souffrant de lèpre tuberculoïde ou dimorphe que chez ceux atteints de lèpre lépromateuse. Un nombre significatif de malades avec la forme tuberculoïde ou la forme dimorphe de la maladie secrétaient des anticorps IgA spécifiques contre *M. leprae* dans la salive, sans qu'il soit possible de détecter des anticorps IgA circulants. La carence en anticorps salivaires IgA constatée dans la lèpre lépromateuse n'était pas dûe à une diminution de la secrétion des IgA dans la salive, car les taux moyens d'IgG et d'IgA ne présentaient pas de différences significatives entre les groupes de malades positifs pour les anticorps IgA et ceux qui étaient négatifs, et ceci sans égard à la classification de la lèpre. Ces observations confirment les résultats d'une étude antérieure menée sur des secrétions nasales recueillies chez des malades de la lèpre; elles suggèrent que les anticorps secrètoires jouent un rôle important dans les mécanismes locaux de défense contre *M. leprae* au niveau de la membrane muqueuse.

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