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ENZYME-LINKED IMMUNOSORBENT ASSAY OF SIgA IN WHOLE SALIVA OF HEALTHY SUBJECTS AND PATIENTS WITH ORAL DISEASES

BY

Kazuko SATO

ABSTRACT

The author tried to determine the secretory immunoglobulin A (SIgA) level in the whole saliva of healthy subjects and patients with oral diseases by the enzyme-linked immunosorbent assay (ELISA) technique. An ELISA system that was previously developed for determining the SIgA level in the pancreatic juice was modified and was proved to be applicable for the SIgA in the whole saliva. It was found that the SIgA in the whole saliva could be determined with excellent reproducibility and linearity when the samples were diluted 1,000-fold. This assay method was superior to the conventional techniques in terms of accuracy, covering a wide range SIgA levels in the saliva.

In the 74 healthy subjects, the SIgA level in the whole saliva did not show any major age- or sex-related difference. However, patients with leukoplakia, lichen planus and carcinoma in the oral cavity showed higher levels of SIgA. The elevated levels of SIgA in the saliva of these patients suggested that some local changes in immunological competence might occur due to prolonged exposure to the antigen in the oral cavity.

Key words: Secretory IgA (SIgA), Enzyme-linked immunosorbent assay, Saliva, Squamous cell carcinoma, Leukoplakia

INTRODUCTION

It is generally believed that the secretion of the saliva into the oral cavity works as a kind of immune system against extrinsic antigenic substances. Among other things, the secretory immunoglobulin A (SIgA), IgA and free secretory component (FSC) in the saliva have been considered to play major roles in this immune system. Thus far, various approaches to quantitate each of these substances have been tried. However, all conventional methods of quantitation have many problems with regard to their accuracy and simplicity. The SIgA levels cannot be determined by means of the single radial immunodiffusion (SRID) method because only the sum of the IgA and SIgA levels is quantitated by this method. The electro-immunodiffusion method is inconvenient as it takes a long time to quantitate, low levels and high levels of SIgA cannot be measured simultaneously and it cannot be used to analyze many samples at one time.

The purpose of the present study is to establish a new method to quantitate the SIgA in the whole saliva by the enzyme-liked immunosorbent assay (ELISA) that can be used for the clinical subjects. The author first performed a methodological evaluation and then determined the actual

佐藤和子：The First Department of Oral and Maxillofacial Surgery (Chief: Prof. S. SHIODA), Faculty of Dentistry, Tokyo Medical and Dental University (Tokyo Ika Shika Daigaku)
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SIgA levels in the whole saliva collected from 74 healthy volunteers and 77 patients with oral diseases.

Abbreviations:

SIgA: Secretory immunoglobulin A
FSC: Free secretory component
ELISA: Enzyme-linked immunosorbent assay

Materials and Methods

I. Collection of saliva and donors

Four to six dental cotton rolls were placed in the oral cavity for 15 to 30 minutes and then removed and centrifuged in specially devised centrifuge tubes developed by Kuboki et al., as shown in Fig. 1 (Kuboki et al., [1]), at 3,000 r.p.m. for 5 minutes. After determining the volume, the supernatant of the saliva sample was frozen at −20°C for storage. Immediately before analysis, the samples were thawed and centrifuged again at 3,000 r.p.m. for 5 minutes.

The materials for this study were obtained from 74 healthy volunteers who had no disease in the oral cavity (52 males and 22 females), 34 patients with squamous cell carcinoma of the oral cacity, 17 with leukoplakia, 18 with lichen planus and 8 with glossodynia (Table 1). The healthy subjects ranged in age from 16 to 82 years (mean: 32.8 years). The mean ages for each disease group were as follows: 56.4 years for squamous cell carcinoma, 56.2 for leukoplakia, 51.7 for lichen planus and 62.5 for glossodynia (Table 1).

II. Determination of SIgA level in saliva

The SIgA levels in the saliva samples were determined by the ELISA method using two commercial ELISA kits (Amano Co., Ltd, Nagoya), both of which were developed by Yamamoto et al. [2]. One kit is designed for SIgA determination in the pancreatic juice (hereafter called the pancreatic juice kit) and the other for SIgA determination in the blood (hereafter called the blood kit). The pancreatic juice kit contains the soluble human SC-specific antibody and the blood kit contains the IgA-specific antibody.

A. Application of column technique for quantitation of salivary SIgA

Two samples were comparatively assayed after dilution to 0.03 μl/tube, 0.1

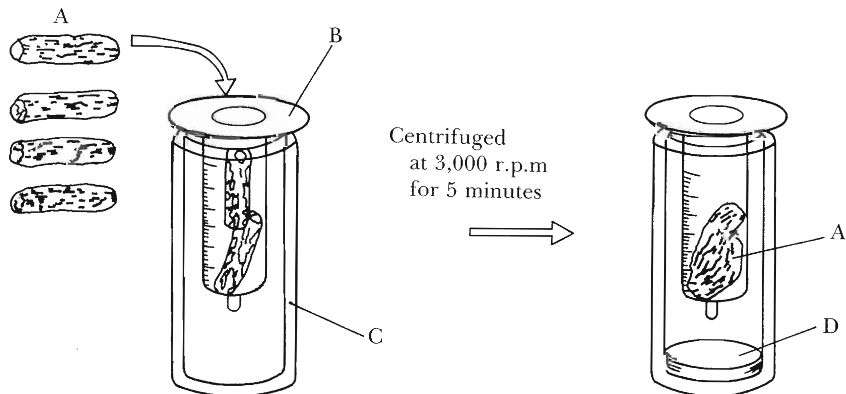

Fig. 1. Collection of Saliva by Kuboki Method
A: Dental cotton rolls containing saliva
B: Disposable syringe
C: Thick glass centrifuge tube
A′: Dental cotton rolls after removing saliva
D: Clear saliva collected

(Kuboki, Y. [1])

Table 1. Subjects and Age Distribution

	Male	Female	Total	Age	
				Min.–Max.	Mean
Healthy subjects	52	22	74	16–82	32.8
Squamous cell carcinoma	23	11	34	33–72	56.4
Leukoplakia	8	9	17	38–73	65.2
Lichen planus	4	12	18	36–70	51.7
Glossodynia	1	7	8	58–66	62.5

μl/tube and 0.3 μl/tube with both the pancreatic juice and the blood kits. The procedures described below are those using the pancreatic juice kit, except for the words or phrases in the square brackets [ ] which indicate the modifications in the method using the blood kit.

1. Aliquots (0.1 ml) of both standard SIgA solution and diluted test samples were reacted with 0.5 ml of enzyme-labeled IgA antibody [or enzyme-labeled SC antibody] solution.

2. After a 90-minute reaction at 37°C, 0.5 ml of the test sample bound to the enzyme-labeled IgA antibody was passed through a column that was packed with a carrier of insoluble human SC-specific antibody. In this way, the SIgA was bound to the enzyme-labeled IgA antibody [enzyme-labeled SC antibody] in the column.

3. The column was eluted with 1.0 ml of elution buffer. Thus, the enzyme-labeled IgA antibodies [enzyme-labeled SC antibodies] not bound to the SIgA were removed.

4. The column was eluted with 0.5 ml of enzyme substrate (o-nitrophenyl-β-D-galactoside) solution. This was followed by 90-minute [overnight] enzymatic reaction at 25°C.

5. One milliliter of the coloring reagent was passed through the column twice. The products of the enzymatic reaction in the columm were eluted with the coloring reagent.

6. The eluate was examined for absorbance at 420 nm with a spectrophotometer.

7. The SIgA levels of the test samples were determined by the calibration of the data using the standard SIgA solution.

B. Interference by FSC

Methods: To examine the degree of the interference caused by FSC, the author determined the extent of the inhibition of the binding of anti-SIgA and IgA with the FSC by assaying a mixture of standard SIgA and FSC (0.4, 0.75, 1.5, 2.9 and 5.0 μg/tube) with the blood and pancreatic juice kits.

C. Determination of optimum dilution

Methods: Four samples were diluted 100, 200, 400, 1,000 and 2,000 times followed by quantitation with the pancreatic juice kit.

D. Linearity of enzyme immunoassay

Methods: For the analysis of SIgA (0–120 ng/ml), a standard solution and a whole saliva sample containing 45 μg SIgA per ml were used. Each test solution (0.01, 0.03, 0.1 and 0.3 μl/tube) was diluted 1,000 times, followed by quantitation of the SIgA level with the pancreatic juice kit.

E. Reproducibility

Methods: Two samples (Sampe 1 and Sample 2) were assayed eight times with the pancreatic juice kit after 1,000-fold dilution with a buffer solution. The mean value and the coefficient of the variation were obtained to examine the reproducibility. Furthermore, standard SIgA solu-

tions (150, 300, 600 μg/ml) were added to two saliva samples containing 45 μg or 183 μg/ml of SIgA. The recovery rates of the SIgA were determined by the same method.

Results

I. Examination for determination of SIgA level

A. Selection of columns applicable for quantitation of salivary SIgA

1. Pancreatic juice kit

The absorbances of Sample 1 were 0.17 nm at a dilution of 0.03 μl/tube, 0.40 nm at 0.1 μl/tube and 0.72 nm at 0.3 μl/tube. The absorbances of Sample 2 were 0.11 nm at a dilution of 0.03 μl/tube, 0.26 nm at 0.1 μl/tube and 0.53 nm at 0.3 μl/tube (Fig. 2). The curve obtained by the assay with the pancreatic juice kit was parallel to the standard curve.

2. Blood kit

The absorbances of Sample 1 were 0.20 nm at a dilution of 0.03 μl/tube, 0.22 nm at 0.1 μl/tube, and 0.55 nm at 0.3 μl/tube. The absorbances of Sample 2 were 0.28 nm at a dilution of 0.03 μl/tube, 0.31 nm at 0.1 μl/tube, and 0.52 nm at 0.3 μl/tube (Fig. 3). The curve obtained by the assay with the blood kit crossed the standard curve.

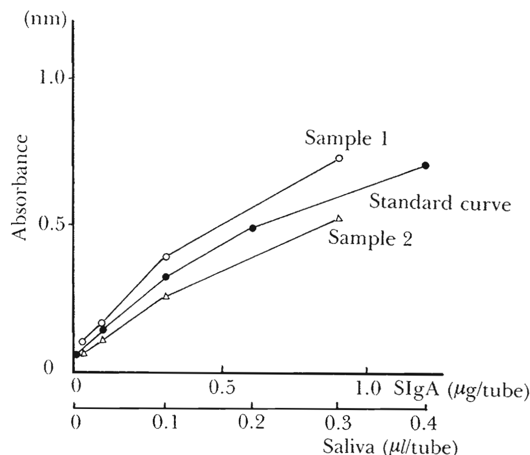

Fig. 2. Comparison With SIgA Standard Curve and Two Saliva Samples Curves (Pancreatic Juice Kit)
The curves of two saliva samples obtained by assay using the pancreatic juice kit are parallel to the standard curve.

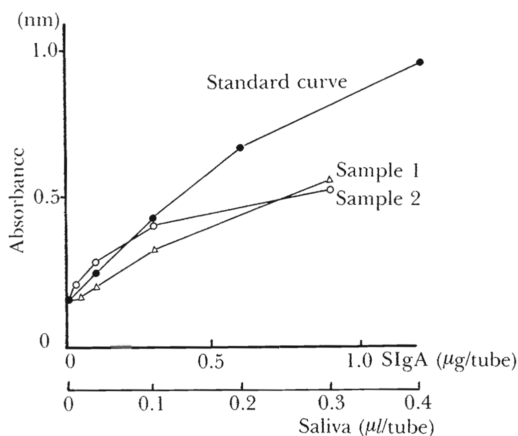

Fig. 3. Comparison With SIgA Standard Curve and Two Saliva Samples Curves (Blood Kit)
The curves of two saliva samples obtained by the assay using the blood kit are not parallel to and cross the standard curve.

B. Interference by FSC

When assayed with the pancreatic juice kit, the extent of the inhibition increased gradually as the FSC content increased stepwise to 0.4, 0.75, 1.5, 2.9 and 5.0 μg/tube: the percent of inhibition was 0% at 0.4 μg/tube, 2.5% at 0.75 μg/tube, 6.0% at 1.5 μg/tube, 12.5% at 2.9 μg/tube and 21% at 5.0 μg/tube, indicating that the interference in the determination of SIgA was at a minimum when there was a small FSC content (Fig. 4). When assayed with the blood kit, on the other hand, the rate of obstructed binding was 81% at an FSC content of 0.4 μg/tube, 83% at 0.75 μg/tube, 86% at 2.9 μg/tube and 86% at 5 μg/tube, indicating that the presence of a small amount of FSC was sufficient to cause interference in SIgA quantitation (Fig. 4).

C. Determination of optimum dilution

The absorbance values of the SIgA contents of four samples could be mea-

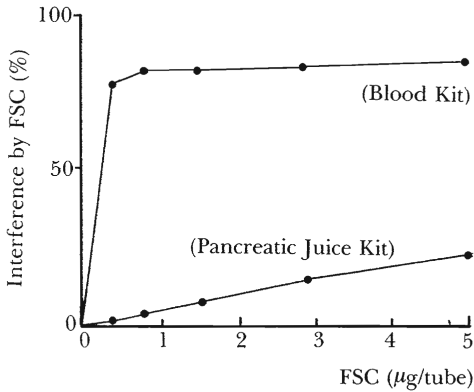

Fig. 4. Interference by Free Secretory Component in SIgA Determination

$$\text{Interference rate (\%)} = \frac{\text{SIgA value (FSC=0\%)} - \text{measured SIgA value (FSC=}\times\text{\%)}}{\text{SIgA value (FSC value=0\%)}} \times 100$$

When assayed using the pancreatic juice kit, interference by an increasing amount of the FSC in the determination of SIgA was at a minimum. However, the presence of a small amount of the FSC was sufficient to cause interference in SIgA determination when assayed using the blood kit.

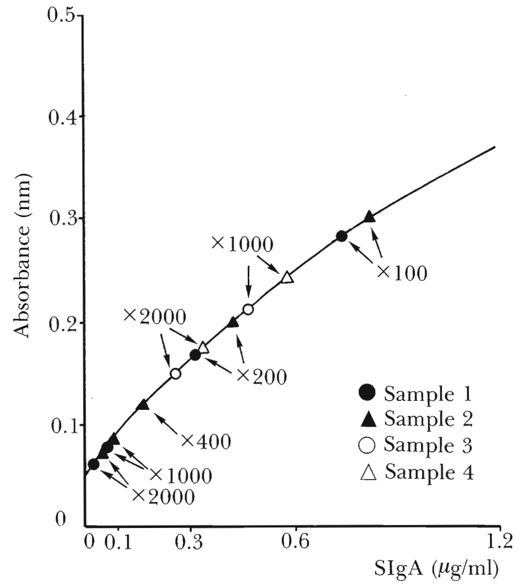

Fig. 5. Determination of Optimum Dilution
Four saliva samples were assayed using the pancreatic juice kit after 100-, 200-, 400-, 1,000-, and 2,000- fold dilutions.
From this result, the 1,000-fold dilution of saliva was selected for the determination of SIgA.

sured with the standard curve after 1,000- or 2,000-fold dilution, but two of the four samples could not be measured with the standard curve after 400-fold dilution or less (Fig. 5).

In addition, the patients' maximum SIgA levels were shown to be about 1,000 μg/ml by the other examinations, so they could not be measured at 500-fold dilution or less.

It was thus concluded that the determination of the SIgA level would be suitable after a 1,000-fold dilution of the samples (Fig. 5).

D. Linearity of enzyme-linked immunosorbent assay

When the SIgA levels of 0 to 120 ng/ml were examined with a standard solution and a sample of the total saliva containing 45 μg of SIgA per ml (diluted to 0.01, 0.03, 0.1 or 0.3 μl/tube), the SIgA contents of

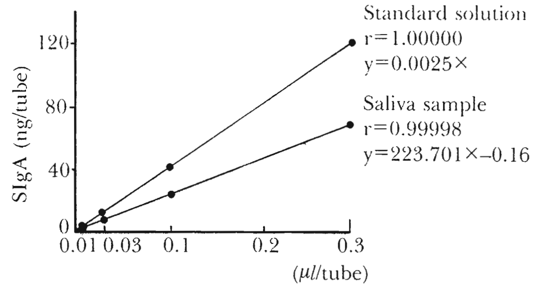

Fig. 6. Linearity of SIgA Determination
Correlations between increasing volumes of the standard SIgA solution or a saliva sample and the values determined.
r=correlation coefficient

these 4 dilutions were 4, 12, 40 and 120 ng/tube, respectively, for the standard solution and 2, 6.7, 22 and 67 ng/tube, respectively, for the total saliva sample. The coefficient of correlation (r) was 1.00000 for the standard solution (Y=0.0025x) and 0.99998 for the saliva

sample (y=223.70x–0.16). Thus, a good linearity was obtained (Fig. 6).

E. Reproducibility

When the two samples were assayed eight times with the pancreatic juice kit, Sample 1 showed a mean level of 243.8 μg/ml and a coefficient of variation of 3.5%, while Sample 2 showed a mean level of 517 μg/ml and a coefficient of variation of 3.9% (Table 2). These results showed an excellent reproducibility. The results of the recovery rates of the added standard SIgA to sample saliva were 93.3% to 100.3% (Table 3). Thus, sufficient recovery rates were obtained.

Regarding the above results, it was decided that each sample of the whole saliva should be diluted 1000 times with a buffer solution followed by the quantitation of the SIgA with the pancreatic juice kit and that this measuring method was simple in operation with excellent accuracy in measuring the SIgA levels.

Table 2. Reproducibility

Number	SIgA (μg/ml) Sample 1	Sample 2
1	250.0	510.0
2	250.0	490.0
3	230.0	530.0
4	250.0	560.0
5	230.0	525.0
6	250.0	510.0
7	250.0	500.0
8	240.0	510.0
Mean value	243.8	517.0
C.V. (%)	3.5	3.9

C.V.: Coefficient of variation

II. SIgA level in healthy subjects

The SIgA level in the whole saliva in the healthy subjects ranged from 30 to 290 μg/ml (mean: 114.7 μg/ml).

In the males, the level ranged from 10 to 250 μg/ml (mean: 116.2 μg/ml) and, in the females, it ranged from 10 to 290 μg/ml (mean: 111.3 μg/ml) (Table 4). The mean level was 107.8 μg/ml for the subjects between 16 and 29 years of age, 126.6 μg/ml for those between 30 and 39 years old, 102.5 μg/ml for those aged between 40 and 49 years and 127.9 μg/ml for those over the age of 50 years (Table 5). The mean levels classified by the volume of the collected saliva were 92.0 μg/ml for the volume between 0.11 and 0.2 ml/min, 98.4 μg/ml for the volume between 0.21 and 0.50 ml/min, 110.7 μg/ml for the volume between 0.51 and 1.00 ml/min and 77.2 μg/ml for the volume of over 1.00 ml/min (Table 6). There were no statistically significant differences among sex, different age groups and different volume groups.

III. SIgA levels in samples from patients (Fig. 7)

The SIgA levels in the whole saliva from those with diseases ranged from 20 to 510

Table 3. Recovery Rates

Standard SIgA added	Sample 1 containing 45 μg/ml	Recovery rate (%)	Sample 2 containing 138 μg/ml	Recovery rate (%)
150 μg/ml	195 μg/ml	100.0	323 μg/ml	93.3
300	345	100.0	478	98.3
600	638	98.9	785	100.3

$$\text{Recovery rate (\%)} = \frac{\text{Total SIgA value measured} - \text{SIgA value in original SIgA value}}{\text{added SIgA}} \times 100$$

Table 4. SIgA Level in Whole Saliva (Healthy Subjects)

Sex	Number	SIgA level (μg/ml) Mean	s.d.
Male	52	116.2	64.8
Female	22	111.3	67.8
Total	74	114.7	65.3

s.d.: Standard deviation

Table 5. SIgA Level in Whole Saliva (Healthy Subjects)

Age	SIgA level (μg/ml) Mean	s.d.
16–29	107.8	66.0
30–39	126.6	57.5
40–49	102.5	15.0
50–82	127.9	71.1

s.d.: Standard deviation

Table 6. SIgA Level in Whole Saliva (Healthy Subjects)

Volume of collected saliva (ml/min)	Number	SIgA level (μg/ml) Mean	s.d.
0.11–0.20	5	92.0	69.6
0.21–0.50	19	98.4	48.6
0.51–1.00	23	110.7	73.2
1.01–2.00	9	77.2	35.6

s.d.: Standard deviation

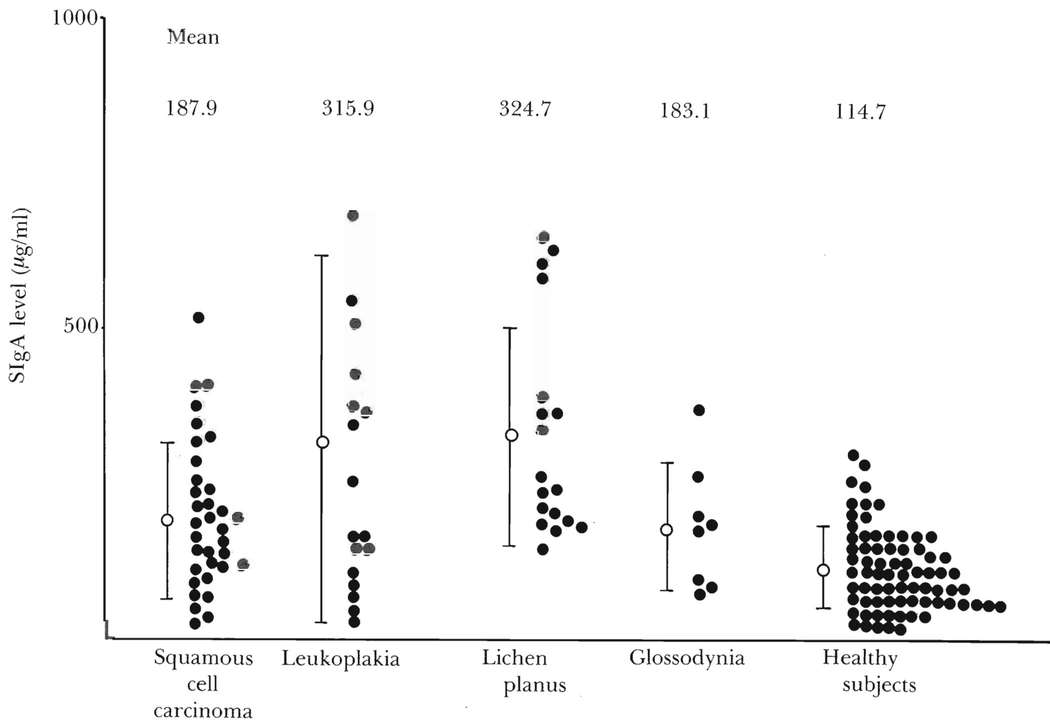

Fig. 7. SIgA Level in Whole Saliva.
(Healthy Subjects and Patients with Oral Diseases)
(Mean ± S.D.)

μg/ml (mean=187.9 μg/ml) for squamous cell carcinoma, from 20 to 670 μg/ml (mean=315 μg/ml) for leukoplakia, from 140 to 620 μg/ml (mean=324.7 μg/ml) for lichen planus and from 75 to 370 μg/ml (mean=100.7 μg/ml) for glossodynia.

There were statistically significant differences between the control and diseased subjects for carcinoma, leukoplakia and lichen planus but not for glossodynia. The SIgA levels of the leukoplakia and lichen planus patients were higher than in the carcinoma patients but the author found no statistically significant differences among them.

Discussion

Enzyme immunoassay is a type of labeled immunoassay that quantifies antigens or antibodies by the antigen-antibody reaction through the use of an enzyme for labeling. Because of its high specificity, this technique has been widely used for the quantitation of various components in the organisms.

In the present study, the author tried to establish a new method for quantifying the salivary SIgA levels using commercially available enzyme immunoassay kits (Amano Kits) designed for the quantitation of the blood or pancreatic juice SIgA (Ishiguro et al. :2]; Yamamoto et al. [3]).

In the selection of columns for the blood kit or pancreatic juice kit, the same sample was assayed with both kits. This additional assay disclosed that the consistency curve was similar to the standard curve when the assays were performed with the pancreatic juice kit but that the consistency curve crossed the standard curve when the blood kit was used. This difference can be explained as follows: The binding of the enzyme-labeled SC antibody to the SIgA may be inhibited in the presence of the FSC when using the blood kit. Therefore, the author determined the inhibition of the anti-SIgA-SIgA reaction with the FSC by assaying a mixture of standard SIgA and FSC with the blood and pancreatic juicekit. When assayed with the pancreatic juice kit, the rate of obstruced binding was less than 3% at the FSC level of 10 μg/ml. As the FSC level in the saliva was found to be less than 0.3 μg/ml in a 1,000-fold dilution in our other study, there was no obstructed binding by the FSC with the pancreatic juice kit. When assayed with the blood kit, on the other hand, the rate of obstructed binding was 81% with an FSC content of 0.4 μg/tube, indicating that the presence of a small amount of FSC was enough to cause interference in SIgA quantiation. These results indicated that the pancreatic juice kit was appropriate for the quantitation of the salivary SIgA. It was also found that the use of this kit allowed quantitation of the salivary SIgA in about six hours, shorter than the time needed with the conventional methods (Brandtzaeg [4, 5]).

When assayed with the pancreatic juice kit, the standard curve was relatively linear at the SIgA levels of 0–0.3 μg/dl (=1.00000, Y=0.0025X) (X: volume of collected saliva, Y: actually measured values of SIgA) but tended to show a slightly convex form at levels of over 0.3 μg/dl. If the quantitation were to be performed utilizing this standard curve, the samples of the whole saliva after a 1,000-fold dilution could be quantified and would be suitable because of the relatively small dilution rate and lack of interference by the FSC.

Sufficient recovery rars of the standard SIgA added to the saliva samples were obtained by this method.

The coefficients of variation for the two samples were 3.5% and 3.9%, respectively, in contrast to the figure of about 20% recorded as the maximum coefficient of variation with the SRID technique, a major

technique used for SIgA quantiation. Thus, the technique in this study is superior to the SRID technique in terms of accuracy and seems to be comparable to the light-scattering method (coefficient of variance: 2.3–3.9%) and the turbidimetric immunoassay (1.2–6.7%), which are considered to be highly accurate techniques for IgA quantitation (Brandzaeg [6]).

In the healthy subjects, the mean and SD of SIgA in the whole saliva were 114.7 and 65.3 μg/ml, respectively. There was no difference between the males and females or between the age groups. Analysis of the relationship between the volume of collected saliva and the SIgA level disclosed that the SIgA level tended to be higher at a salivary volume of 0.11–2.00 ml/min than at volumes below 0.1 ml/min or over 0.61 ml/min. However, no statistically significant difference was noted in the SIgA level among the different volumes of the collected saliva. In the past studies, the mean of the SIgA level in the whole saliva of healthy subjects was 24.7 mg/dl by the immunoelectrophoretic determination by Tsukuda [7] and 73.6–128.0 mg/l by enzyme immunoassay by Ishiguro et al. [2]. The results of this study were almost the same as those of Ishiguro but were a little less than of those of Tsukuda. The reason for these differences could not be determined but it might depend upon the small number of healthy subjects in the other studies.

In the past studies that compared the IgA levels in the whole saliva and parotid saliva between the cancer patients and healthy controls, the IgA levels were higher in the cancer patients (Brown et al. [8]). Furthermore, Rajendran reported that the levels of serum IgA, IgD and IgE were found to be elevated both in oral submucous fibrosis and in oral cancer and that the oral submucous fibrosis can be an intermediate stage in the transformation of a normal cell to oral malignancy (Rajendran et al. [9]). The serum IgA levels in the patients with nasopharynegeal carcinoma were elevated more than in the healthy controls (Henle et al. [10]). However, they evaluated the IgA levels that contained both IgA and SIgA. There were no reports on the SIgA levels in the saliva of patients with oral cancer, leukoplakia or lichen planus. The SIgA level in this study was, on the whole, higher in the patients with oral diseases, except for those with glossodynia, than in the healthy ones. Patients with carcinoma, leukoplakia and lichen planus showed SIgA levels that were significantly higher than those in the healthy ones. Among the disease groups, the SIgA levels of the leukoplakia patients were the same as those of the lichen planus patients and the SIgA levels of the leukoplakia and lichen planus patients were higher than those of the carcinoma patients. But there were no statistically significant differences among the subjects with carcinoma, leukoplakia and lichen planus.

From these results, it has been concluded that measuring the SIgA by enzyme immunoassay is both simple and superior to the conventional techniques in terms of accuracy and application and that the immune reaction in the oral cavity may vary between the healthy subjects and patients with oral diseases and among the disease groups.

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References

1) Kuboki, Y.: Method of collected saliva and biochemical examination of saliva. Den. Journal, 16: 725–732, 1982 (in Japanese).
2) Yamamoto, R., Kimura, S., Hattori, S., Ishiguro, Y., and Kato, K.: Column enzyme immunoassay for secretory immunoglobulin A in serum. Clin. Chem., 29: 151–153, 1983.
3) Ishiguro, Y., Kato, K., and Ito, T.: Sensitive solid phase enzyme immunoassay for human IgA, secretory IgA and secretory component. Clinical Chimica Acta, 116: 237–243, 1981.
4) Brandtzaeg, P.: Human secretory immunoglobulins. 4. Quantitation of free secretory piece. Acta Path. Microbiol. Scand., Section B, 79: 189–203, 1971.
5) Brandtzaeg, P.: Human secretory immunoglobulins. 3. Immunochemical and physicochemical studies of secretory IgA and free secretory piece. Acta. Path. Microbiol. Scand., Section B. 79: 165–188, 1971.
6) Brandtzaeg, P., Fjellanger, I., and Gjeruldsen, S. T.: 1. Salivary secretions from individuals with normal or low levels of serum immunoglobulins. (Human secretory immunoglobulins). Scand. J. Haemat., Suppl. No. 12, 1970. Munksgaard, Copenhagen.
7) Tsukuda, T.: Development of new quantitative methods for assays of SIgA, IgA and FSC and results of the quantitative analyses of these components in oral diseases. Shikoku Acta Med., 37: 253–265, 1981 (In Japanese).
8) Brown, A. M., Lally, E. T., and Frankel, A.: IgA and IgG content of the saliva and serum of oral cancer patients. Archs. Oral Biol., 20: 395–398, 1975.
9) Rajendran, R., Sugathan, C. K., Remani, P., Ravindran, A., and Vijayakumar, T.: Cell-mediated and humoral immune responses in oral submucous fibrosis. Cancer, 58: 2628–2631, 1986.
10) Henle, G., and Henle, W.: Epstein-Barr virus-specific IgA serum antibodies as an outstanding feature of nasopharyngeal carcinoma. Int. J. Cancer, 17: 1–7, 1976.