Determinant of Immunoglobulin A in Saliva by Immunobead Enzyme-Linked Immunosorbent Assay: Comparison with Single Radial Immunodiffusion

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For the quantitative measurement of immunoglobulin A in human saliva, an immunobead enzyme-linked immunosorbent assay was investigated in detail. The method proved to be fast and sensitive, and it gave reproducible results. There was a strong correlation with results obtained by a single radial immunodiffusion method. The immunobead method gave 1.8 times higher values than the single radial immunodiffusion method with a lower correction factor for low-immunoglobulin A saliva and a higher factor for immunoglobulin A-rich saliva.

Secretary immunoglobulin A (s-IgA) is the principal immunoglobulin in the external secretions of humans and is of importance in the prevention of a variety of local and systemic diseases (3, 7). To quantify this important protein, the Mancini technique (5), a radial immunodiffusion assay (RID), has been widely used. However, several investigators have pointed out problems in using immunodiffusion methods owing to difficulties in preparing standards with exactly the same molecular size as the IgA present in the secretions (1, 4). The presence of polymeric forms, including J-chains and secretory components, complicates the procedure. One approach sometimes used to overcome the problem is to reduce the s-IgA polymers into monomer units before immunodiffusion or electrophoresis, but this technique also has its drawbacks as pointed out by Peppard (8). Therefore, quantifying IgA by techniques other than immunodiffusion should be of particular value. Some such methods have recently been described. Peppard (8), working with IgA from the body fluids of rats, has obtained accurate results with a radioimmunoassay system. Sack et al. (9), using mostly human colostrum and only a few saliva samples, have presented an immunobead enzyme-linked immunosorbent assay (ELISA). Since the ELISA is used often in our laboratories for several other purposes, we decided to adopt the method of Sack for our routine determinations of IgA in human saliva. In trying to do this, we found it necessary to evaluate the method in much deeper detail. This report describes the methodological standardization of the immunobead ELISA as it relates to measuring salivary IgA.

Whole saliva was collected from three groups of subjects: 9 children aged 3 to 12, 14 adults aged 26 to 59, and 4 hypogammaglobulinemic patients (2). The saliva samples, obtained after paraffin chewing for 5 min, were immediately frozen. Before use, the samples were thawed, heated at 56°C for 30 min, and centrifuged at 1,300 × g for 20 min. Also included was a saliva pool obtained after mixing the individual samples from eight other healthy adults.

The following reagents were used. Human serum immunoglobulin (Behringwerke AG, Marburg, West Germany) containing 2.39 mg of IgA per ml was used as the standard. The immunobeads, consisting of polyacrylamide beads with anti-human IgA (α-chain specific), were obtained from Bio-Rad Laboratories (Richmond, Calif.). Swine anti-human IgA serum (α-chain), labeled with alkaline phosphatase, was obtained from Orion Diagnostica (Helsinki, Finland), and the substrate p-nitrophenylphosphate was purchased from Sigma Chemical Co. (St. Louis, Mo.).

For the Mancini RID technique (5), agarose (Miles Laboratories, Ltd., Stoke Poges, England) containing 25 μl of anti-human IgA per ml (anti-α-chain, Behringwerke AG) was used. Each well was filled with a 5-μl sample. The mean standard deviation (SD) for 23 saliva samples run in quadruplicate was 0.14 with a range of 0 to 0.66 (Table 1).

According to Sack et al. (9), the immunobead ELISA consists of the following main steps: the sample is diluted in phosphate-buffered saline (PBS) containing fetal calf serum (FCS) and mixed with the immunobead suspension. After incubation, the beads are washed, and the en-

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zyme-labeled anti-human IgA is added. Incubation and washing are again performed, and then the substrate, p-nitrophenylphosphate, is added. The reaction is stopped with NaOH. After centrifugation, the absorbance of the supernatant is measured at 405 nm.

Several steps of the procedure were investigated in detail. After numerous preliminary experiments, we decided to use disposable plastic tubes (14 by 110 mm; Nunc, Roskilde, Denmark) for the experiments and to read the absorbance in a Beckman model 35 spectrophotometer. Glass test tubes were also acceptable, but Mini-sorp tubes (Nunc) were not suitable because the beads were lost too easily after centrifugation. Trials with a more time-efficient system combining micro-ELISA plates and Titertek Multiskan (Flow Laboratories, Inc., McLean, Va.) technology were abandoned because the range of values for quadruplicate samples was widened significantly.

When absorbance was plotted against titrated concentrations of the serum IgA standard, Sack et al. (9) obtained a straight-line relationship between 10 and 100 ng of IgA per ml. In our hands, 100 ng of IgA yielded an absorbance which was too low. Consequently, readings over 75 ng/ml were not allowed. This was true also when the 1% FCS was excluded from the PBS. Dilution of the saliva samples to 1:500 or 1:1,000 usually yielded a concentration of IgA appropriate for these assays.

Two variants of the ELISA were used for comparison with the Mancini RID: with and without FCS in the PBS. The 23 samples were tested in quadruplicate. The results showed a strong correlation between the methods: \( r = 0.77 \) for the Mancini RID compared with the ELISA with FCS, and \( r = 0.86 \) for the Mancini RID compared with the ELISA without FCS.

Higher values for IgA were obtained with the ELISA methods than with the Mancini RID. Based on identical saliva samples, the mean values for the ELISA without FCS were 1.8 times higher than for the Mancini RID, and values for the ELISA with FCS were 1.9 times higher (Fig. 1). IgA in the four saliva samples from the hypogammaglobulinemic patients was not detected by the Mancini RID but was measured at 0, 0.18, and 0.46 mg/100 ml by the ELISA. These four samples were not included in the calculations of the regression curves.

After some further methodological controls, the following procedure was adopted as our standard. Details different from the procedure of Sack et al. (9) or not mentioned by them are italicized. Saliva was diluted in PBS (pH 7.2) without FCS. For adults, a dilution of 1:1,000 was used, and for children, who usually have lower levels of IgA, the dilution was 1:500. A 1-ml sample was mixed with 100 \( \mu l \) of immuno-bead suspension (2.5 mg/ml of PBS). After incubation for 2 h at 37°C, the beads were washed three times in 2 ml of PBS-Tween (0.5 ml of Tween 20 and 0.2 g of NaN3 per liter of PBS). Each time, refrigerated centrifugation was performed at 500 \( \times \) g for 3 min. A 500-\( \mu l \) sample of the enzyme-labeled anti-human IgA, usually at a

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**TABLE 1. Concentrations of IgA in saliva samples from children and adults as determined by RID and by immunobead ELISA performed with or without FCS in dilution buffer**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>RID</th>
<th>Immunobead ELISA</th>
<th>Immunobead ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>With FCS</td>
<td>Without FCS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>X</td>
<td>SD</td>
</tr>
<tr>
<td>Children</td>
<td>( n = 9 )</td>
<td>1.96*</td>
<td>0.12</td>
</tr>
<tr>
<td>Adults</td>
<td>( n = 14 )</td>
<td>2.48</td>
<td>0.15</td>
</tr>
<tr>
<td>Total</td>
<td>( n = 23 )</td>
<td>2.27</td>
<td>0.14</td>
</tr>
</tbody>
</table>

* Means of all means of quadruplicates.

**FIG. 1. Comparison between determination of IgA in saliva by immunobead ELISA (no FCS) and by single RID. Dots represent single saliva samples from adults (●), from children (▲), and from hypogammaglobulinemic subjects (▲).**
concentration of 1:400 in PBS-Tween, was added, and incubation was performed for 45 min at 37°C. The beads were again washed three times. Then, 1 ml of p-nitrophenylphosphate (1 mg/ml in 10% diethanolamine buffer, pH 9.8) was added and mixed. Incubation time was usually 20 min at 37°C unless the visible color reaction was so weak that the incubation time had to be prolonged. The reaction was stopped by adding 250 μl of 3 M NaOH, and the suspension was cleared by refrigerated centrifugation for 5 min at 1,300 × g. Absorbance was measured at 405 nm. Always included were a frozen stock saliva pool standard and a serum standard of 60 ng of IgA per ml.

In tests performed in this way, the SDs based on 10 parallel samples were 0.47, 0.30, and 0.18 for three different saliva samples. The mean SD for 23 saliva samples tested in quadruplicate on different days was 0.29, with a range of 0.07 to 0.65 (Table 1).

As mentioned above, the ELISA yielded higher values than the Mancini RID. These results were expected since in the ELISA, both IgA molecules of the secretory dimer should be available for the binding of anti-human IgA antibodies. Moreover, with the ELISA, there is no gel diffusion stage during which differences in the sizes and configurations of molecules may affect the results. It is a well-known fact that the RID underestimates the IgA content in secretions when a 7-S standard is used (1). To convert the RID values to an approximate 11-S standard, multiplication with a factor of 3 is often used. This type of conversion has been opposed by Mandel (6). He has pointed out that standard curves for immunodiffusion based on serum IgA and s-IgA are not parallel. Therefore, a lower correction factor should be used for samples with low s-IgA content, and a higher factor should be used for s-IgA-rich samples. Mandel recommended a range of correction factors from 1.2 to 4.6 instead of the fixed factor of 3. Our data support this suggestion. Our overall correction factor for the RID was calculated to be 1.8. However, if only saliva samples from the children were included, the mean factor would be 1.6. For the adults, who have saliva richer in IgA, the mean factor would be 1.9 with a range of 0.98 to 2.67. The lowest value was obtained for a subject with only 1.48 mg of IgA per 100 ml; the highest correction factor was for a subject with a high amount of IgA, 6.15 mg/100 ml. To illustrate this point, Fig. 2 shows the distribution of samples when the different mean correction factors have been used for samples from children (1.6) and from adults (1.9). It should be emphasized, however, that the immunobead ELISA is a much more sensitive and correct technique than the RID and that the calculations comparing correction factors are of limited interest when the ELISA is used as the sole technique for IgA determinations. Instead, because of the sensitivity of the ELISA, it is of the utmost importance to control carefully variables such as collection procedures, sample handling, etc., to avoid significant discrepancies in the results.

Collectively, our data strongly support the immunobead ELISA as a rapid and highly sensitive method for the determination of IgA in secretions. They also illustrate that similar results are not always obtained from RID assays after correction with the commonly used factor of 3. Moreover, in addition to being both time and cost efficient, the immunobead ELISA offers the opportunity to use greatly diluted samples in comparison to the undiluted saliva samples required for the Mancini RID, our previous standard method.

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LITERATURE CITED


