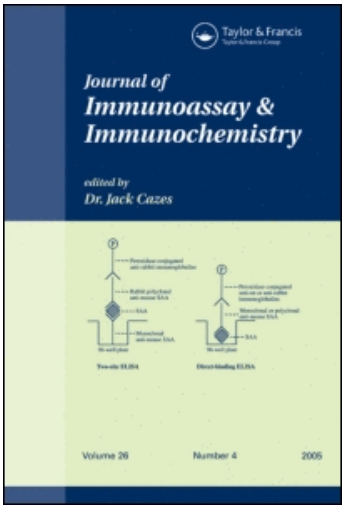

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Roy B. Johnson Jr.[ab]; Junda Liu[ab]
[a] Department of Pathology, Hospital of Scripps Clinic, La Jolla, CA [b] Beijing Medical College, Beijing, Peoples Republic of China

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THE APPLICATION OF ENZYME IMMUNOASSAY TO THE STUDY OF SALIVARY IGA

Roy B. Johnson, Jr. and Junda Liu
Hospital of Scripps Clinic, Department of Pathology
10666 North Torrey Pines Road, La Jolla, CA. 92037 and
Beijing Medical College, Beijing, Peoples' Republic of China

ABSTRACT

In the study of salivary IgA, solid-phase enzyme immunoassay has been able to show that 1) the assay is reliable, 2) minimal loss of IgA occurs from centrifuging or by elimination of insoluble mucins, 3) IgA can be easily measured in fractions from sucrose gradient sedimentation, 4) freezing alone is sufficient to preserve salivary IgA, 5) the concentration in normal saliva in 11 persons ranged from 15 to 240 mg/L ($\bar{x}$ = 81), and 6) an abnormal saliva contained less than 6 ug/L, the detection limit for this EIA.

INTRODUCTION

The purpose of this report is to demonstrate the power of solid-phase enzyme immunoassay (EIA) as a tool to detect and to quantitate an antigen under conditions adverse to many other means of assay.

Salivary IgA is such an antigen: It occurs in nonhomogeneous, often viscous fluids, in low concentration, and in a variety of molecular sizes for which serum IgA cannot always be used as standard. In addition, definition of its absence can be

diagnostically important in patients with recurrent infection.

In solid phase EIA, an excess of immobilized anti-human IgA traps various amounts of IgA or secretory IgA (sIgA). This bound IgA is then reacted with an enzyme-labeled anti-human IgA to cause the enzyme to become immobilized in proportion to the amount of bound IgA. Potentially interfering materials are removed by washing before addition of a low background-producing buffer-substrate which interacts with bound enzyme.

In this report we shall demonstrate the good correlation of EIA with an accepted method of assay; compare a variety of methods for preparing saliva for assay of IgA; demonstrate the presence of IgA in fractions obtained after sucrose gradient sedimentation of saliva; define the range of concentrations of salivary IgA in normal individuals, and show that the detection limit of the assay is adequate to identify patients with virtual absence of IgA in saliva.

The results will demonstrate the ability of EIA to substitute for radial immunodiffusion (1), electroimmunodiffusion (2), nephelometry (3), and radioimmunoassay (4) as a means for detecting and quantitating a protein antigen simply and reliably.

MATERIALS AND METHODS

The assay requires:

1) Antibodies to human IgA and sIgA which can function in the system described.

2) Control of the coating process in order to deposit adequate and reproducible amounts of first antibody.

3) A reliable standard

4) Demonstrated reliability of the result, including freedom from interference by sample constituents or by changes in sample volume or viscosity.

Antisera: The coating antibody selected was the IgG fraction of goat antihuman IgA (lot # 14506) from Cappell Laboratories, Cochranville, PA. 19330. The antibody to be labeled with enzyme was prepared at our institution using as immunogen pooled IgA isolated from the sera of patients with myeloma. One hundred ug mixed with an equal volume of Freund's complete adjuvant to make 1 ml was injected subcutaneously at 4 sites on rump and shoulders of the goat at four weekly intervals. Blood was collected for testing on day 28 and thereafter at bi-weekly intervals before booster injections until antibodies reached an equivalence titer $\geq$ 1:8 by Ouchterlony analysis. The IgG fraction was isolated using octanoic acid followed by batch diethylaminoethyl cellulose absorption (5), then further purified by absorption with solid-phase human IgG (6) to obtain a highly specific antibody.

Enzyme-labeled antibody: Glucose oxidase (GOx) type II, Sigma Chemical Co. St. Louis, MO. 63178 was combined with goat IgG containing antibody using the method of Wilson and Nakane (7). Briefly 12 mg GOx was reacted with 0.04 mol/L periodate at pH 4, 20°C, for 20 min to produce aldehyde groups which combined with

free amino-groups in 10 mg (8) goat IgG at pH 9.5, 18 h., to form a Schiff base. This weak linkage was converted to a strong covalent bond by reduction with 5 mg. borohydride (72 h, 20°C, in dark). After three 30 minute dialyses against phosphate-buffered saline (PBS) and concentration in a colloidin bag to 1 ml by negative pressure, the conjugate was preserved with an equal volume of glycerol. The material was used at 1:100 dilution.

Glucose oxidase (GOx) is stable, nonmammalian, and may be used with stable reagents to yield a colored product. The reaction chosen was:

$$\beta\text{-D-Glucose} + O_2 \xrightarrow{GOx} H_2O_2 + \text{Glucuronic acid}$$

$$H_2O_2 + \text{ABTS} \xrightarrow{\text{Peroxidase}} \text{Color at 410 nm}$$

2,2'-Azino-di- [3-ethyl-benzthiazoline sulfonate (6)] (ABTS), obtained from Boehringer-Mannheim, Indianapolis, IN. 46250 is oxidized by hydrogen peroxide in the presence of horseradish peroxidase (type VI, Sigma Chemical Co., St. Louis, MO. 63178). All other chemicals were reagent grade or the purest obtainable.

Coating of microtiter wells: Eighty micrograms of goat IgG per ml carbonate-bicarbonate buffer, 0.01 mol/L, pH 9.5 was dispensed in 50 ul portions to all 96 wells of a microtiter plate (M129A, flat-bottom, Dynatech Laboratories, Alexandria, VA. 22314) and dried for 48 h in a vacuum desiccator. This process causes a large and reproducible amount of the applied protein to adsorb to the well surface, as determined by ^{125}I-labeled protein. The application

of 4 ug per well provides nearly maximum adsorption and has a capacity to bind more than 100 ng human IgA.

Solutions required: 1) A wash and diluent solution (PBS/T/BSA) contains 5 g/L bovine serum albumin (BSA), 5 g/L Tween-20 [polyoxyethylene (20) monolaurate] and 1 g/L sodium azide in PBS. 2) A second wash solution (PBS/T) is similar to the above but lacks BSA. 3) Buffer-substrate is prepared as needed by combining:

120 volumes phosphate buffer, 0.1 mol/L, pH 6

1 volume ABTS, 20 g/L H_2O

1 volume Peroxidase, 1 g/L H_2O

15 volumes Glucose, 180 g/L in H_2O, several hours old.

All solutions are best kept at 5°C.

Standards: Commercial sera provided as immunoglobulin standards for the RID assay of human sera (Meloy Laboratories Inc., Springfield, VA. 22151) proved to be equivalent to three IgA isolates ($IgA_1\kappa$, $IgA_1\lambda$, $IgA_2\kappa$) which were gifts from Hans Spiegelberg of the Research Institute of Scripps Clinic.

Sera: Twelve human sera were assayed for IgA by both RID and EIA to test reliability of this EIA method.

Saliva: Samples were collected in 10 ml amounts from laboratory personnel (ages 24 to 65). Some used chewing gum to aid collection. One series of 10 samples was collected without preservatives but kept frozen at -13°C between assays. A second series was preserved with proteinase inhibitors (Trasylol, 10000 units/L; benzamidine, 1 mmol/L; phenyl methyl sulfonyl fluoride,

10 mg/L and ethylenediamine tetracetate, sodium salt 500 mg/L. This last series was also kept frozen between assays.

Pre-treatment of saliva: The following include methods used by other workers. (9,10).

1) Centrifuging at 1000 x g for 2 min to obtain a relatively clear supernate.
2) One hour dialysis versus tris (hydroxy methyl) methylamine (TRIS)-acetate buffer, 0.1 mol/L, pH 4.5 followed by centrifugation at 14000 RPM for 30 min as described by Virella et al (9).
3) Vigorous vortexing for 30 sec to obtain a temporary homogeneity followed by immediate dilution of sample. This provided a control value.

Separation of sIgA and marker proteins by sucrose gradient sedimentation: Approximately 10 ug each of ^{125}I-thyroglobulin, or ^{125}I-human IgG, together with galactosidase (3.2.1.2.3) or glucose oxidase (1.7.3.4) were added to 200 ul aliquots of dialysed saliva (9) readjusted to pH 7 and layered onto 12 ml of 50-200 g/L linear sucrose gradients in TRIS-acetate buffer, pH 7 and centrifuged (160000 x g 17 h, 5°C) in a Beckman SW 41 Ti rotor. Eleven drop fractions were collected from a hole punched in the tube bottom and analyzed without removal of sucrose for ^{125}I, enzyme activity, and IgA concentration using EIA. Galactosidase was assayed in 20 ul of each fraction using p-nitrophenol-beta-galactosidase as substrate (11) and glucose oxidase in 20 ul of each fraction using the buffer-substrate described in this report.

EIA protocol:

1. Antibody coated plates are best used within 1 week.
2. Prior to use, wells are washed 3 min with PBS/T/BSA and twice with PBS/T for 3 min each time. Fluid is flicked from the wells over a sink after each wash and finally the plate is made semi-dry by striking inverted against toweling.
3. Sera and saliva are diluted in PBS/T/BSA. Normal human sera require 1:1000; normal saliva require 1:50, 1:200, and 1:400 dilution. Samples suspected of deficiency are tested undiluted.
4. Standards and samples: 50 ul, are dispensed into duplicate wells.
5. After covering with Parafilm and incubating 2 to 16 h with gentle agitation (Aliquot mixer/Miles-Ames, Elkhart, IN. 46515), the wells are washed with PBS/T/BSA and PBS/T as before.
6. Enzyme-labeled antibody is diluted in PBS/T/BSA and 50 ul distributed into each well. The plate is incubated 1 h as before and washed 3 times with PBS/T.
   (Note: The adequacy of this washing process was confirmed by use of ^{125}I-labeled protein.)
7. Freshly prepared buffer-substate, 200 ul is added to each well. In the absence of a plate-reader this can be done at 15 sec intervals to duplicate wells. If a plate-reader is used, there should be minimum time delay between wells. After

incubation 30 min at about 20°C, duplicate well fluids are transferred into clean tubes at 15 sec intervals to maintain equal incubation times. Enzyme action stops on transfer.

8. The color produced with ABTS has a broad absorbance between 408 and 417 and a lesser but narrower absorbance at 642 nm. Without a plate-reader, absorbancies can be determined at 410 nm using 300 ul capacity cuvets and a spectrophotometer-240 (Gilford Instrument Laboratories, Oberlin, OH. 44070).

9. The standard curve is prepared over the range of zero to 100 nanograms using data obtained with standard sera or pure IgA.

10. Absorbance values falling within range of the standard curve are converted to nanograms (ng).

Then,

$$\text{ng found} \times \frac{10^3 \text{ ul/ml}}{50 \text{ ul}} \times \frac{\text{dilution}}{10^3 \text{ng/ug}} = \text{mg/L}$$

or: ng found x 0.02 x dilution = mg/L

RESULTS

1. The standard curve: The relationship between absorbancy at 410 nm and mass of IgA over the range 6.25 to 100 ng is shown in Figure 1. This relationship was extremely reproducible day after day for the range 6.25 to 50 ng and tended to vary with room temperature above 50 ng.

2. The reliability of assay: Twelve sera were assayed by both EIA and RID. (Table 1). The correlation was 0.95 but the EIA values were 15% higher than those by RID. This is not due to

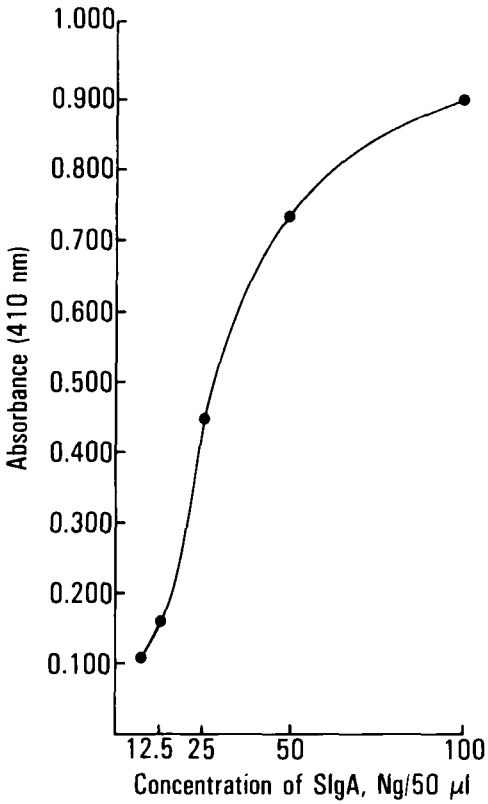

Figure 1 Standard curve relating absorbance at 410 nm with amounts of human IgA added. This curve was prepared using pure human IgA but is nearly identical to one prepared from commercial standard sera.

TABLE 1

Comparison of EIA and RID Values (mg IgA/L) in Human sera

Sample	1	2	3	4	5	6	7	8	9	10	11	12
EIA*	1.17	4.44	3.92	4.20	4.68	1.84	1.88	1.20	2.20	1.76	2.00	0.06
RID*	0.86	3.90	3.40	3.20	4.40	1.30	1.55	1.57	2.20	1.10	1.93	B.L.

B.L. Below limit of detection
* times 10^{-3}

IgA or IgM which would have influenced the result on Sample 12.

3. Comparison of various methods of pretreatment of whole saliva for assay of total IgA: All methods gave results that differed only within limits of precision of the assay. (Table 2).

4. Range of IgA concentration among 11 "normal" whole saliva, varied from 15 to 239 ($\bar{x}$ = 81) mg/L which compares well to those found using other methods. (Table 3).

4b. Intraindividual reproducibility of salivary IgA levels over 1 month. Salivary samples collected from seven individuals on two occasions 50 days apart showed minimal changes (Table 4).

5. Use of EIA to detect and semi quantitate IgA in fractions from sucrose gradient sedimentation. The IgA in 10 ul of each eleven drop fraction is shown in Figure 2A. Estimation of the various molecular sizes found in this saliva were obtained using the separation of marker proteins included in the sample (Figure 2B). Similar molecular sizes of IgA have previously been reported (15). Although, the saliva sample had a total IgA content of 240 mg/L, there is little IgA of molecular size below 11S. Sucrose gradient studies on a second sample of saliva yielded similar results.

6. Detection limit of the method: Increasing dilutions of standard were made to give concentrations below 20 ug/L. In order to enhance sensitivity, 400 ul (instead of 50 ul) of

TABLE 2

Comparison of Effects of Various Pretreatments of Saliva

Sample	Centrifuging	Removing insoluble mucins (9)	Vortexing
1	14 ± 2 mg/L	18 ± 2 mg/L	15 ± 2 mg/L
2	30 ± 13	21 ± 5	30 ± 4
3	160 ± 42	102 ± 5	136 ± 24
5	25 ± 9	21 ± 2	26 ± 8
7	13 ± 3	21 ± 3	17 ± 3
8	76 ± 15	92 ± 14	110 ± 29

TABLE 3

Comparison of Various Reported Levels for Salivary IgA

Study	Method	Samples	Range (Av.) mg/L
Virella (9)	Nephelometry	12	22 - 150 (65)
Akerlund (10)	EIA	10	7.8 - 25.6 (14.9)
Claman (13)	Rocket	16	20 - 150 (95)
Sack (14)	EIA	4(?)	to 250[a]
Tomasi (15)	RID	6	63 - 121(90)[b]
Present	EIA	11	15 - 239(81)

a. Calculated from figure
b. Corrected from original data to compensate for use of 7S standard

TABLE 4

Constancy of Salivary IgA (mg/L)

Donor	1st Collection	2nd Collection 50 days later
1	13	16
2	25	28
3	96	96
5	15	21
6	67	56
7	18	14
8	104	100

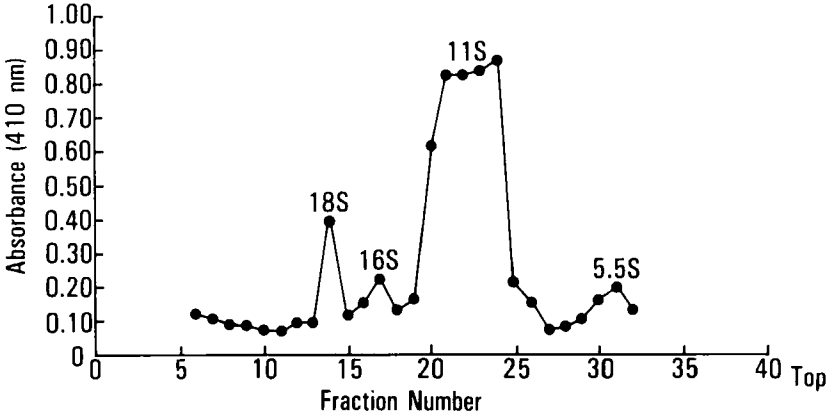

Figure 2a Separation of IgA (sIgA) by sedimentation in a 5 to 20% sucrose gradient onto a 30% sucrose cushion. IgA presence was determined by EIA. The numbers on the peaks are sedimentation coefficients determined from marker proteins included in the 200 ul portion of saliva initially layered onto the gradient. The blunted quality of the 11S peak was caused by lack of capacity of the immobilized goat anti-human antibody to bind all the IgA in fractions 21 through 23. This sample of saliva had the greatest concentration (240 mg/L) of the eleven studied.

these were added to duplicate wells and incubated 36 h (instead of 2-16 h). Absorbancy twice background was given by 6 ug/L.

7. Demonstration of absence of IgA in a saliva. A saliva sample, from a patient known to be deficient in serum IgA was

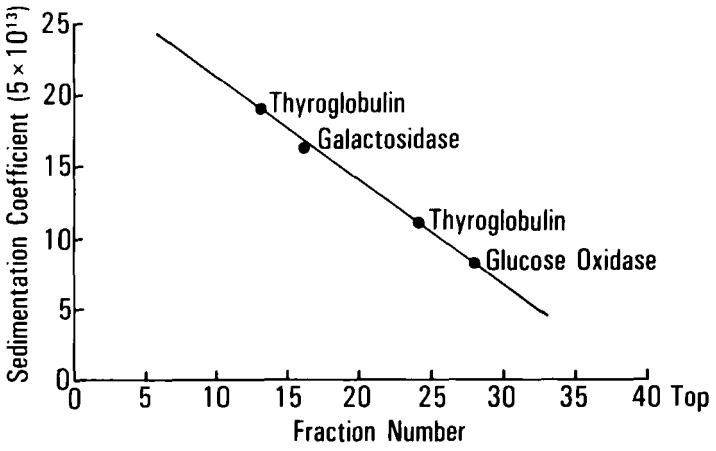

Figure 2b Separation of marker proteins in 5-20% sucrose gradient with a 30% cushion. ^{125}I-thyroglobulin was used within 5 days of labeling and has 19S and 12S components. Galactosidase, 15.9S and glucose oxidase, 7.9S, were detected by enzymatic activities.

pretreated according to Virella et al (9) to reduce viscosity which retards diffusion. It was added undiluted 400 ul per well and incubated 36 h at 37°C. After enzyme reaction it showed an absorbance no greater than background.

8. Effect of preservatives. Samples kept 1 month at -13°C retained their IgA levels as well as samples containing numerous proteinase inhibitors.

DISCUSSION

Salivary IgA varied from 15 to 239 mg/L among the eleven adult donors (ages 25 to 65) and showed little intra-individual change

on resampling after 50 d. The salivary concentrations compare well with previous reports (9,12,13,14). Two samples of saliva examined by sucrose gradient sedimentation showed the IgA to exist with a sedimentation coefficient predominately of 11S with minor amounts of 18S, 16S, 14S, and 5.5-6S.

Other assay methods (9,10) have required pretreatment of the sample to make it acceptable for assay. The effects of such pretreatment on IgA concentration have been examined here by EIA and found to introduce minimal changes.

An IgA deficient saliva sample assayed by EIA was found to have less than 6 ug/L IgA. In contrast the minimum salivary IgA among the 10 normals was 15 mg/L.

EIA being a reagent-excess assay, is not restricted by need for uniformity of antigen, similarity of its fluid matrix with that of the standard or an optimum ratio of antigens to antibody. For example, the size of the molecule, which profoundly influences RID and "rocket" assay results, has no effect on EIA. Lack of sample homogeneity and optical clarity are unacceptable for nephelometry but cause minimal difficulties with EIA. As a result, EIA was compatible with three increasingly complex pretreatments of saliva and with sucrose gradient sedimentation fractions.

The antigen binding to excess antibody in EIA obviates the need to optimize the antigen-antibody ratio that is required for reliable nephelometry. EIA merely requires dilution of sample to

keep absorbancy within the limits of the standard curve. EIA is also capable of yielding semiquantitative information by visual inspection in situations where colorimety is not possible.

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Roy B. Johnson, Jr.

Department of Pathology

Hospital of Scripps Clinic

10666 North Torrey Pines Road

La Jolla, CA. 92037

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