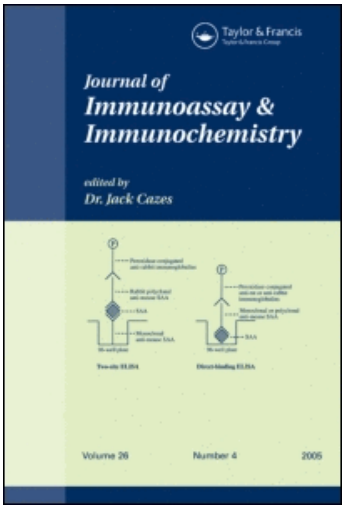

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Adler-storthz, Karen , Dreesman, Gordon R. , Graham, David Y. and Evans, Dolores G.(1985) 'Biotin-Avidin Amplified Elisa for Quantitation of Human IgA', Journal of Immunoassay and Immunochemistry, 6: 1, 67 — 77
To link to this Article: DOI: 10.1080/01971528508063021
URL: http://dx.doi.org/10.1080/01971528508063021

JOURNAL OF IMMUNOASSAY, 6(1&2), 67-77 (1985)

BIOTIN-AVIDIN AMPLIFIED ELISA FOR QUANTITATION OF HUMAN IgA

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ABSTRACT

A biotin-avidin amplified enzyme-linked immunosorbent assay (ELISA) for the measurement of human IgA in serum, colostrum, gastric juice, and supernatants of in vitro mitogen-stimulated human peripheral blood mononuclear cells is described. The ELISA reproducibly detects as little as one nanogram of IgA in a fifty microliter sample (20 ng/ml). (KEY WORDS: biotin-avidin, ELISA, IgA detection)

INTRODUCTION

Since its development (1), the ELISA has been used extensively with numerous applications. The sensitivity of the ELISA can be enhanced by the use of fluorogenic substrates (2), chemiluminescence (3,4), and the biotin-avidin system (5-8). The advantages of biotin-avidin amplification are: (i) avidin has an extremely high affinity for biotin with a dissociation constant of 10^{-15}M (9), (ii) each avidin molecule can bind four molecules of biotin (9), and (iii) biotin can be covalently coupled to antibody at a high specific activity without affecting the antigen-binding capacity of the antibody (5,6).

0197-1522/85/0601-0067$3.50/0

In the current paper we describe a biotin-avidin amplified ELISA for the detection of nanogram quantities of human IgA in clinical samples. The ELISA has been applied for measurement of IgA in human serum, breast milk, colostrum, duodenal fluid specimens, and supernatants of in vitro mitogen-stimulated human peripheral blood mononuclear cells (PBMNC).

MATERIALS AND METHODS

Antibodies

Affinity purified goat anti-human IgA (GAHIgA; alpha chain specific) and rabbit anti-human IgA (RAHIgA) were obtained commercially (Kirkegaard and Perry Laboratories, Gaithersburg, MD and Cappel Laboratories, Cochranville, PA, respectively). Ouchterlony analysis showed the GAHIgA to be alpha chain specific whereas the RAHIgA reacted with IgA, IgG, and IgM indicating both alpha and light chain specificity. Therefore, the GAHIgA was used as the capture antibody in the ELISA and the RAHIgA was used as the probe antibody.

Preparations of IgA, IgG, and IgM

IgA was purified from the serum of a patient with an IgA-secreting multiple myeloma. Any IgG in the serum was undetectable by Ouchterlony analysis with a gamma chain specific reagent. Ten ml of serum was equilibrated with 0.15M PBS (pH 7.2) and applied by reverse flow to an Ultrogel AcA 34 molecular sieve column (2cm X 90cm, LKB Instruments, Rockville, MD). The column had been previously calibrated with Blue Dextran 2000, chymotrypsinogen A (molecular weight 25,000), albumin (molecular weight 67,000), and aldolase (molecular weight 158,000) (Pharmacia Fine Chemicals, Piscataway, NJ). The peak that eluted at an approximate molecular weight of 160,000 was concentrated and shown to be pure by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in that only 2 bands were noted,

one with a molecular weight of approximately 53,000 to 55,000 and another of approximately 22,000 to 25,000. Ouchterlony analysis indicated the immunological purity of the IgA by a reaction of identity with an alpha chain specific reagent and no reaction with gamma and mu chain specific reagents.

IgG was purified from normal human serum by DEAE-cellulose chromatography (2cm X 10cm). Two ml of serum was equilibrated with 0.0175M phosphate buffer (pH 6.8) and applied to the top of the column. Elution was continued until the unbound peak of UV-absorbing material was completely eluted. This peak was concentrated and shown to be pure by SDS-PAGE and Ouchterlony analysis.

IgM was a commercially available preparation (Pel Freez Biologicals, Rogers, AR) and was pure by SDS-PAGE and Ouchterlony analysis.

The concentrations of IgA, IgG, and IgM were determined by the Lowry method for protein quantitation using bovine serum albumin as a standard (10).

Milk and colostrum samples were obtained from the Milk Bank of Baylor College of Medicine. Serum and duodenal fluid samples were obtained from normal volunteers as part of another study. A standard serum of known IgA concentration (2.45 mg/ml) was obtained from Hyland (Costa Mesa, CA). Duodenal fluids were heated at 56°C for 30 minutes and stored at -70°C. Colostrum and breast milk samples were stored at -70°C.

Biotin-Avidin Reagents

Biotin was covalently conjugated to RAHIgA as previously described (5). Briefly, 57 ul of a 0.1M solution of biotin-N-hydroxy-succinimide (Calbiochem, San Diego, CA) in distilled dimethyl formamide (Burdick and Jackson Laboratories, Muskegon, MI) was added to 1.0 ml containing 10 mg of RAHIgA in 0.1M sodium bicarbonate. After incubation for 1 hr at 22°C, the reaction mixture was dialyzed against several changes of 0.5 M PBS (pH 7.2). After dialysis, an equal volume of glycerol was added, and the biotin-labeled RAHIgA was stored at -20°C. The reagent has remained stable for 14 months, to date. Avidin conjugated to alkaline phosphatase was obtained from Vector Laboratories, Rollingwood, CA.

In Vitro Culture of Human Peripheral Blood Mononuclear Cells

PBMNC were separated from the blood of normal healthy donors. Heparinized peripheral blood was separated by centrifugation on a Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient (11). PBMNC (1×10^6 cells/ml) were cultured (round bottom microtiter plates) in RPMI 1640 buffer with 10 mM HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid) and supplemented with 2 mM L-glutamine, 100 IU/ml of penicillin, 100 ug/ml streptomycin, 15% heat-inactivated fetal calf serum, with 20 ug/ml pokeweed mitogen (Sigma Chemical Co., St. Louis, MO) added. After six days of incubation at 37°C in a 5% CO_2 atmosphere, supernatants were collected and assayed for IgA.

ELISA

All assays were done in rigid, non-sterile, flat-bottomed, 96-well, polystyrene microtiter plates (Linbro/Titertek, Hamden, CN). Wells were coated overnight at 4°C with 200 ng of GAHIgA in 50 ul of 0.1M sodium carbonate-sodium bicarbonate buffer (pH 9.6), followed by a post-coating step with 200 ul of 0.5% gelatin in PBS for 30 min at room temperature. To determine optimal dilutions of biotin-labeled RAHIgA and avidin conjugated to alkaline phosphatase, checkerboard titrations were done. Human IgA was diluted to 5 ug/ml in 0.1% gelatin in PBS and 50 ul was added to the wells. Fifty ul of 0.1% gelatin in PBS was added to a separate group of wells as the negative control. The plates were incubated 2 hr at 45°C, followed by three washes with 200 ul of 0.01% gelatin in PBS. Fifty ul of biotin-labeled RAHIgA, diluted in 0.1% gelatin in PBS, was added to duplicate wells of both IgA and the negative control. Dilutions ranging from 1:50 to 1:1600 were tested. The plates were incubated 1 hr at 37°C and washed three times. Fifty ul of dilutions of avidin conjugated to alkaline phosphatase were added and the plates incubated 20 min at room temperature. Dilutions of avidin conjugated to alkaline phosphatase ranged from 1:5 to 1:50. Following three

additional washes, 200 ul of p-nitrophenyl phosphate (Sigma 104 phosphatase substrate tablets; Sigma Chemical Co., St. Louis, MO) in 10% diethanolamine buffer (pH 9.8) containing 0.5 mM $MgCl_2$ was added. After 30 min at 37°C, the reaction was stopped by the addition of 50 ul of 3 M NaOH and the extent of the reaction determined spectrophotometrically by reading the optical density (O.D.) at 405 nm in an automatic plate reader (Titertek Multiskan, Flow Laboratories, McClean, VA). The optimal dilutions of biotin-labeled RAHIgA and avidin conjugated to alkaline phosphatase were 1:800 and 1:25, respectively, based on the strongest reaction with the IgA and the weakest reaction with the diluent.

All subsequent ELISAs were done as above using the stated reagent concentrations. Starting dilutions of test samples were determined based on normal physiological concentrations of IgA in the various sources (12).

RESULTS

Specificity and Sensitivity of ELISA

The IgA specificity of the ELISA was determined by titrating preparations of IgA, IgM, and IgG (Figure 1). While reaction with the dilutions of IgA resulted in a typical titration curve, the reaction with IgG and IgM were nearly identical to that with the diluent at all concentrations tested. We considered an $O.D._{405}$ value twice that of the diluent to indicate a positive reaction. Based on this, the estimated sensitivity of the ELISA is 20-25 ng IgA/ml or approximately 1 ng in 50 ul. An essentially identical titration curve was obtained using a standard serum with an IgA concentration of 2.45 mg/ml (data not shown).

Reproducibility of ELISA

The ELISA was done twice monthly for a period of 4 months so that there were a total of 8 readings of the 5 different concentrations of IgA. The

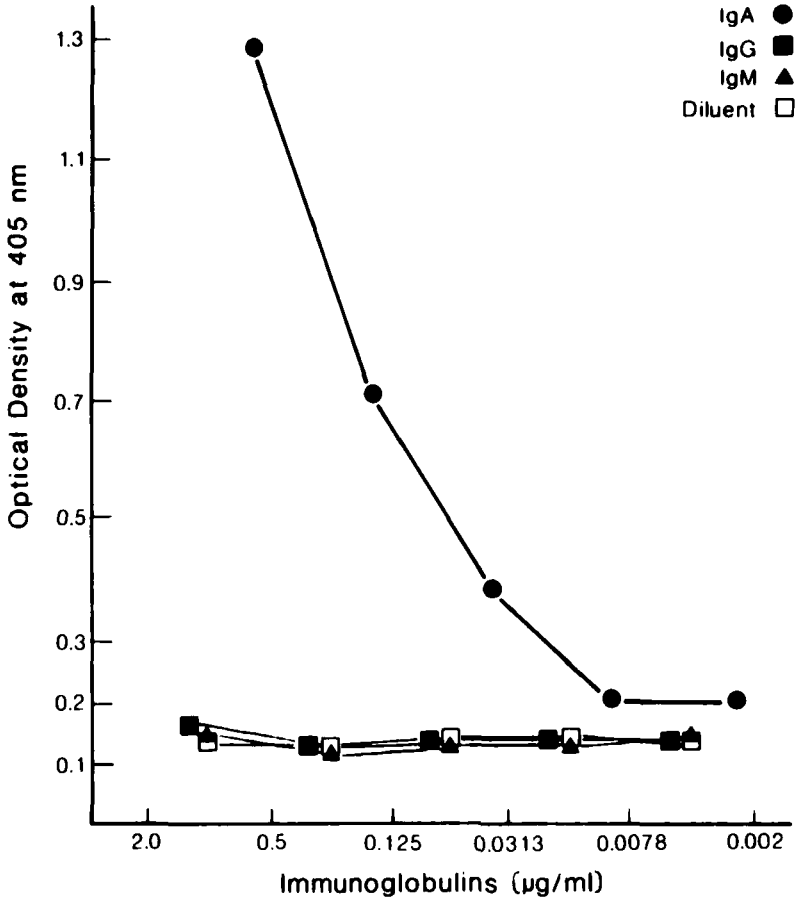

FIGURE 1. Specificity of the IgA ELISA.

coefficient of variation (standard deviation/mean) was between 0.03 and 0.09 for each of the 5 concentrations.

Determination of IgA Concentration in Test Samples

A standard curve was prepared on the day that the samples were tested. A representative standard curve is shown in Figure 2. Serum, breast milk, colostrum, duodenal fluid, and supernatants from PBMNC cultured in vitro with

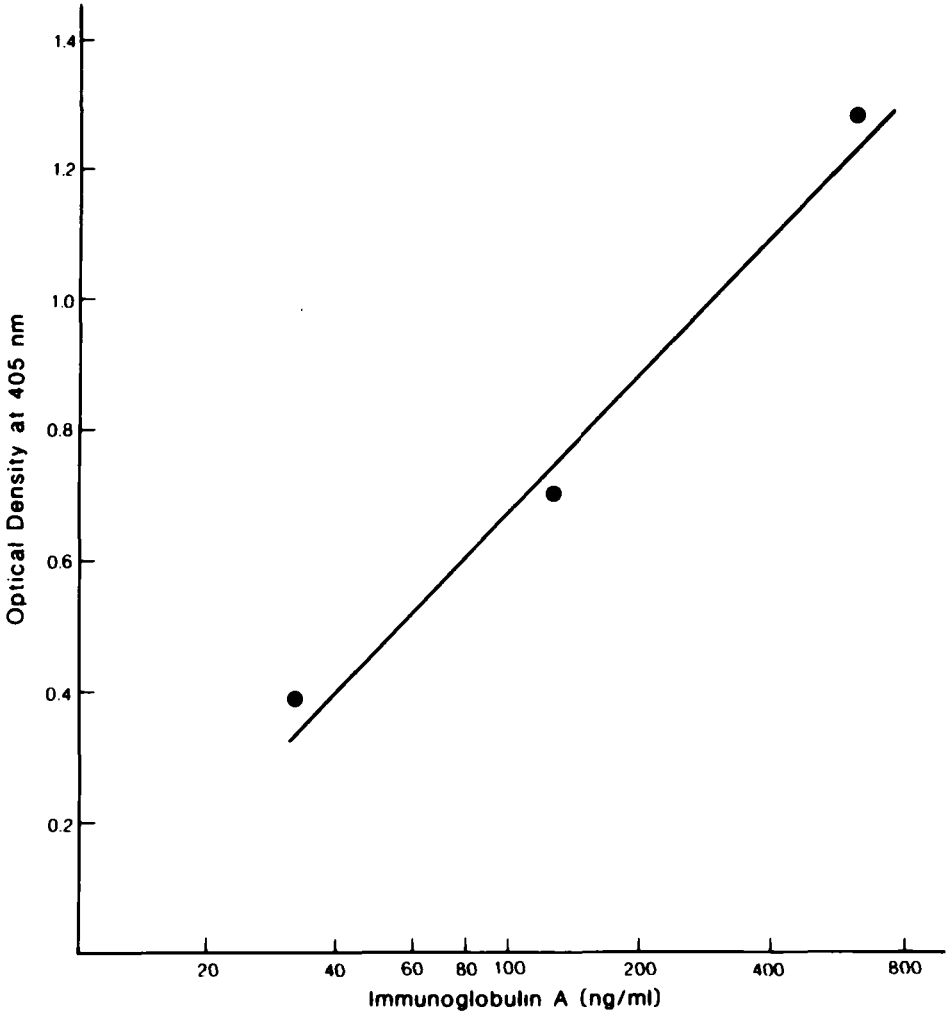

FIGURE 2. Standard curve for determining IgA concentration in test samples.

TABLE 1

Concentration of IgA in Test Samples

Specimen	Dilution	O.D.	Concentration
Gut Juice 5[a]	1:4000	1.09	1.32 mg/ml
Gut Juice 18	1:1600	1.01	480.0 ug/ml
Breast Milk 2	1:40,000	0.87	7.7 mg/ml
Colostrum 21	1:160,000	0.87	30.8 mg/ml
Colostrum 22	1:160,000	0.70	17.3 mg/ml
Colostrum 23	1:160,000	0.65	14.7 mg/ml
Colostrum 29	1:40,000	1.03	12.1 mg/ml
Serum 1	1:16,000	0.70	17.0 mg/ml
Serum 2	1:16,000	0.84	2.8 mg/ml
Serum 3	1:16,000	0.81	2.5 mg/ml
Sup 1[b]	1:16	0.90	3.4 ug/ml
Sup 2	1:16	0.71	1.8 ug/ml
Sup 3	1:16	0.76	2.1 ug/ml

a = Numbers refer to subject or patient numbers

b = PMMNC Supernatant

pokeweed mitogen were assayed. The standard curve was plotted and use to determine the IgA concentration of the samples (Table 1). The values correlate with those considered normal for the various sources.

DISCUSSION

The biotin-avidin amplified ELISA described in this paper is a simple, rapid, reproducible method for quantitation of IgA. The method is as specific and sensitive of that reported previously (13), but offers the advantage of biotin-labeling of antibody rather than glutaraldehyde conjugation of enzyme to antibody. Theoretically, the sensitivity of this assay for IgA detection (20 ng/ml) could be be influenced by our use of a single, purified myeloma protein as the standard, i.e., the use of the single protein might allow the variations of apparent potency between the individual protein and other idiotypes or subclass variations or normal polyclonal protein. However, since titration of a standard serum of known IgA concentration yielded essentially identical sensitivity, this appears unlikely. In addition, the capture antibody was alpha chain specific and should have bound any idiotype or subclass variation. While others have used the ELISA to measure IgA in serum, saliva, and PBMNC culture supernatants (13), to the best of our knowledge, this is the first report of the use of the ELISA to quantitate IgA in colostrum, breast milk, and duodenal fluid specimens.

ACKNOWLEDGEMENTS

The authors thank the Milk Bank at Baylor College of Medicine for supplying breast milk and colostrum, and Dr. Gerald Cyprus for the serum from the patient with IgA myeloma. This work was supported by the Veterans Administration and by a National Research Service Award, CA 09197, from the National Cancer Institute (KA-S).

The authors also thank Ms. Linda Carpenter for preparation of the manuscript.

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