Development and validation of a fluorescent microsphere immunoassay for anti-IgA

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Anti-IgA may cause anaphylactic transfusion reactions in IgA-deficient individuals. Testing for IgG anti-IgA is useful to identify persons at risk. This report describes an immunoassay for anti-IgA that uses polyclonal IgA coupled to fluorescent microspheres as an immunosorbent. Anti-IgA is detected by phycoerythrin-labeled anti-IgG. The assay is calibrated in arbitrary units by use of a serum that contains anti-IgA. Dose-response studies with sera that contain anti-IgA showed positive responses at dilutions up to 32-fold greater than the dilution used to test patients' samples. Inhibition studies with purified IgA and IgA-deficient serum showed no inhibition with IgA-deficient serum and complete inhibition with soluble IgA. Clinical tests performed in more than 90 assays had a CV of 13.6 percent for measurements of an internal positive control. The fluorescent immunoassay method is rapid, reproducible, and sensitive to low concentrations of IgG anti-IgA.
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Selective IgA deficiency is the most common humoral immunodeficiency.[1,2] The reported frequency varies widely in different populations. The frequency of IgA deficiency in Caucasians of Northern European ancestry is estimated to be approximately 1 in 600 with lesser frequencies reported in Asian populations.[2] The definition of IgA deficiency is determined in part by the analytic sensitivity of immunoassay methods used to measure IgA. Commonly used nephelometric immunoassays are capable of detecting IgA at concentrations greater than 0.3 mg/dL, and 0.5 mg/dL is often cited as a cutoff concentration to define IgA deficiency in adults. A lower concentration, 0.05 mg/dL, is used to define IgA-deficient blood donors and blood products.[3–5] Approximately one third of persons with IgA deficiency have detectable anti-IgA.[2,6] The mechanisms responsible for development of anti-IgA in IgA-deficient individuals are unknown.

Anti-IgA has been reported to be associated with severe anaphylactic transfusion reactions in IgA-deficient patients, and antibodies of both the IgG and IgE isotypes have been described.[6–8] Although it is assumed that IgE antibodies may be capable of eliciting anaphylactic transfusion reactions, not all investigators have been able to identify such antibodies, and the mechanism(s) by which anti-IgA produces anaphylactic reactions remains unclear. Although several studies suggest that IgG anti-IgA is associated with transfusion reactions, reported data also indicate that some patients with anti-IgA fail to react to IgA and reactions have been described in patients who have no demonstrable antibodies.[9–11] Nevertheless, accurate identification of IgA-deficient persons with anti-IgA is important to investigate the possible etiology of immediate hypersensitivity transfusion reactions and to facilitate the optimal use of blood products obtained from IgA-deficient donors.

A variety of analytical methods have been used to detect anti-IgA, including double immunodiffusion with purified IgA paraproteins, hemagglutination, two-site immunometric assays, flow cytometric immunoassays, and enzyme immunoassays.[12–17] In this report we describe the development, analytical validation, and clinical performance of a fluorescent microsphere immunoassay for IgG anti-IgA. The assay has excellent analytic sensitivity and reproducibility and a rapid turnaround time, and makes use of commercially available reagents and instrumentation.

Materials and Methods

Institutional Review Board approval was obtained for use of residual patients' sera and to review the medical records of all patients tested as part of this study. Previously tested sera (n = 7) shown to contain IgG anti-IgA were available from the Division of Transfusion Medicine.[15] These sera were from healthy blood donors who had fulfilled all donation criteria defined by the U.S. Food and Drug Administration and who had been found to be negative on blood donor infectious disease testing. The donors were identified by a routine screening process used in our collection center to identify IgA-deficient donors as previously described.[18] None of the seven donors had previously been transfused, and therefore none had a clinical history of anaphylaxis to IgA. All represented selective IgA deficiency with anti-IgA. Serum from a patient with IgA deficiency, a history of previous anaphylactic transfusion reactions, and high levels of IgG anti-IgA was tested at serial dilutions (described later) and was used as the assay calibrator (1 in 1280 dilution) and internal control (1 in 640 dilution) in all subsequent assays. Serum samples from 133 healthy adults were tested to determine background binding of IgG to the IgA-coupled fluorescent microspheres (described later), and specimens submitted for testing in the clinical laboratory obtained from 724 patients suspected of having immediate hypersensitivity transfusion reactions were used to determine the frequency of IgG anti-IgA in patients. Additional clinical information for the 724 patients, beyond the fact that they were suspected of having an immediate hypersensitivity

reaction to transfusion, was not available. The IgA status of the 133 healthy subject and 724 patient samples was determined by nephelometry using a Dade Behring BN II Nephelometer (Siemens Healthcare Diagnostics, Deerfield, IL) and Dade Behring IgA reagents according to manufacturer's instructions.

Preparation of IgA-Coupled Fluorescent Microspheres

Purified, human polyclonal IgA (Fitzgerald Industries International Inc., Concord, MA) was coupled covalently to fluorescent microspheres (SeroMap D microspheres, Luminex Corp., Austin, TX) by a modified carbodiimide method as follows[19]: An aliquot of 50×10^6 microspheres was reacted in the dark for 20 minutes with 100 µL of 50 mg/mL *N*-hydroxysulfosuccinimide and 100 µL of 50 mg/mL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in a final volume of 1 mL of 100 mM monobasic sodium phosphate, pH 6.2. After activation, the microspheres were washed twice with 50 mM MES, pH 5.0 (2-[*N*-morpholino] ethanesulfonic acid), and 1 mg of purified IgA in 1.5 mL of MES, pH 5.0, was added. The microspheres were then incubated in the dark for 2 hours at room temperature. The IgA-coupled microspheres were washed, resuspended in 0.1 M PBS with 0.05% Tween 20, and incubated in the dark for an additional 30 minutes. After this incubation, the IgA-coupled microspheres were diluted 1 in 1600 in PBS with 1% BSA, 10% sodium azide, and 100 µL/L of protease inhibitor cocktail #P2714 (Sigma Chemical Co., St. Louis, MO) to a final concentration of 31,350 microspheres/mL.

Dose-Response and Dose-Inhibition Analyses of IgA-Coupled Fluorescent Microspheres

Maximum specific binding of IgG antibodies to the IgA-coupled microspheres was tested in a standard assay (described later) by incubating 100-µL aliquots of diluted microspheres with decreasing serial twofold dilutions (1 in 20 through 1 in 81,920) of positive serum. The specificity of binding of IgG to the IgA-coupled microspheres was tested in inhibition assays with purified polyclonal IgA (Fitzgerald Industries International Inc.) and IgA-deficient serum inhibitors. Aliquots of diluted, purified IgA or diluted, IgA-deficient serum (100 µL in PBS with 1% BSA diluent) were incubated overnight with an equal volume of diluted (1 in 40) positive serum. The incubated mixtures were then tested in a standard assay, and inhibition by purified IgA or IgA-deficient serum was calculated by comparing the fluorescent responses of the incubated mixtures to results for the uninhibited positive serum. Nonspecific binding of IgG protein to the IgA-coupled microspheres was determined by testing sera from healthy adults in standard assays. A limit of inhibition was not chosen as inhibition is dose dependent (see Results section).

Standard Assay Format

Tests for IgG anti-IgA were performed in filter microtiter plates (MABV N1250, Millipore Inc., Billerica, MA) as follows: 100 µL of the positive calibrator, internal control, or test sera diluted 1 in 40 in PBS with 1% BSA was incubated with 100 µL of IgA-coupled microspheres in darkened, covered microtiter plates for 30 minutes at room temperature. After incubation, the microspheres were washed five times with 1% BSA in PBS, then 100 µL of diluted, R-phycoerythrin conjugated, goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA) was added to each well, and the plates were again incubated in the dark for 1 hour at room temperature. Binding of IgG antibodies to the microspheres was measured using a Luminex 100 platform (Luminex Corp.) according to the manufacturer's instructions. Results were calculated by STATLia version 3.2 (Brendan Scientific, Carlsbad, CA). The raw fluorescent scores for the calibrator and internal control in fluorescent response units (FRU) were assigned arbitrary values of 100 and 200 units (U), respectively, and results for patients' sera were calculated by comparison to the calibrator as follows: Test result (U) = FRU patient / FRU calibrator × 100. Results greater than 200 U and less than 100 U were reported as positive and negative, respectively. Results between 100 and 200 U were reported as equivocal. Inhibition studies with soluble IgA were not performed on routine clinical specimens that gave positive or equivocal results.

Results

Specific binding of IgG antibodies to the IgA-coupled microspheres was determined by testing serial twofold dilutions of known positive sera. Results of a typical dose-response analysis for one strongly positive serum are shown in Figure 1. The maximum response for this serum exceeded 20,000 arbitrary FRU at a 1 in 40 dilution (Fig. 2). This particular positive serum was chosen as a calibrator and internal control for all subsequent assays. The serum was used as a calibrator at a 1 in 1280 dilution (100 U, described earlier) and as an internal control at a 1 in 640 dilution (200 U, previously described).

Nonspecific binding of IgG to the IgA-coupled microspheres was determined by testing sera from 133 healthy, non–IgA-deficient adults. The results are shown in Figure 2 for tests performed on serial twofold dilutions of sera from 1 in 20 to 1 in 80. The mean, standard deviation, and maximum response for sera in the normal group displayed in Figure 2 were 984, 819, and 5884 FRU, respectively. All but five sera from these healthy adults had fewer than 3000 FRU at a 1 in 40 dilution. The upper limit of nonspecific binding in the fluorescent microsphere immunoassay (5079 FRU) was calculated as the mean plus five standard deviations for tests of normal sera at a 1 in 40 dilution using the standard assay format. This level of binding corresponded approximately to the response of the positive serum calibrator (4206 FRU), which was assigned an arbitrary value of 100 U. The 100-U value for the assay calibrator obtained by testing this serum at a 1 in 1280 dilution represents a 32-fold greater dilution of the positive serum calibrator than is used routinely to test patients' specimens. The cutoff for

defining a positive result was established at 200 U (a 1 in 640 dilution of the positive serum, 7165 FRU) to avoid misclassifying any patients' sera as positive that did not contain IgG anti-IgA. Results between 100 and 200 U were classified as equivocal. In this classification, serum from one healthy adult shown in Figure 2 would be classified as equivocal, and no sera would be classified as positive.

The specificity of binding of IgG antibodies to the IgA-coupled microspheres was determined by testing a number of positive sera in sequential dose-inhibition assays with purified polyclonal IgA and IgA-deficient serum inhibitors (Fig. 3). Excess polyclonal IgA abolished binding of IgG antibodies in all positive sera, but IgA-deficient serum had no effect on binding of IgG antibodies. Significant inhibition of binding of IgG antibodies to the IgA-coupled microspheres (up to 40%) was produced by adding as little as 1 ng of purified polyclonal IgA as an inhibitor.

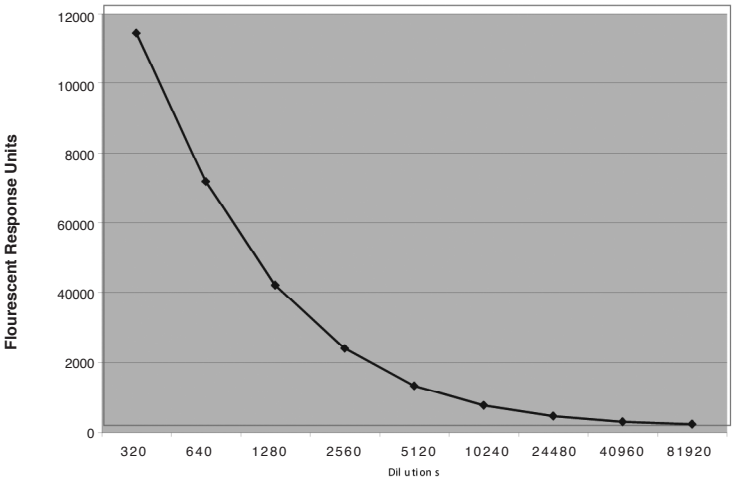

Fig 1. Dose-response analysis of a positive serum used as assay calibrator and internal control. Fluorescent response units are plotted versus dilutions of test serum (see text).

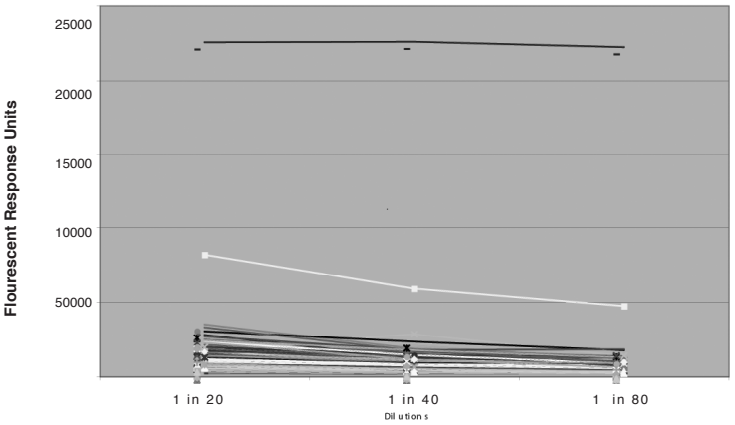

Fig. 2. Determination of nonspecific binding of sera from healthy adults. Fluorescent responses for sera from healthy adults tested at serial twofold dilutions are plotted and can be compared with the positive serum used as the assay calibrator (top line). One serum from a healthy control produced binding intermediate to the positive serum and the majority of other sera from healthy adults (see text).

Since this immunoassay was approved for routine use in the clinical laboratory, we have performed more than 90 assay runs for IgG anti-IgA on 724 persons during a period of approximately 18 months using two different lots of IgA-coupled microspheres. Interassay reproducibility across all 90 assay runs as measured by the CV of replicate measurements of the internal control was 13.6 percent (data not shown). Tests performed on sera from these 724 persons yielded 46 results greater than 100 U (6.3%). In this group, 38 sera had results greater than 200 U, and 8 sera yielded equivocal results. The medical histories of four patients seen at this institution who had anaphylactic reactions and results greater than 200 U were available for review: all had IgA deficiency as revealed by serum IgA levels of less than 0.5 mg/dL.

Discussion

The fluorescent microsphere immunoassay described in this report compares favorably with several previously reported methods for detecting anti-IgA.[12–17] The assay method has excellent analytical sensitivity as revealed by dose-response studies with positive sera and inhibition studies with soluble IgA. In addition, the results show excellent discrimination between nonspecific binding and binding produced by IgG anti-IgA. Only slightly more than 1 percent of sera tested in the clinical laboratory with this

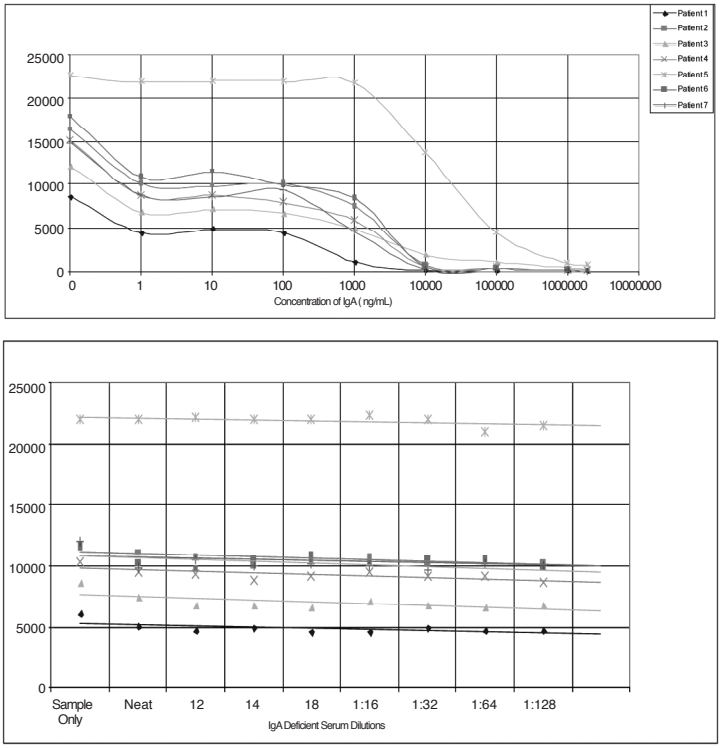

Fig. 3. Inhibition studies with polyclonal IgA and IgA-deficient serum inhibitors. Sera from seven patients with IgG anti-IgA were tested. Fluorescent responses are plotted versus the log IgA inhibitor in nanograms per milliliter (**upper frame**) or dose of IgA-deficient serum inhibitor **(lower frame**; see text).

assay yielded equivocal results during more than 18 months of use, and those positive sera, obtained from four patients whose medical histories were available for review, were confirmed as being associated with immediate hypersensitivity transfusion reactions and IgA deficiency.

Overall, the frequency of positive test results among the 724 patient samples tested during 18 months of use was 5.2 percent. In the paper published by Sandler et al.,[11] anti-IgA was detected in 18 percent of sera submitted for testing. How can this discrepancy with our results be explained? The frequency of positive results is determined primarily by the selectivity of physicians in choosing sera for testing. It is worth noting that our institution accepts specimens from laboratories around the world for testing, and it must be assumed that not all requests are supported by strong clinical evidence of prior transfusion reactions or even diminished levels of IgA in blood. Therefore, no direct comparison can be made with the experience reported by Sandler et al.[11]

The initial methods for detecting anti-IgA described in the medical literature used an immunodiffusion technique that was sensitive to microgram per milliliter concentrations of antibodies, required purified IgA myeloma proteins as reagents, and took several days to generate test results.[12,20] Subsequent generations of immunoassays, which included hemagglutination assays, modified radioimmunoassays, and sandwich immunoradiometric assay methods, had improved analytical sensitivity and were capable of detecting nanogram per milliliter concentrations of anti-IgA.[11,15] Despite having improved analytical sensitivity, these methods also used purified IgA myeloma proteins as reagents. The radioassay methods took days to perform and had the added disadvantage of using radionuclides in the assay procedures, which limited the shelf life of test reagents and required monitoring of testing personnel and disposal of radioactive wastes. Hemagglutination assays were more rapid, but had high levels of interassay variability and generated occasional false-negative results attributable to prozone phenomena. Sandwich immunoradiometric assays could be adapted to the use of purified polyclonal IgA as an antigen source, but these methods still required the use and disposal of radionuclides. More recently, enzyme immunoassay, flow cytometric immunoassay, and particle gel immunoassay methods have been used to measure anti-IgA.[13,14,16] Compared with enzyme immunoassay, the flow cytometric assay is reported to be more reproducible as large numbers of latex microbeads are used to test each individual serum specimen compared with one or two wells in a typical microtiter enzyme immunoassay. The fluorescent microsphere immunoassay we have developed is similar in design to a flow cytometric immunoassay. Both assays rely on optical measurement of fluorescence generated by the binding of fluorochrome-conjugated anti-IgG to specific antibodies bound to IgA-coupled beads. In the fluorescent microsphere immunoassay, IgA is coupled covalently to fluorescent microspheres. Covalent coupling of IgA to the microsphere immunosorbent reagent contributes to a long shelf life and very low nonspecific binding. We have noted stable binding of calibrator and control sera to the IgA-coupled microspheres for several months of use. In other respects, the fluorescent microsphere and flow cytometric assays appear quite comparable, although it is not possible to compare the analytic sensitivity of these methods without performing tests on the same sera.

As noted earlier, the fluorescent microsphere immunoassay for IgG anti-IgA we developed has proved reliable for more than 18 months of routine use in the clinical laboratory. Compared with the immunoradiometric assay we used for several years, the fluorescent microsphere immunoassay has improved our practice by markedly reducing the time required to evaluate patients who might be at risk of anaphylactic transfusion reactions or might require blood products free of IgA. The results of the microsphere immunoassay are available within 4 hours, whereas those of the immunoradiometric assay require 48 hours to be available. This has the potential to decrease utilization of the rare IgA-deficient plasma product inventory maintained at our institution.

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