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An enzyme-linked immunosorbent assay to screen blood donors for IgA deficiency

CRISTINA SANZ, CAROLINA FREIRE, ANTONI ORDINAS, ARTURO PEREIRA
Service of Hemotherapy and Hemostasis, IDIBAPS, Hospital Clínico, Barcelona, Spain

ABSTRACT

Background and Objectives. **In order to build panels of IgA deficient blood donors, an assay is described that is sensitive, inexpensive and easily adaptable to the automated sample processors and turnaround times of blood banks.**

Design and Methods. **We developed a two-step enzyme-linked immunosorbent assay (ELISA) carried out in microwell plates coated with rabbit anti-human IgA antibody. Captured IgA was revealed with the same antibody conjugated to horseradish-peroxidase. The assay was adapted to the automatic pipetting system and ELISA processors used in routine blood donor screening.**

Results. **The assay sensitivity was 0.1 µg/mL. Intra-assay coefficient of variation (CV) for IgA concentrations between 0.1 and 100 µg/mL ranged from 0.69% to 3.80%. The median interassay CV was 3.05% (range: 1.2-7.9%). Coated plates can be stored frozen for at least 3 months without any loss in performance. The assay takes around 80 min to be performed. By using this ELISA we found 32 IgA-deficient individuals among 20,000 blood donors (prevalence 1:625).**

Interpretation and Conclusions. **The ELISA has a good sensitivity, is reproducible, precise and time-saving. It is easily adaptable to the automated sample processors and operating procedures used in blood banks. This facilitates the building of panels of IgA-deficient blood donors.**

Key words: IgA, enzyme immunoassay, IgA deficiency, transfusion

IgA deficiency is a common finding in healthy people, since its prevalence ranges from 1:18,500 in Japanese blood donors[1] to 1:328 in American ones[2] to 1:163 in Western Spain,[3] which is the highest prevalence so far reported. IgA deficiency is usually asymptomatic, but occasionally it may be associated with other immune diseases or may predispose patients to recurrent infections, mainly of the respiratory tract.[4] Some IgA-deficient patients have anti-IgA antibodies in plasma and develop severe anaphylactic reactions when they are transfused with IgA-containing blood components. Although the frequency of anaphylactic reactions was estimated to be between 1:20,000 and 1:47,000 transfusions[5-7] they constitute one of the most frequent non-hemolytic causes of transfusion-related mortality.[8] Patients with anti-IgA should receive blood components poor in IgA, ideally containing less than 0.5 µg/mL,[9] a recommendation that some authors extend to all IgA-deficient blood recipients, in order to avoid the induction of anti-IgA antibodies.[10,11]

It is possible to obtain IgA-poor red blood cells and platelets by extensive cell washing, but this is a long and laborious procedure, that may be insufficient for highly sensitized patients,[12] and unsuitable for those requiring fresh frozen plasma or cryoprecipitates. Transfusing blood components obtained from IgA deficient donors seems to be a better alternative. However, building panels of IgA deficient individuals entails massive screening of a large number of blood donors, which requires a sensitive and inexpensive assay that could be performed in the turn-around time of donor screening and product release, and that could be adapted to the automated sample processors used in blood banks.

We describe a microtiter plate enzyme-linked immunosorbent assay (ELISA) for determination of low concentrations of IgA that fulfils the above conditions. Since this assay was included in our blood donor screening protocol, 32 IgA deficient individuals have been found in the 20,000 donors tested.

Design and Methods

Reagents and equipment

Microtiter plates were obtained from Nunc (MaxiSorp F96, Roskilde, Denmark). Unconjugated and horseradish peroxidase-conjugated rabbit anti-human IgA were from Dako (Glostrup, Denmark). Bovine serum albumin (BSA), O-phenylenediamine dihydrochloride (OPD) and phosphate-citrate buffer tablets with urea hydrogen peroxide were from Sigma Chemicals (St. Louis, MO, USA). Phosphate-buffered saline (PBS), 150 mM pH 7.2, was supplied by Biomérieux (Marcy, l'Etoile, France). Tween 20

Correspondence: Cristina Sanz , MD, Service of Hemotherapy and Hemostasis, Hospital Clínico, Villarroel 170, 08036 Barcelona, Spain. Phone and Fax: international +34-93-2275448 – Email: csanz@medicina.ub.es

was purchased from Merck (Schuchardt, München, Germany). The purified human serum IgA preparation used as reference for the assay standardization was from Sigma. The IgA concentration in this standard was confirmed by nephelometry.

The coating buffer was PBS. The blocking solution consisted of 3% BSA in PBS. The washing solution contained 0.1% BSA and 0.05% Tween 20 in PBS. The substrate was prepared by dissolving 10 mg of OPD in 25 mL of phosphate-citrate buffer with urea hydrogen peroxide (0.05 M phosphate-citrate buffer, containing 0.014% H_2O_2, pH 5.0).

Assays were performed using an automatic pipetting system (Ortho Summit plus, manufactured by Hamilton Company, Bonaduz, Switzerland) and an automated ELISA processor (Ortho Summit processor, manufactured by Hamilton Company).

Procedures

The microtiter plates were coated with 100 µL per well of a 1/1,000 dilution of rabbit anti-human IgA in the coating buffer, and incubated overnight at 4°C in a wet chamber. After coating, plates were emptied, filled with 225 µL of blocking solution per well and held for 3 hours at room temperature. Thereafter, plates were emptied, covered with plastic films and stored frozen at –80°C for up to three months.

Microtiter plates were allowed to thaw at room temperature and washed six times just before use. One hundred microliters of control and test samples (both diluted 1/100 in washing solution) were dispensed into each well and incubated at 37±1ºC for 30 min. After six washes, 100 µL per well of HRPO-conjugate rabbit anti-human IgA, diluted 1/4,000 in washing solution, were added and incubated for 30 min at 37ºC. After washing the plate six times, 200 µL of substrate solution were dispensed to each well and incubated at 22ºC for 20 min in the dark. The reaction was stopped with 25 µL of 4 N H_2SO_4 and the optical density (OD) of each well was measured at 492 nm, with the reference wavelength set at 620 nm. Absorbance values were corrected for the sample blank, which consisted of human IgA-deficient plasma.

For the assay standardization, known concentrations of IgA were obtained by diluting the reference IgA preparation in IgA-deficient human plasma. Each IgA dilution was processed 6 times as described above, and the mean, standard deviation (SD), 99% confidence intervals (CI) around the mean, and coefficient of variation (CV) of OD were calculated.

Results

Standardization and assay sensitivity

A standard curve relating OD to IgA concentration was made by using dilutions of the reference IgA preparation (Figure 1). No differences were found between plates used just after coating and those which were stored frozen for three months. As can be seen in the figure, the OD for an IgA concentration of 0.01 µg/mL overlapped with that of IgA-deficient plasma. In contrast, the lower limit of the 99% CI around the mean OD for IgA concentrations of 0.1 µg/mL was 3.1 times higher than the upper limit of the 99% CI around the mean of the IgA-deficient plasma (30.5±7.2). Therefore, the assay sensitivity is 0.1 µg/mL, since this was the lowest IgA concentration that was clearly discriminated from the blank.

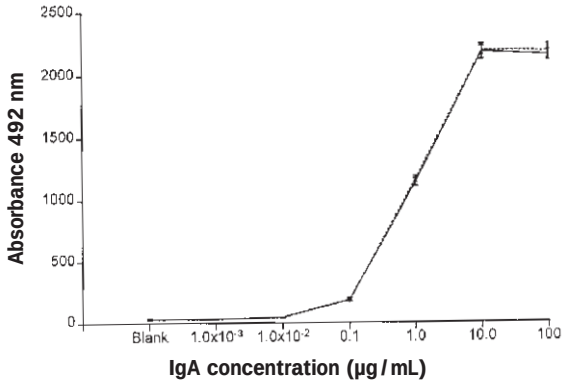

Figure 1. Absorbance at 492 nm as a function of IgA concentration in fresh (continuous line) and frozen stored (dotted line) microtiter plates. Values are represented as the mean and 99% CI of 6 determinations.

Assay imprecision

The intra-assay CV for IgA concentrations between 0.1 µg/mL and 100 µg/mL ranged from 0.69% to 3.8% (Table 1). Inter-assay CV was assessed by analyzing 20 samples from normal donors in six assays runs. The median for the inter-assay CVs of OD was 3.05% (range: 1.2-7.9%)(Table 2).

High-dose hook effect

In order to test the possibility of false negative results due to very high IgA levels in donor's plasma (high-dose hook effect), we investigated the assay's response to serial dilutions of a human monoclonal IgA in IgA-deficient plasma. Final IgA concentrations on the plate ranged from 6.7 mg/mL to 100 µg/mL. In all cases, the OD values remained high, proving that the assay was not prone to the high-dose hook effect.

Table 1.

IgA concentration (µg/mL)	OD_{492}*	CV (%)
100	2184±58	2.65
10	2191±32	1.46
1	1158±8	0.69
0.1	184±7	3.80

**Mean of 6 values. Coefficient of variation (CV) of the optical density at 492 mm (OD_{492}) for different plasma concentrations of IgA.*

Table 2.

Sample	*OD_{492}**	*C.V. (%)*
1	1805.7±91.5	5.06
2	1842.7±74.9	4.06
3	1837.2±40.7	2.2
4	1831.0±92.2	5.0
5	1856.2±53.6	2.8
6	1862.8±36.7	1.97
7	1839±63.13	3.43
8	1905.7±39.2	2.05
9	1910.3±38.7	2.0
10	1866.2±23.3	1.2
11	1839.7±59.6	3.2
12	1860.8±51.8	2.7
13	1855.5±50.6	2.7
14	1829.5±82.3	4.4
15	1839.5±69.4	3.7
16	1841.8±108.5	5.8
17	1863.8±55.6	2.9
18	1864.3±73.0	3.9
19	1826.7±145.4	7.9
20	1896.8±85.3	4.4

**Mean of 6 values. Inter-assay precision. Samples from 20 healthy donors were tested in six different assay runs, and the mean optical density at 492 nm (OD_{492}), standard deviation around the mean, and coefficient of variation (CV) were calculated.*

Routine screening of blood donors

In the routine screening of blood donors a working cut-off was set in the mean absorbance of negative controls plus 0.200, since the resulting figure corresponds to the assay's sensitivity threshold. Thirty-two IgA deficient individuals were found in 20,000 blood donors tested in the last three years, so the prevalence of IgA deficiency was 0.16%, or one case per 625 blood donors. The IgA deficient plasma samples were confirmed twice by our ELISA, and they were also submitted to nephelomety, where the IgA content was below the detection threshold of the assay. The mean ± SD absorbances at 492 nm for IgA deficient and IgA-containing samples were 0.026± 0.053 and 2.738±47.4, respectively.

Since the screening of blood donors for IgA deficiency was started, two units of IgA deficient fresh-frozen plasma have been transfused uneventfully to a patient with anti-IgA antibodies, who had an history of a severe post-transfusion anaphylactic reaction.

Discussion

Assays usually employed to quantify serum IgA have several handicaps to being applied to the massive screening of blood donors. Nephelometry, radial immunodiffusion and radiommunoassay either have low sensitivity, take a long time to be performed, cannot be easily automated or require costly instrumentation that is unfamiliar to most blood banks.[9,11,13] Hemagglutination inhibition assays are used in some blood banks to screen donors for IgA deficiency.[14] These assays have the advantage that they can be adapted to the automated blood grouping machines, their sensitivity is around 0.5 µg/mL and they can be performed in the lapse of time usually spent for blood donor screening. However, hemagglutination assays require a tedious red blood cell coating procedure and relatively specialized skills for its performance and results' interpretation. In addition, false positive results can occur due to anti-RBC antibodies in the donors' serum.[6,13,14]

Our goal was to develop a sensitive and inexpensive assay that could be easily adapted to the procedures and equipment routinely used in blood donor screening, in order to take advantage of technicians' skills and the already installed instrumentation and operating procedures. Microtiter ELISA fulfils these prerequisites since it is the method used in most blood banks to screen donors for viral diseases. In addition, the sensitivity level of our ELISA compares favorably with those reported for similar assays, which ranged from 0.5 µg/mL to 5 µg/mL,[11,13,15] and is five-fold lower than the 0.5 µg/mL threshold suggested for IgA poor blood components.[9] The precision is also satisfactory, since the CV for IgA concentrations in the sensitivity limit of the assay was only 3.8%, whereas in the inter-assay reproducibility assessment, the CVs were always below 7.9%. IgA deficient donors were clearly discriminated, since differences in OD between normal and IgA deficient samples were around 105-fold. This might theoretically lead to some IgA deficient donors passing undetected, because of carryover from neighboring wells in the microtiter plate. However, both in reproducibility assays, in working intra-assay negative controls, and in repeated tests of IgA-deficient individuals, no carryover was detected. A more serious possibility would be that of taking as IgA-deficient a donor with abnormally high serum levels of IgA (i.e. unrecognized monoclonal gammapathy of uncertain significance) because of the high-dose hook effect. This effect can occur in one-step assays in which samples and conjugate are incubated together, so that most conjugate is washed out with the excess antigen that was not captured by the coating antibody.[16] The high-dose hook effect is very rare in two-step assays, such as the one described in this work, and it was ruled out after testing samples with very high IgA concentrations.

Although the assay was home-made, plates can be prepared in bulk by using automated sample processors to dispense the coating antibody, and the washing and blocking solutions. Moreover, microtiter plates can be stored frozen without any loss in performance, at least for as long as three months. It is therefore comparable to a commercial kit in regards to ease of handling, stability and reproducibility. Plates and reagents are readily available from commercial sources and their cost was estimated to be around $0.1 per test. Since the assay can be per-

formed in 1.5 hours and takes advantage of the operating procedures already in use for viral screening, its implementation did not imply a significant increase in the technicians' workload.

The assay conditions represent a trade off between maximizing sensitivity and minimizing costs and incubation times. They were not chosen to quantify serum IgA accurately across a wide range of values. Therefore, the relation of IgA concentration to OD is linear only in the interval between 0.1 µg/µL and 10 µg/mL. However, it is plausible that linearity may be extended to higher IgA concentrations after minor modifications in dilution of samples or reagents and incubation times.

Our ELISA is comparable to that reported by Hirvonen *et al.*,[15] from the Finnish Red Cross Blood Service, and to some commercial assays.[13] Ours, however, is quicker to perform (1.5 hours vs 4 hours), which is a valuable advantage in the workloaded scenario of blood bank laboratories. It also has demonstrated to be a robust and useful assay under daily laboratory conditions, which are less controlled than those during the assay's development. In the routine screening of blood donors we found that the prevalence of IgA deficiency in our donor population is 0.16%, or 1 case per 625 individuals. This figure is similar to those reported from other European countries or the USA,[2] but is significantly lower than the prevalence of IgA deficiency found by Pereira *et al.* in Western Spain.[3] Differences in assay sensitivities or in population characteristics probably account for this discrepancy.

Contributions and Ackonwledgments

This study was designed and co-ordinated by AP. CS and CF carried out the validation studies and the assay adaptation to automated processors. CS wrote the manuscript, which was critically reviewed by CF, AO and AP.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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