Quantitation of red cell-bound IgG, IgA, and IgM in patients with autoimmune hemolytic anemia and blood donors by enzyme-linked immunosorbent assay

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This paper describes an enzyme immunoassay for the quantitative determination of IgG, IgA, and IgM immunoglobulins on RBCs. Ether eluates made from RBCs were followed by an enzyme-linked immunosorbent assay of immunoglobulin concentration. Calibration curves were derived from immunoglobulin standards and the number of molecules of each isotype per RBC was calculated. The assay was carried out in 200 healthy blood donors and 62 patients with warm autoimmune hemolytic anemia (AIHA), two of them with a negative DAT. For healthy blood donors, mean values were 58 IgG, 16 IgA, and 3 IgM molecules per RBC. For patients with a positive DAT, the mean values were 3435 IgG, 157 IgA, and 69 IgM molecules per RBC. An increased level of IgA was found in 12 patients without IgA autoantibodies demonstrable in RBC eluates. Increased IgG levels were also observed in patients with a negative DAT and, in one case, an increased level of IgA was also found. The enzyme-linked immunosorbent assay using ether eluates is a sensitive method for quantitating RBC autoantibodies in patients with AIHA as well as immunoglobulins bound to RBCs in healthy individuals. 

Key Words: enzyme-linked immunosorbent assay, autoimmune hemolytic anemia, IgG, IgA, and IgM immunoglobulins, RBCs, quantitative method

Autoimmune hemolytic anemia (AIHA) is characterized by immune-mediated hemolysis associated with the presence of IgG, IgA, or IgM immunoglobulins on the RBC membrane. The most common type of AIHA is associated with warm-reactive antibodies and in most cases they are detectable by the DAT. The presence of immunoglobulins can also be revealed on the surface of RBCs of DAT-negative healthy individuals by more sensitive methods. Several methods to quantitate RBC-bound immunoglobulins have been reported, including an immunoradiometric assay, autoanalyzer assays, antiglobulin consumption assays, and enzyme-linked immunosorbent assay (ELISA). Quantitation of immunoglobulins in a patient with AIHA would allow a more precise method of monitoring the disease or defining the synergistic effect of the different immunoproteins in causing RBC destruction.

We have developed an enzyme immunoassay for the quantitative determination of immunoglobulin isotypes in ether eluates made from RBCs. This method can be used to study RBC-associated immunoglobulins in patients with AIHA and in healthy individuals.

Materials and Methods

Blood samples and ether elution

After informed consent had been obtained from patients and blood donors, blood samples were collected from venous blood in EDTA (Merck). The samples were centrifuged at 270 × g for 8 minutes at room temperature, and the plasma anduffy coat were removed.

Ether eluates were prepared after washing 1 mL of packed RBCs × 10 using 10 volumes of saline. Elution was performed by adding 1 mL of PBS, pH 7.2, containing 0.4% BSA (Sigma Chemical Co.) and 1 mL of diethyl ether (Merck) to 1 mL of RBCs (an average of 1.1 × 10^10 RBCs). The mixture was incubated in a 37°C water bath for 10 minutes, with frequent mixing. After centrifugation, the upper layer of ether was discarded and the hemoglobin-stained eluate was transferred into a test tube. The residual ether was evaporated at 37°C for 15 minutes. An average of 1 mL of eluate was recovered. Eluates were kept frozen at −30°C until used.
ELISA for the quantitation of IgG, IgA, and IgM on RBCs

IgG, IgA, and IgM standard curves were prepared from reference sera (Nor-Partigen, Behring) in concentration ranges from 0.0025 to 0.1 µg/mL in PBS, 0.05% Tween (Tw) 20 (Merck), and 0.4% BSA (PBS/Tw/BSA). Flat-bottomed 96-well plates (Maxisorp, Nunce Immunoplates) were precoated with 100 µL per well of 5 µg/mL goat anti-human IgG, IgA, or IgM (Sigma) in 0.05M carbonate buffer, pH 9.6, at 4°C temperature overnight. After washing × 4 in PBS/Tw, 0.2%, 100 µL per well of standards or diluted eluates in PBS/Tw/BSA were incubated at 37°C for 60 minutes. The plates were washed × 4 in PBS/Tw, 0.05%, before addition of 100 µL per well of peroxidase conjugated (Sigma) goat anti-human IgG or IgM diluted 1 in 5000 or goat anti-human IgA diluted 1 in 1000. The plates were incubated with the conjugate at 37°C for 60 minutes and then washed as above. A substrate solution (100 µL per well) of 0.24 mg/mL of O-phenylenediamine (Merck) in 0.05M phosphate citrate solution (100 µL per well) of 0.24 mg/mL of O-phenylenediamine (Merck) in 0.05M phosphate citrate buffer, pH 5.0, with 0.024% of hydrogen peroxide (BDH) and absorbance was read at 492 nm on the Titertek Multiscan MC plate reader. Absorbances of duplicate determinations were plotted against the concentration of the standard points (linear scale). The amount of IgG, IgA, and IgM was calculated and the results were expressed as the approximate number of molecules of immunoglobulin per RBC, using Avogadro’s constant and the molecular weights of IgG or IgA (160,000 daltons) and IgM (970,000 daltons). According to Avogadro’s constant, 1 µg contains 376 × 10^10 molecules of IgG or IgA and 62 × 10^10 molecules of IgM. The number of molecules of immunoglobulins on RBCs is equal to the concentration in µg/mL of Igs in eluates, multiplied by the molecules of Igs in 1µg, and divided among the number of RBCs in 1 mL.

Effect of test sample milieu on IgG, IgA, and IgM quantitation by ELISA

We obtained free hemoglobin from human RBCs as follows: To 6 mL × 10 washed RBCs an equal volume of hypotonic lytic solution (5M sodium phosphate buffer, pH 7.4) was added. We placed the mixture in a 37°C water bath for 10 minutes. The hemoglobin solution was collected by centrifugation at 370 × g for 30 minutes at room temperature and dialyzed overnight against 20 volumes of PBS. IgG, IgA, and IgM standard curves from reference serum (Nor-Partigen, Behring) were prepared in the hemoglobin solution and one sample without immunoglobulins was used as a blank. To 1 volume of each point of standard curves prepared in the hemoglobin solution and the blank, an equal volume of diethyl ether was added and mixed. The mixture was incubated at 37°C for 10 minutes. The upper layer of ether was carefully discarded and the residual evaporated at 37°C for 15 minutes. BSA and Tw 20 were added to each sample to obtain a concentration of 0.4% and 0.05%, respectively. The ELISA was carried out with standard curves with this treatment and with standard curves prepared in PBS/Tw/BSA. The slopes of the curves were compared with the t test.

Analytical evaluation of the ELISA

The limit of detection was calculated as the concentration of the dilution of the first point of the curves that gave an absorbance more than the mean absorbance of the blank, plus 3 standard deviations (SDs). The undiluted and diluted 1 in 5, 1 in 10, 1 in 100, and 1 in 500 in PBS/Tw/BSA of hemoglobin solution treated with ether and the PBS/Tw/BSA were used as blanks and also as a control for nonspecific binding by the assay. The mean and SD were calculated on 20 repeat estimation assays.

The working range of the assay was determined by testing in replicates of 10 at each point of the standard curves of IgG, IgA, and IgM and the coefficient of variation (CV) was calculated. The working range was accepted as that over which CV was no greater than 10%.

Parallelism was investigated after serial dilution in PBS/Tw/BSA of seven eluates made from RBCs with reactions of 4+ (IgG, IgA) or 3+ (IgM), 1+, and negative in the DAT with anti-IgG (BPL; Elstree, UK), -IgA, and -IgM (CLB; Amsterdam).

The eluate of a pool of ten DAT-negative RBCs as well as two eluates with IgG, IgA, and IgM autoantibodies, and a standard human serum with known concentration of immunoglobulins (Immunocontrol, Bio-Mérieux, France) adjusted to a concentration of 0.052 µg/mL of IgG, IgA, and IgM, were used to determine the precision of the ELISA. The intra-assay and the inter-assay coefficient of variation on separate days were calculated on 20 determinations.

Test samples

The ELISA was carried out in duplicate in ether eluates from 200 healthy blood donors with a negative
Quantitation of red cell immunoglobulins

DAT, 60 DAT-positive patients with warm AIHA, and two patients with AIHA and a negative DAT. Hemolytic anemia was documented by a low hemoglobin concentration, increased reticulocytes, increased unconjugated bilirubin concentration, the presence of marrow erythroid hyperplasia, and the exclusion of other hemolytic disorders. Thirty-nine patients had idiopathic AIHA; the hemolytic anemia in the remainder was associated with other conditions: four patients had systemic lupus erythematosus, four had unspecified respiratory infections, four had chronic lymphocytic leukemia, three were on methyldopa, three had chronic active hepatitis, two had multiple myeloma, two had acute lymphocytic leukemia, and one had non-Hodgkin's lymphoma. Patients' ages ranged from 1 to 84 years (median 45 years) and 41 were female and 21 were male. The eluates from blood donors were tested undiluted and diluted 1 in 5; those for patients were tested after serial dilution in PBS/Tw/BSA to ensure that the tests were carried out within the working ranges for the assay.

Immunohematologic studies

The immunoglobulin classes of the autoantibodies were determined by the DAT using monospecific anti-IgG (BPL), -IgM, and -IgA reagents (CLB, Amsterdam) and in the eluates by a microplate test.

The microplate test was performed using microtiter plates with V-shaped bottom wells (Greiner) as follows: 10 µL of the mixture of three kinds of packed group O RBCs (R₁R₁, R₂R₂, and rr), were incubated with 100 µL of the eluate at 37°C for 1 hour in a tube. The RBCs were washed × 4 and 0.5% of RBC suspensions in saline were made. Anti-IgG, -IgA, and -IgM were diluted 1 in 2, 1 in 5, 1 in 10, and 1 in 20 in PBS with 5% of fetal calf serum (Gibco, UK). Then 25 µL of the antiserum dilutions and the diluent (as negative control) were transferred into appropriate microtiter wells and an equal volume of the sensitized RBC suspension was added. After overnight incubation at 4°C, the microtiter plate was placed at a 60° angle for 10 minutes, then inverted for 5–10 minutes and read. When the result was positive, the cells remained together in a tight button, when negative they ran down in a smear.

All the patients with a positive DAT had IgG autoantibodies. In 11 and six patients, IgA and IgM autoantibodies, respectively, were also present. Similar patterns of immunoglobulin classes were found in the eluates and with the DAT. Antibodies were not detected in eluates from patients with AIHA and a negative DAT.

Results

Curves of IgG, IgA, and IgM represent the mean of ten consecutive standard curves, each performed in duplicate. Immunoglobulin standards made up in hemoglobin and treated with ether did not significantly affect the standard curves (p > 0.49) (Fig. 1). The hemoglobin solution treated with ether used as a blank gave values of optical density from 0.053 to 0.061 similar to the PBS/Tw/BSA (0.051). The working ranges for the ELISA were found to be 0.0025 to 0.07 µg/mL for IgG and IgM and 0.0025 to 0.06 µg/mL for IgA. The limit of detection for IgG corresponded to 0.00062 µg/mL for IgG (Fig. 1A) and IgA (Fig. 1B) with immunoglobulin standards prepared in PBS/Tween 20 (0.05%), and BSA (0.4%) (●) and in hemoglobin treated with ether (○). The curve for IgM is similar to that for IgG.
µg/mL, for IgA to 0.0011 µg/ml, and for IgM to 0.0068 µg/ml (approximately 1 molecule of Igs per RBC). The ELISA showed excellent parallelism for all the samples diluted in the measuring ranges with interdilutional coefficient of variation from 5.9% to 16% for IgG samples, from 2.9% to 13% for IgA samples, and from 2.4% to 8.6% for IgM samples (Fig. 2). The intra-assay variation ranged from 3.6% to 6.7% for IgG samples, from 3.1% to 6.5% for IgA samples, and from 5.5% to 6.7% for IgM samples. The inter-assay variation ranged from 9.2% to 10% for IgG samples, from 7.5% to 9.5% for IgA samples, and from 8.2% to 9.3% for IgM samples.

It was established that the immunoglobulins present in the eluates were not contaminated by serum immunoglobulins after ten washes of the RBCs (as described in Materials and Methods). The supernatant of the last wash gave values of optical density similar to those of the blank in the ELISA.

The ELISA on healthy blood donors gave mean and standard deviation values of 58 ± 35 molecules of IgG per RBC (range from 2 to 146), 16 ± 11 molecules of IgA per RBC (range from 1 to 81), and 3 ± 2 molecules of IgM per RBC (range from 1 to 11).

In AIHA patients with a positive DAT, the range of results for IgG was from 206 to 20,000 molecules per RBC, with a mean of 3435 molecules. In the group of patients with IgA autoantibodies the range of results was from 90 to 250 molecules per RBC, with a mean of 157 molecules. An increased level of IgA, with a range from 94 to 385 molecules per RBC, was found in 12 patients with a negative DAT and in the test of eluates by microplate with anti-IgA. In the remainder of cases, the values of molecules of IgA per RBC were within the range found in blood donors. A mean value of 69 with a range from 26 to 109 molecules per RBC was obtained in patients with IgM autoantibodies and results were within the normal range in the rest of the patients. The results are shown in Figure 3.

Increased IgG levels of 370 and 460 molecules per RBC were observed in two patients with AIHA and a negative DAT. In one case an increased level of IgA (119 molecules per RBC) was also found.

Discussion

The sandwich ELISA described provides a method for the quantitation of RBC-bound IgG, IgA, and IgM immunoglobulins. The assay was carried out in ether eluates because our laboratory routinely performs elution in the investigation of autoantibodies in AIHA and because the testing of eluates is a more sensitive method to detect immunoglobulins than direct testing of the RBCs.14

Differences in the milieu in test samples and immunoglobulin standards can produce nonspecific effects, which modify the kinetics of antigen-antibody reactions and could affect the estimation of Ig concentration from the standard curve.11 However, the presence of hemoglobin and the ether treatment of immunoglobulins did not affect standard curves. Therefore, all the test samples were investigated with immunoglobulin standards prepared in PBS/Tw/BSA. Hemoglobin has a peroxidase activity and obscures the antibody assay if a peroxidase conjugate is used.15 In this ELISA the hemoglobin in eluates was removed by washing and did not interfere with the peroxidase
Quantitation of red cell immunoglobulins

The unaffected immunoglobulin detection after ether treatment was not surprising in view of the known use of this elution method for recovering antibody attached to RBCs, although Dumaswala et al. found decreased detectability of IgG after treatment with another organic solvent such as xylene, using an immunoblotting technique with peroxidase-labeled anti-IgG.

The ELISA showed a wide measuring range; the lower limit corresponded approximately to one molecule of immunoglobulin per RBC. Accordingly, this assay is suitable for the quantitation of immunoglobulins on RBC eluates from DAT-positive patients and from normal subjects. Although the working range had an upper limit of 0.07 µg/mL for IgG and IgM, and 0.06 µg/mL for IgA, it could be extended by dilution of high concentration samples with PBS/Tw/BSA. This was in agreement with the excellent dilution parallelism over the measuring range showed by assay with all sample types tested.

We found the mean amount of 58 IgG, 16 IgA, and 3 IgM molecules per RBC by using the eluates from healthy donors. Other methods gave similar results for IgG and IgM. There has been one previous report of the number of RBC-associated IgA molecules on normal human RBCs. That study reported a median of < 29 molecules per RBC, which is comparable to the amount quantitated by our assay.

There was considerable variation in the number of IgG, IgA, and IgM molecules per RBC in patients with AIHA, demonstrating the ability of the assay to measure widely different degrees of sensitization in clinical samples. The results are comparable with previous studies where the reported ranges in patients with AIHA were from 230 to about 30,000 IgG molecules, from 20 to 168 IgM molecules, and from < 29 to 4500 IgA molecules per RBC.

We encountered IgA autoantibodies in 18 percent of DAT-positive patients, which is similar to the 14 percent reported previously by Sokol et al. An increase of RBC-bound IgA by ELISA was also found in 12 patients without IgA autoantibodies demonstrable on RBCs by the DAT and in the eluates by a microplate test. The clinical significance of antibodies only detected by ELISA should be further elucidated because an increased amount of cell-bound immunoglobulins may also be due to immune complexes and nonspecific adsorption of Igs onto RBCs rather than as a result of autoantibodies.

Similar considerations are applicable to the results found in two patients with AIHA and a negative DAT without antibodies demonstrated in eluates. However, in these cases there were findings to suggest that IgG and IgA (in one case) antibodies detected by ELISA were responsible for the hemolysis. The hemolytic anemia in these patients was associated with chronic lymphocytic leukemia and non-Hodgkin’s lymphoma, respectively. It is known that the incidence of AIHA is higher in patients with these hematological malignancies than in the general population. Previous investigators have demonstrated IgG and IgA autoantibodies on RBCs with more sensitive methods.

Fig. 3. The number of IgG (A), IgA (B), and IgM (C) molecules per RBC in patients with AIHA. The increased values for IgA and IgM were found in patients with positive DATs with anti-IgA and -IgM, respectively. An increased level of IgA was also found in 12 patients without IgA autoantibodies demonstrable on their RBCs. A heavy horizontal line inside Figure 3 (A, B, and C) shows the highest value of molecule per red cell obtained in healthy blood donors.
than the DAT in patients with a DAT-negative AIHA. The patients were diagnosed by clinical manifestations including the exclusion of other hemolytic disorders and a positive clinical course following administration of corticosteroid.

These results showed that the quantitative determination of immunoglobulins on eluates by ELISA appears to be useful for the serologic diagnosis of DAT-negative AIHA, although the assay should be evaluated with a larger number of cases.

Previous investigation identified RBC autoantibodies of the IgM class in approximately 30 percent of patients with warm AIHA by enzyme-linked DAT. We found a frequency (around 10%) of warm IgM autoantibodies that is similar to those found in earlier studies in both the DAT and the ELISA. This difference may be attributable to multiple washing required by our assay, which carries the possibility for loss of small quantities of IgM autoantibodies prior to elution. In addition, it can be difficult to obtain IgM autoantibodies in eluates. An investigation showed that heat elution was the better method compared to acid stromal, chloroform-trichlorethylene, and freeze-thaw methods. We detected IgM antibodies in ether eluates of only three of the six patients with IgM autoantibodies, when a conventional IAT with anti-IgM was used (data not shown). However, all the IgM autoantibodies revealed by the DAT were detected in the eluates by the microplate test and ELISA. As described previously, ELISA also quantitated a very small quantity of IgM on RBCs of healthy blood donors. We have no explanation for our different findings from a previous report.

In essence, the ELISA, using ether eluates, is a sensitive method to follow patients with RBC autoantibodies as well as immunoglobulins bound to RBCs in healthy individuals. The assay can be also extended to measure IgG subclasses or C3 by using appropriate specific antiserum.

References

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