Direct Quantitation of Platelet-Associated IgG by Electroimmunoassay

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An electroimmunoassay was applied to the quantitation of platelet-associated IgG (PAIgG). Protein solubilized by Triton X100 from washed platelets was electrophoresed at pH 5.0 in agarose gels containing carbamoylated rabbit anti-human IgG (pl = 5.0). Because the rabbit antibody is immobilized under these conditions, while PAIgG is freely mobile, rocket precipitates were produced, the heights of which were directly proportional to the amount of antigen (PAIgG) present. By this method, PAIgG for normal individuals was found to be 4.3 ± 1.7 fg/platelet (mean ± 2 SD; n = 35). Increased PAIgG levels (direct assay) were found in 27 of 29 patients with a diagnosis of clinically active idiopathic thrombocytopenic purpura (ITP), ranging from 10.5 to 101.5 fg/platelet. Moderately elevated PAIgG was found in 8 of 10 patients in an early stage of recovery from ITP (range 7.5–9.5 fg/platelet) and in 3 of 6 patients with apparent nonimmune thrombocytopenia (range 14.5–24.0 fg/platelet). Electroimmunoassay for PAIgG can be performed on patients with platelet counts as low as 2000/μl, yields results in less than 24 hr, is highly reproducible, and appears to provide a useful tool for the evaluation of patients with immunologically mediated thrombocytopenia.

SUBSEQUENT to the introduction of a quantitative antiglobulin consumption technique by Dixon et al.,1 several methods for the direct quantitation of IgG bound to platelets have been proposed. The level of platelet-associated IgG (PAIgG) in patients with idiopathic thrombocytopenic purpura (ITP), as determined by such direct assays, correlates reasonably well with the clinical status of the patient. Measurements of PAIgG can be useful in the effective management of patients with this disorder.

In this article, we have adapted the quantitative electroimmunoassay method of Laurell,2 as modified by Bjerrum et al.,3 to the quantitation of PAIgG in Triton-X100-solubilized preparations of washed platelets. We now provide evidence to show that this approach is simple, sensitive, relatively rapid, and highly reproducible.

MATERIALS AND METHODS

Carbamoylated Rabbit IgG

The IgG fraction of rabbit antiserum specific for gamma chains of human IgG was obtained commercially (Dako no. 10-090; Accurate Chemical and Scientific Corp., Westbury, N.Y.) and carbamoylated as described by Bjerrum et al.4 Briefly, to 89.8 ml of distilled water, 0.2 ml of 2 N HCl, 38.14 mg of sodium boroate, and 10.0 ml (~140 mg) of rabbit IgG (as supplied by the manufacturer) were added. The reaction vessel was placed in a water bath at 45°C, and the pH of the mixture was monitored constantly. The carbamoylation reaction was initiated by addition of 1.622 g of potassium cyanate, and the reaction was allowed to proceed for 220 min. The pH and ionic strength of the reaction mixture were maintained at 8.0 and 0.22, respectively, by drop-wise addition of a solution of 0.2 N HCl, 0.2 M KCl, 0.02 M NaCl, when necessary. The reaction was terminated by transferring the mixture to an ice bath, and the mixture was dialyzed for 24 hr against 2 4-liter changes of 0.01 M phosphate buffer, pH 6.0, containing 0.145 M NaCl. The carbamoylated IgG was finally dialyzed against 4 liters of 0.1 M sodium acetate, pH 5.0, containing 0.05% NaN3, and stored at 4°C until used. Based on initial concentration and final dilution following carbamoylation, the final IgG concentration was approximately 120 μg/ml.

Platelets

Platelets were isolated from EDTA-anticoagulated whole blood by differential centrifugation and washed in 0.013 M sodium-potassium phosphate, 0.145 M NaCl, 0.003 M EDTA, pH 7.4 (EDTA-PBS). The number of platelets in the final pellet was determined by counting (manually) the platelets in a known volume of EDTA-PBS prior to the final centrifugation. Two-tenths milliliter of 0.1 M sodium acetate, pH 5.0, was added to the pellet, and the platelets were resuspended by repeated gentle aspiration. Fifty microliters of 10% Triton X100 (v/v) in 0.1 M sodium acetate pH 5.0 were then added, and the mixture was agitated at 4°C for 30 min. Solubilized samples were either immediately assayed (without prior ultracentrifugation) or frozen at −30°C.

Electroimmunoassay

Electrophoresis was performed at pH 5.0 in 0.1 M acetate buffer. Solutions of molten 1% (w/v) agarose (Standard Low—m; Bio-Rad, Richmond, Calif.) in 0.1 M acetate pH 5.0 containing 0.5% Triton X100 (v/v) were kept at 56°C. Into a prewarmed glass test tube, 0.15 ml of the carbamoylated rabbit IgG suspension was mixed with 12.6 ml of the 1% agarose solution. The agarose–antibody mixture was poured onto a 10.2 x 8.3 cm section of Gel-Bond (Marine Colloids, Rockland, Me.), resulting in a gel of 1.5 mm thickness. When the gel had solidified, 4 mm diameter (15 μl volume) wells were punched to hold antigen (platelet) solutions. Electrophoresis was performed at 2 v/cm on platforms (Bio-Rad Model 1415) cooled to 15°C with an circulating water bath (Model 2095; Forma Scientific, Marietta, Ohio). Following electrophoresis, gels were pressed for 15 min, washed in 0.145 M NaCl for 15 min, pressed again for 15 min, then air-dried. Dried gels were stained by

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incubation for 3–5 min in a solution of 45% ethanol; 10% acetic acid containing 0.5% (w/v) Coomassie blue R (Bio-Rad), and destained by rinsing briefly in the same solution without Coomassie blue R.

**Human IgG Standards**

IgG was isolated from pooled normal human plasma by ammonium sulfate precipitation and DEAE-cellulose chromatography as described by Harboe and Ingold.4 Stock solutions of purified IgG (1.5 mg/ml) were stored in 0.1 M acetate buffer pH 5.0 at −70°C. Working solutions of IgG were prepared by dilution of stock solutions to final IgG concentrations of 30, 20, 10, and 5 g/ml with 0.1 M acetate buffer, pH 5.0, containing 10% (w/v) bovine serum albumin (Sigma) and 0.05% (w/v) NaN₃, and stored at 4°C until used.

**Patients**

The following groups of subjects were studied: (1) healthy normal volunteers with normal platelet counts (n = 35); (2) patients with thrombocytopenia at the time of testing who satisfied the diagnostic criteria of ITP (acute or chronic) as described by Lacey and Penner5 (n = 29); (3) patients with a history of acute or chronic ITP who were asymptomatic at the time of testing and who had normal or nearly normal platelet counts (n = 10); and (4) patients with thrombocytopenia of apparently nonimmune origin (n = 6). The last group included two patients with chronic alcoholism and associated liver damage, one patient with myelogenous leukemia, one patient with preleukemia (and splenomegaly secondary to portal hypertension), one patient with idiopathic nephrotic syndrome, and one patient with membranoproliferative glomerulonephritis, type I.

**RESULTS**

**Electrophoretic Characteristics of Carbamoylated Rabbit Immunoglobulin G**

Rabbit IgG that had been carbamoylated for 220 min at pH 8.0, 45°C was subjected to agarose gel electrophoresis at pH 5.0 alongside nontreated rabbit IgG (Fig. 1). Even after electrophoresis for only 2 hr at 4 V/cm, it is evident that the nontreated IgG (Fig. 1A) had migrated substantially toward the cathode, while the carbamoylated IgG (Fig. 1B) was essentially immobilized. The slight anodic migration of the carbamoylated IgG is attributable to electroendosmosis.

**Establishment of Standard Curves**

Standard curves for purified human IgG were determined by electroimmunoassay at pH 5.0 using carbamoylated rabbit IgG concentrations within the agarose gel ranging from 210 to 840 ng/sq cm (Fig. 2). It is apparent that a linear correlation exists between peak height (cm) and the amount of human IgG added to
the well at each of the three carbamoylated rabbit IgG concentrations. In order to conserve reagents, a concentration of rabbit IgG of 210 ng/sq cm was used in all subsequent assays. The addition of Triton X100, at a final concentration of 2% (v/v), to purified IgG standards did not affect the measurement of IgG by carbamoylated anti-IgG.

Normal Subjects

Platelet-associated IgG of 35 normal individuals was found to be 4.3 ± 1.7 fg/platelet (mean ± 2 SD) with a range of 2.5–7.2 fg/platelet. Duplicate measurements of platelet samples from several normal donors assayed on different occasions varied by ≤1.2 fg/platelet. Multiple determinations of individual platelet samples performed simultaneously varied by ≤10%.

Additional experiments using platelet samples from normal subjects were performed to establish conditions for optimal sample processing. These preliminary studies demonstrated that: (1) a minimum of two washes in EDTA-PBS was necessary to remove detectable plasma IgG contamination from platelet samples; (2) no increase in the level of detectable IgG was observed when washed platelet preparations were sonicated and/or frozen and thawed repeatedly either prior to or after Triton X100 solubilization; (3) lymphocyte contamination (≤5/100 platelets) or erythrocyte contamination (≤2/100 platelets) did not contribute significantly to the total IgG content of washed platelet samples; (4) the number of platelets, over a range of 2–50 × 10⁶/well, did not in itself significantly affect the efficiency of precipitation of the associated IgG by the carbamoylated rabbit antibody; (5) whole blood samples anticoagulated with EDTA could be stored for up to 96 hr at 4°C with no greater than a 25% increase in the platelet-associated IgG level, provided that visible hemolysis had not occurred; and (6) visible hemolysis in PRP isolated from stored EDTA-anticoagulated blood was routinely associated with an elevation of PAIgG ranging from 3 to 4 times normal levels. In view of the last observation, patient samples that had undergone visible hemolysis were excluded from analysis.

Patients

PAIgG was significantly elevated in 27 patients with clinically active idiopathic thrombocytopenic purpura, ranging from 10.5 to 101.5 fg/platelet (Fig. 3). In this group, whole blood platelet counts ranged from 1700 to 71,000/cu mm, a rough inverse correlation existing between whole blood platelet count and PAIgG. PAIgG was within the normal range in 2 other patients with ITP.

In a group of 10 ITP patients clinically responsive to steroid therapy, PAIgG levels ranged from 1.9 to 10.5 fg/platelet (Fig. 3). Whole blood platelet counts of these patients ranged from 38,000 to 215,000/cu mm, and no correlation between platelet count and PAIgG was found.

Six patients with nonimmune thrombocytopenic purpura were also studied. PAIgG levels in three of the patients were within normal limits (Fig. 3), while the remaining three patients were found to have significantly elevated PAIgG levels. The latter patients were diagnosed as having chronic myelogenous leukemia (22.0 fg/platelet) and chronic alcoholism with associated hepatic dysfunction (14.5 and 24.0 fg/platelet).

A typical dried, stained electrophoretic gel is shown in Fig. 4, containing samples of IgG standards (A–D), solubilized normal platelet protein (E, F), and solubilized protein from two patients previously diagnosed as having chronic (G) and acute (H) ITP.
PLATELET IgG ELECTROIMMUNOASSAY

Fig. 4. A typical electrophoretic gel. Preparations of purified human IgG (A–D) and Triton-X100-solubilized platelets (E–G) were added to 15-μl sample wells and electrophoresed at 2 V/cm for 18 hr into 1% agarose, 0.5% Triton X100, 0.1 M sodium acetate, pH 5.0 containing 210 ng/sq cm of the carbamoylated rabbit anti-human IgG. Following electrophoresis, the gels were washed and stained with Coomassie blue R. Single samples of A through D were analyzed; duplicate samples of E through G. (A–D) 300, 200, 100, and 50 ng of purified human IgG, respectively; (E) 2.5 x 10⁶ platelets from a normal subject; (F) 2.5 x 10⁶ platelets from a second normal subject; (G) 10⁵ platelets from a patient with chronic ITP; (H) 10⁵ platelets from a patient with acute ITP. Based on the height of each precipitate and the number of platelets per sample well, the total PAIgG (fg/plt) in samples E through G was calculated to be 4.0, 3.0, 12.5, and 25.5, respectively. The bar in the upper right-hand corner of the figure represents 1 cm. Cathode (–) is at the top.

DISCUSSION

Demand for reliable methods of detecting and quantitating platelet-associated antibodies in sera of patients with autoimmune thrombocytopenia has led to a profusion of techniques for the direct assay of PAIgG. For an analysis of the limitations and advantages of some of these assays, the reader is referred to recent reviews by Karpatkin and McMillan.

The electroimmunoassay described in this report is a simple and sensitive technique that provides a highly reproducible estimate of PAIgG. Using this assay, we have found the normal level of PAIgG to be 4.3 ± 1.7 fg/platelet (mean ± 2 SD), in close agreement with previously published values for PAIgG of normal platelets. The quantitative radial immunodiffusion assay for PAIgG recently introduced by Morse et al. is based on the same principle of immunoprecipitation in agarose, yields virtually identical values for PAIgG (3.8 ± 1.9 fg/platelet), and is comparatively simple and reproducible. Nevertheless, the electroimmunoassay reported here is certainly more rapid, requiring less than 24 hr for completion, as compared to the >60 hr preparation and incubation time period required for the immunodiffusion test.

With this method we observed a positive relationship between elevated platelet-associated IgG, reduced circulating platelet count, and clinical symptoms in each of 27 patients with either acute or chronic ITP. Platelet-associated IgG levels were within the normal range in two other patients who satisfied the diagnostic criteria of ITP, having platelet counts of 43,000 and 52,000/cu mm, respectively. According to the system of classification recently suggested by Karpatkin, these two patients can be considered to have "true" ITP, while the previously mentioned 27 patients can be considered to have autoimmune thrombocytopenic purpura (ATP). In 8 of 10 additional patients with ITP who were responding to steroid therapy, platelet-associated IgG levels were only slightly elevated (less than 10.5 fg/platelet). The two remaining patients had normal levels of PAIgG. These findings are similar to previous reports using other techniques for PAIgG determination.

Our finding of elevated platelet-associated IgG in 3 of 6 patients with apparently nonimmune thrombocytopenia is consistent with recent reports by Pfeuller et al. and Mueller-Eckhardt et al. who found modest elevations of PAIgG in some patients with thrombocytopenia apparently of nonimmune etiology. Other workers have uniformly observed normal PAIgG levels in such patients. Whether these differences relate to
criteria used for diagnosis, differences in methodology, patient selection, absorption of immune complexes onto platelets of some patients, or other factors not yet recognized remains a subject for further investigation. Our finding of elevated PAIgG levels in two patients with chronic alcoholism and associated hepatic dysfunction is consistent with the recent report of Barrison and coworkers. In that study, raised levels of PAIgG were observed in 9 of 14 patients with alcoholic cirrhosis. Four of these 9 patients had elevated levels of circulating immune complexes, but no correlation between elevated PAIgG and the level of circulating immune complexes was observed.

McMillan and coworkers recently reported that anticoagulation with EDTA resulted in falsely elevated surface-associated IgG levels when platelets were isolated from whole blood stored for 48 hr or longer at 4°C, while anticoagulation with ACD-A did not exert such an effect. Although we observed an increase in PAIgG (≤25%) on platelets isolated from EDTA-blood stored at 4°C for 96 hr, the extent of this increase was such that PAIgG would normally not exceed the range of control values. In our hands, no advantage of using ACD-A-anticoagulated blood was apparent.

An intriguing fact is that a significant increase in total PAIgG (3–4-fold) was observed in whole blood samples stored at 4°C when visible hemolysis had occurred. At this time, we are investigating a possible cause and effect relationship between the two phenomena.

In addition to its apparent suitability for the direct assay of PAIgG, the electroimmunoassay described in this report is useful for the indirect determination of the presence of human alloantibodies. Work is now in progress using this method to quantitate human platelet alloantigens such as PI^A^ and other blood cell alloantigens such as the lymphocyte DR, MB, and MT markers. These studies will be the subject of a subsequent report.

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REFERENCES
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