Cumulative Experience With a Simplified Solid-Phase Radioimmunoassay for the Detection of Bound Antiplatelet IgG, Serum Auto-, Allo-, and Drug-Dependent Antibodies

By Douglas Faig and Simon Karpatkin

A simplified, sensitive, solid-phase radioimmunoassay employing 125I-staphylococcal protein A has been developed that is capable of detecting bound antiplatelet IgG as well as serum auto-, allo-, and drug-dependent antiplatelet antibodies. The simplified assay employs a ratio of test over control platelet counts per minute (cpm) for detection of positive results. All reagents are commercially available. The assay can be performed with as little as 10^6 washed platelets (10 μL of whole blood) that have been stored for as long as 8 wk at 4°C in microtiter plates. The assay time, employing stored platelets, is 4 hr. Bound platelet IgG is positive in 93% of 46 thrombocytopenic patients with autoimmune disease and correlates inversely with their platelet count, r = -0.65, p < 0.001. The ability of this assay to detect serum antibody was studied with a rabbit anti-human platelet antibody capable of giving optimal immunoprecipitation with solubilized platelet membranes at a titer of 1:10. The present assay increases the sensitivity of antibody detection 256-fold to a titer of 1:2560. Human serum antiplatelet membrane antibody was positive in 2 of 2 patients with anti-PLA-1 antibody (titers of 1:256 and >1:64); 7 of 12 multiply transfused patients who were refractory to platelet transfusion (2 had titers of >1:256 and 1:32); 5 of 19 patients with autoimmune thrombocytopenic purpura (2 had titers of 1:64 and 1:32); and 10 of 14 patients with clinical histories of drug-dependent antiplatelet antibody (2 had titers of 1:1280 for quinidine and 1:384 for phenazopyridine).

In a previous report we described a highly sensitive solid-phase radioimmunoassay for the detection of bound platelet IgG, which employed 125I-labeled staphylococcal protein A. The advantage of this technique was its sensitivity (picogram detection level) and its ability to store the eluted IgG at -20°C. Bound platelet IgG was found to be inversely related to the platelet count in patients with autoimmune thrombocytopenic purpura (ATP). The disadvantages of this technique included: (1) the detection of bound IgG from a total platelet extract, rather than an intact platelet surface; (2) the inability to measure serum antibody; and (3) the time factor required for three cycles of freezing and thawing, followed by sonication and centrifugation of a platelet extract (approximately 3 hr).

The purpose of this study was to develop a simple assay that directly measured IgG on the platelet surface and that could be employed to measure serum antiplatelet antibody or drug-dependent antiplatelet antibody. This was accomplished by developing a procedure where washed platelets were adsorbed to a plastic microtiter plate. The data were quantified by employing a ratio of IgG on test platelets divided by IgG on control platelets. Bound platelet IgG was found to be inversely related to platelet count in patients with autoimmune thrombocytopenic purpura (ATP). Serum antiplatelet antibody and drug-dependent antiplatelet antibody were detectable with great sensitivity. In this article we present our cumulative experience in the detection of bound platelet IgG in 46 patients with autoimmune disorders, and serum antiplatelet antibody in 20 patients with autoimmune disorders, 13 patients with multiple platelet transfusions, and 14 patients with histories of drug-dependent thrombocytopenias.
MATERIALS AND METHODS

Preparation of Platelets

Platelets were prepared as described previously. Platelets were then washed once in 1% ammonium oxalate and 3 times in human-Ringer solution, pH 7.1, containing 2 mM EDTA, 10 mM benzamidine, and 100 µg/ml soybean trypsin inhibitor and adjusted to 2.5 x 10^8 platelets/ml in modified human-Ringer solution.

Solid-Phase Radioimmunoassay

Forty microliters (10^4–10^5) platelets of control or test platelets were applied to each of 12 wells of a plastic microtiter plate (u-shaped flexible microtiter plates, Scientific Products, McGraw Park, Md.) containing 96 wells. The platelets were allowed to incubate in the wells for 1 hr at room temperature. The microtiter plate was then either used immediately or stored at 4°C after being tightly wrapped with parafilm (American Can Co., Greenwich, Conn.). The wells were then rinsed with 1% bovine serum albumin (BSA) in phosphate-buffered saline, 0.01 M, pH 7.4 (PBS). The plate was inverted for 5 min to drain. This wash procedure was repeated on two more occasions. Then, 40 µl of a 1:80 or 1:160 dilution of rabbit anti-human IgG in 1% BSA-PBS (γ heavy chain specific, Miles Research Products, Elkhart, Ind.) was added to each well. The 1:80 dilution was added to the first 6 wells and the 1:160 dilution to the last 6 wells. This suspension was allowed to incubate for 1 hr at room temperature. The wells were then rinsed and inverted 3 times with 1% BSA-PBS, as above. Forty microliters of 125-staphylococcal protein A (prepared as described previously or purchased from Amersham, Arlington Heights, Ill., 55 mCi/mg) was diluted in 1% BSA-PBS, added to each well (50,000 cpm/well), approximately 2 x 10^7 cpm/µg protein of laboratory prepared protein A or 150,000 cpm/well of Amersham protein A), and allowed to incubate for 1 hr at room temperature. The wells were again rinsed and incubated 3 times. Each well was then separated from its plastic plate with a custom-made cutting machine under a hood (D. Lee, 932 Kintyre Way, Sunnyvale, Calif.) and placed in 12 x 75 mm plastic tubes for assay of radioactivity in a Beckman Biogamma II Counter (Beckman Instruments, Inc., Irvine, Calif.). Background counts were 50–100 cpm. Control wells, in the absence of platelets, contained 200–300 cpm.

Results were expressed as the ratio of the highest cpm obtained with test platelets (i.e., at 1:80 or 1:160 dilution of rabbit anti-human IgG) divided by the comparable titer employed for control platelets. At least 2–3 controls were run on each day of determination of test samples.

Detection of Number of Platelets Bound to Plastic Plates

Platelets from 20 ml of whole blood were washed as above and resuspended in 200 µCi of 51Cr (New England Nuclear, Boston, Mass., 100 Ci/g) for 30 min at room temperature. The platelets were then sedimented at 3000 g for 15 min at 4°C and washed 3 times with human-Ringer solution. Platelets were then resuspended at an appropriate cell concentration and 10^7 platelets applied to each of 6 wells. After 1 hr of incubation, the cells were washed as above and the wells removed and assayed for radioactivity. The percent adherent cells as calculated by dividing the radioactivity of the isolated wells by the total radioactivity applied to the wells (minus the radioactivity of the supernatant of the platelet suspension). The radioactivity of the supernatant was generally less than 10% total radioactivity (indicating nonappreciable elution of label).

Detection of Serum Antiplatelet Antibody (Rabbit Anti-Human Platelet Antibody, PLA-1 Antibody, Alloantibodies, Drug-Dependent Antibodies)

Washed platelets were prepared and placed onto wells of a plastic plate as described above. Antisera were serially diluted 1:2, 1:4, etc., in 1% BSA-PBS and 40 µl placed on top of the platelets adhering to the well for 1 hr at room temperature. The nonadherent antisera were washed away, as above, in 1% BSA-PBS (3 times). 125-staphylococcal protein A (40 µl, containing 50,000–150,000 cpm) was then added to each well and incubated for 1 hr. The wells were then washed as above and assayed for radioactivity. Similar results were obtained with 56°C heat-inactivated sera as well as unheated sera.

For measurement of drug-dependent antibody, serial dilutions of antisera were made in a solution of the drug to be tested, with the final concentration of drug being 1 µM.

RESULTS

Number of Platelets Adhering to the Plastic Wells

In order for this test to be feasible, it was first necessary to demonstrate that aliquots of washed platelet suspensions from different donors adhered to the same extent. The average adherence ± SD of aliquots from 5 different subjects was 9.4% ± 2.6%. Similar results were obtained with 3 ATP patients having platelet counts of 20,000/cu mm, 25,000/cu mm, and 130,000/cu mm.

Detection of Bound Platelet IgG

Variation of IgG Bound to Control Platelets

Platelets from 6 different control subjects were assayed on the same day with the same batch of reagents at a rabbit anti-human IgG titer of 1:80 and 1:160. Each of these 6 platelet suspensions was divided into 6 different aliquots, which were assayed independently on separate wells. The error of 6 determinations on the same aliquot, from 6 different subjects, averaged ± 12.4%. The mean cpm ± SD obtained for all 6 subjects at a titer of 1:80 was 3296 ± 157. At a rabbit anti-human IgG titer of 1:160, the mean cpm was 3775 ± 482. These represent 2 SD changes of ± 19% and ± 26%, respectively. A difference between experimental and control subjects of greater than 26% or 1.26-fold was therefore considered abnormal (this was rounded off to 1.3-fold or greater).

Bound Platelet IgG Ratios of Ten Patients With Thrombocytopenia of Nonautoimmune Origin

Ten patients were studied with thrombocytopenia of nonautoimmune origin. These included three patients with decreased megakaryocytes, two with hypersplenism, one with multiple myeloma, one with acute myelocytic leukemia, one with adenocarcinoma of the
breast, one with disseminated intravascular coagulation, and one with orthopedic trauma. Their mean ± SEM platelet count and ratio was 59,000 ± 5376/cu mm and 0.97 ± 0.07, respectively.

**Bound Platelet IgG Ratios of 50 Determinations on Platelets of 46 Patients With Autoimmune Disease**

An inverse relationship was noted between bound platelet IgG and the platelet count, \( r = -0.65, p < 0.001 \) (Fig. 1). This could be expressed by the equation
\[
\log R = 0.503 - 0.003p
\]
where \( R \) is the ratio of experimental cpm per platelet divided by control cpm per platelet, and \( p \) is the platelet count \( \times 10^3/cu \) mm. Positive results were obtained in 41 of 44 determinations (93%) on platelets from patients with platelet counts less than 140,000/cu mm. Three of six determinations (50%) were positive from platelets of patients with platelet counts of 140,000/cu mm or greater.

Four of the 46 patients had thrombocytopenia associated with SLE (see Fig. 1), one had thrombocytopenia associated with rheumatoid arthritis.

**Storage of Samples**

Washed platelets could be stored in U-shaped flexible microtiter plates, tightly covered with parafilm for at least 8 wk. Platelet samples stored on microtiter plates, from control subjects as well as patients, tended to increase their ability to bind protein A in a parallel fashion, after 1 wk of storage at 4°C, by approximately 20%.

**Detection of Serum Antibody**

**Rabbit Antiplatelet Antibody**

A rabbit anti-human platelet membrane antibody was prepared as described previously and assayed against fresh platelets applied to the wells, as well as platelets applied to the wells 10 days previously and stored at \(-20°C\). This antibody, which forms optimum immunoprecipitates on crossed immunoelectrophoresis with 40 g of Triton X-100-solubilized platelet membranes at a titer of 1:10, was detectable by solid-phase radioimmunoassay at a titer of 1:2560 (Fig. 2). Similar results were

*When studied in Dr. Aster’s laboratory, this antibody had a titer of 1:50 by \(^{51}Cr\) release assay and >1:100 by indirect immunofluorescence assay.
obtained with serum from a patient (G.A.) who had a normal platelet count and gave birth to a thrombocytopenic child. Her sera had a titer of >1:64.

**Alloantibody**

Sera from 12 multiply transfused patients who were refractory to platelet transfusions were tested for alloantibody. Seven were positive: one with a titer of 1:4, three with titers of 1:8, one with a titer of 1:32, and two (A.C. and C.A.) with titers of >1:256.

**Autoantibody**

Sera from 5 of 19 patients with autoimmune thrombocytopenic purpura were positive for antiplatelet antibody. C.A. and W.O. were positive at titers of 1:64 and 1:32, respectively. C.A. had a bound platelet IgG ratio of 5.0. W.O. was not tested. Control sera often gave a plateau pattern with greater adherence of nonspecific IgG titers of 1:4 to 1:16 compared with titers of 1:2. One patient with Hashimoto's thyroiditis, who had a history of easy bruising and a platelet count of 250,000/cu mm, had a bound platelet IgG ratio of 3.5 and a serum antibody that was detectable at a titer of 1:32.

**Detection of Drug-Dependent Antibody**

Fourteen patients have been studied with good clinical histories for drug-dependent immunologic purpura (onset of purpura and thrombocytopenic purpura while ingesting a drug with rapid return [7–14 days] to normal following discontinuance of the drug). Positive results were obtained with sera from 10 of these patients (Table 1). A particularly high titer was obtained with the drug phenazopyridine for patient M.I., titer of 1:384 (Fig. 4A). A high titer of >1:64 was also obtained with the drug quinidine from patient P.I. (Fig. 4B). A 50% ammonium sulfate fraction was made of this serum, as well as a control serum. When they were assayed under identical conditions, the titer for quinidine was 1:1,280 with P.I., whereas control serum cpm was not enhanced by quinidine (data not shown).

**DISCUSSION**

Since the original report of Rosse and coworkers on the successful application of a technique for the measurement of bound antiplatelet IgG, several additional assays have been reported employing different immunologic techniques. Although these techniques are sensitive and reliable in various investigator's laboratories, they are time-consuming and not easily adaptable to a routine clinical laboratory or blood bank.

The presently described simplified, sensitive, solid-
Simplified Solid-Phase Radioimmunoassay

Fig. 4. Titer of human drug-dependent antiplatelet sera. (A) Phenazopyridine. Patient plus 1 mM drug (○-○). Patient without drug (O-O). Control plus drug (O-O). Control without drug (●-●). (B) Quinidine (same symbols as in A).

Phase radioimmunoassay can easily be performed by a routine laboratory in possession of a gamma counter. 125I-staphylococcal protein A is now commercially available and can be successfully employed for at least 8 wk after purchase. The bound antiplatelet antibody assay can be performed on washed intact platelets (rather than lysates) in 4 hr, saving 3 hr of laboratory time. The platelets can be freshly prepared or stored on plastic microtiter plates at 4°C for as long as 8 wk. The assay requires as little as 10⁶ washed platelets per well, which can be obtained from approximately 10 μl of blood. The simplified bound platelet assay circumvents the necessity of utilizing a standard IgG curve, since data are expressed as a ratio of cpm for patients' platelets divided by cpm for control platelets. The clinical value of this ratio is substantiated by the inverse relationship that was noted between platelet count and cpm ratio for most patients with autoimmune disease. The correlation coefficient of -0.65, p < 0.001, for 50 determinations on patients with autoimmune platelet disorders is similar to the correlation coefficient of -0.71, which we previously reported for platelet lysate IgG content versus platelet count. Three of six patients with platelet counts greater than 140,000/cu mm had an elevated ratio, compatible with a compensated thrombocytolytic state. Of interest was the positive ratio of 3.4 noted in a patient with Hashimoto's thyroiditis, easy bruising, and normal platelet count. Similar observations were also noted in our previous studies employing platelet lysate IgG for ATP patients and patients with Hashimoto's thyroiditis with normal platelet counts. Positive results were obtained in 93% of patients with autoimmune disease and platelet counts of <140,000/cu mm, indicating the diagnostic clinical usefulness of this procedure.

This assay can also be employed to detect serum
antiplatelet antibody. A rabbit antiplatelet antibody could be assayed at a titer of 1:2560, which was 256-fold greater than that noted with immunoprecipitation. This assay could easily be applied to the screening of cell clones for hybridoma antibody production. Alloantibodies could be detected in 7 of 12 multiply-transfused individuals, with titers of >1:256 in two individuals. Similarly, anti-PLA-1 antibody could be detected in two of two individuals at titers of 1:256 and >1:64.

This assay is particularly adaptable to the detection of drug-dependent antiplatelet antibody, since it was positive in 12 of 16 cases with good clinical histories. Two patients had titers detectable at 1:384 and 1:2560 for phenazopyridium and quinidine, respectively, in the presence of 1 mM drug; negative results were obtained with the drug in the presence of control sera. Furthermore, the incubation of all three components—drug, sera, and platelets—were required to obtain positive results. Sequential addition and washing gave negative results. It is of interest that staphylococcal protein A, which binds to the Fc portion of IgG, was capable of detecting bound drug-Ab complex, since there is circumstantial evidence that drug-dependent antibody binds to the platelet Fc receptor by the Fc domain of the antibody. If drug-dependent antibody does bind to the platelet Fc receptor via the Fc domain of the antibody, then protein A must obviously bind to another region of the Fc domain of the antibody.

Serum antiplatelet antibody was detectable in 26% of patients with autoimmune thrombocytopenic purpura who had positive bound antiplatelet antibody. The reason for this low detection rate for serum autoimmune antiplatelet antibody is obscure, particularly since we previously reported positive serum antiplatelet antibody in 65% of ATP patients, employing a PF-3 immunoinjury technique. Since protein A does not bind to human IgG subclass IgG3, it is conceivable that this could account for part of this discrepancy. A similar low incidence of 35% was obtained by Engelfriet and coworkers who employed an immunofluorescent technique utilizing rabbit anti-human IgG that measures all subclasses. What then could be the explanation? It is conceivable that the PF-3 assay, although indirect, is more sensitive for serum antiplatelet antibody, since in this procedure it is not necessary to wash platelets after the binding of serum antibody. Washing could conceivably release low affinity antibody that might still be pathophysiologic in vivo. A positive "autoimmune" serum antiplatelet antibody titer of 1:32 was also obtained in the patient with Hashimoto’s thyroiditis.

The work of Kekomaki and collaborators came to our attention while our manuscript was in preparation. They have also employed a 125I-staphylococcal protein A assay for the detection of serum antiplatelet antibody on stored washed platelets and have obtained positive titers of 1:160 with anti-PLA-1 antibody and 1:40, 1:80, and 1:80 in three patients with quinine-dependent antiplatelet antibody.

Thus, a simplified solid-phase assay employing 125I-staphylococcal protein A can be utilized to detect bound antiplatelet antibody as well as serum antiplatelet antibody. The serum assay, which has been employed by two laboratories, is particularly useful and sensitive in the detection of PLA-1 antibody and drug-dependent antiplatelet antibodies. It can easily be applied to the detection of hybridoma antibodies.

ADDENDUM

This method can be further quantitated by employing a standard IgG curve with each determination as described previously, assuming that approximately 10% of platelets adhere to the wells. Under these conditions, our control value from 50 determinations on 19 different subjects is 1.28 ± 1.18 (2 S.D.) ng/10⁶ platelets.

ACKNOWLEDGMENT

The authors are indebted to Michael Nardy for expert technical assistance and to Bea Grofe for the illustrations.

REFERENCES

SIMPLIFIED SOLID-PHASE RADIOIMMUNOASSAY


Cumulative experience with a simplified solid-phase radioimmunoassay for the detection of bound antiplatelet IgG, serum auto-, allo-, and drug-dependent antibodies

D Faig and S Karpatkin