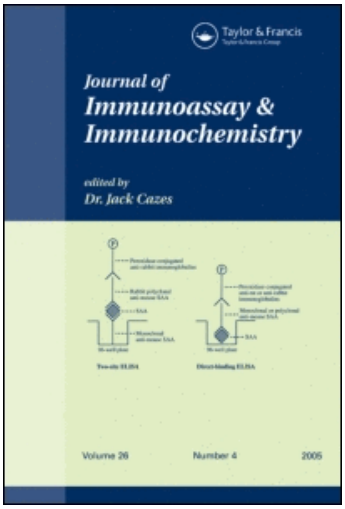

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Kelton, John G. , Denorame, Greg , Walker, Chris , Horsewood, Peter and Gauldie, Jack(1983) 'The Measurement of Plateiet-Associated IgG Using an Immunoradiometric Assay', Journal of Immunoassay and Immunochemistry, 4: 1, 65 — 82
To link to this Article: DOI: 10.1080/15321818308057000
URL: http://dx.doi.org/10.1080/15321818308057000

JOURNAL OF IMMUNOASSAY, 4(1), 65-82 (1983)

THE MEASUREMENT OF PLATELET-ASSOCIATED IgG USING AN IMMUNORADIOMETRIC ASSAY

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ABSTRACT

The amount of IgG on the surface of washed platelets from healthy individuals varies according to the assay used. An immunoradiometric assay (IRMA) for IgG was developed, validated and used to measure platelet associated IgG. The platelet-associated IgG (PAIgG) on washed platelets was allowed to react with excess ^{125}I-labelled anti-IgG. The unbound ^{125}I-anti-IgG was then quantitated by the addition of sepharose beads to which IgG had been covalently bound. The ^{125}I-anti-IgG/IgG-beads were separated from the platelet suspension by passage across a density gradient. The mean amount of IgG present on washed platelets from healthy individuals was 0.8 fg IgG/platelet, or approximately 4,000 molecules of IgG per cell. Inverse Scatchard analysis confirmed the validity of the assay calibration and identical results were obtained when five different anti-IgG antibodies were used. The specific binding of ^{125}I-anti-IgG to platelet-bound IgG was complete by 30 min, but non-specific binding of radioactivity continued thereafter. This non-specific binding occurred not only with anti-IgG but with all antibodies tested and could give elevated estimates of PAIgG in direct binding assays.

INTRODUCTION

The development of techniques for quantitating IgG on the surface of platelets has aided in the investigation of idiopathic

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thrombocytopenic purpura (ITP) and provided insight into its pathogenesis. Depending upon the assay used, the mean amount of IgG on the surface of normal washed platelets has been reported to range from 2 to 11 fg IgG per platelet (1-4). One explanation for the variety of normal ranges is the tendency for platelets to non-specifically adsorb proteins, including those in assay reagents, and as a result, some techniques could overestimate the amount of platelet-bound immunoglobulin. To overcome this difficulty, some investigators have used paraformaldehyde fixation of test platelets (5), others have used two-stage procedures such as the antiglobulin consumption assay (1,6), and still others have solubilized the platelets and measured IgG in the lysate (3,4,7).

In this report we describe an immunoradiometric assay (IRMA) for quantitating IgG on platelets. Studies were performed using this technique to define possible reasons for the varied normal ranges.

METHODS

Patients and Controls. All patients had idiopathic thrombocytopenic purpura (ITP). This was defined as: (a) isolated thrombocytopenia; (b) normal or increased bone marrow megakaryocytes; and (c) no other serological or clinical evidence of a secondary cause for the thrombocytopenia such as systemic lupus erythematosis or hypersplenism.

The controls were healthy non-thrombocytopenic laboratory personnel.

Preparation of Test Platelets. Twenty ml of whole blood was collected into 3.3 ml of acid citrate dextrose (ACD) using a plastic syringe. The platelet rich plasma was separated by centrifugation at 260g for 10 minutes. A platelet pellet was obtained by further centrifugation at 2000g for 15 minutes and the platelets were washed three times with 0.015M EDTA and resuspended in veronal buffered saline (VBS), pH 7.4. Dilutions of the platelet suspension were made in VBS and counted in a Coulter-S-Counter (Coulter Electronics, Hialeigha, Fla.). Lymphocytes and granulocytes were separated from whole blood from healthy donors and 2×10^7 cells incubated with 100 uCi of ^{51}sodium chromate (New England Nuclear, Lachine, Quebec), and then washed. To determine the number of leukocytes contaminating the platelet pellet, varying concentrations of radiolabelled lymphocytes or granulocytes were added to fresh whole blood specimens, and the platelets isolated as described. The lymphocyte and granulocyte contamination in the pellet was determined by relating the radioactivity of the pellets to the specific activity of each leukocyte preparation.

Measurement of Platelet-Associated IgG (PAIgG)

A. The Antiglobulin Consumption Assay. Platelet-associated IgG was measured using the antiglobulin consumption assay

(1,6,8). In this assay, sheep erythrocytes coated with human IgG are lysed by anti-IgG and complement. The lysis can be quantitatively inhibited by prior incubation of the anti-IgG with washed test platelets or known concentrations of IgG. The number of platelets that causes 50% inhibition of erythrocyte lysis is related to the concentration of IgG standard that produces the same inhibition.

B. The Immunoradiometric Assay (IRMA). The principle of this assay is illustrated in Figure 1. Varying dilutions of washed test platelets or known amounts of IgG standard are incubated with a fixed concentration of radiolabelled anti-IgG. The unbound anti-IgG is reacted with an excess of IgG-coated beads. The labelled anti-IgG that binds to the IgG-beads is counted after separation of the beads from the platelets by passage across a density gradient.

Preparations of anti-IgG. Anti-human IgG was raised in sheep by the injection of pooled human IgG purified by column chromatography (DEAE-cellulose and Sephadex G-200, [Pharmacia Chemicals, Dorval, Quebec]). The specificity of the anti-IgG, confirmed by immunodiffusion and immunoelectropheresis, was directed to the Fc fragment of human IgG. The antibody was partially purified by 33% ammonium sulfate precipitation and the precipitate redissolved in 0.15M, pH 7.4 phosphate buffered saline (PBS), dialysed for 12 hours at 4°C against PBS and stored at -70°C. The anti-IgG, prepared as described, together with

four commercial anti-IgG reagents (Atlantic Antibodies, Scarborough, ME; Behring Diagnostics, Montreal, Quebec; Meloy Laboratories, Springfield, VA; and Cedarlane Laboratories, Hornby, Ontario) were labelled with ^{125}Iodine using the chloramine-T method (9), and the radiolabelled protein separated from the iodine by passage through a Sephadex G-75 column. The

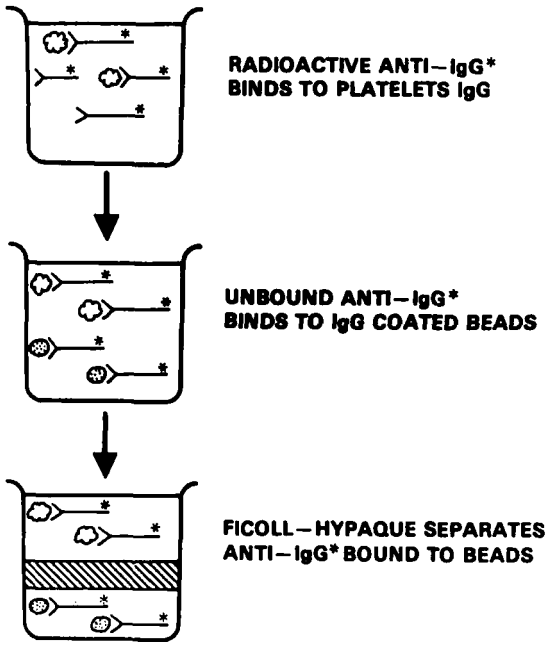

FIGURE 1. A schematic representation of the principles of the immunoradiometric assay for quantitating platelet-associated IgG. A fixed concentration of radiolabelled anti-IgG ( ) is incubated with varying concentrations of washed test platelets ( ) or known amounts of human IgG. IgG-beads ( ) are added to the mixture and the radiolabelled anti-IgG bound to the IgG-beads is measured after centrifugation of the suspension across a Ficoll-Hypaque density gradient.

radiolabelled antiserum was diluted in PBS containing 5% normal sheep serum and stored at -70°C.

Preparation of IgG-beads. Human IgG (Cohn II, Sigma Chemical Co., St. Louis, Mo.) was covalently linked to Sepharose beads Sephasorb HP-ultrafine, 10-23 micron [Pharmacia Fine Chemicals, Dorval, Quebec] using carbonyl-diimidazole (10). One gram of dried beads was mixed for 30 minutes with 10 ml of dioxane (1,4 dioxane, BDH Poole, England) and 0.48 g of 1,1-carbonyl-diimidazole (CDI) was added and incubated for 15 min at 22°C. The beads were washed with dioxane followed by 0.1M borate buffer (pH 8.5) and mixed with 27 mg of soluble IgG for 12 hours at 4°C. The beads were washed once with 1M NaCl and twice with PBS before resuspension to a final count of 10^6 beads per 100 ul in PBS containing 0.1% sodium azide. "Control" beads were subjected to the same procedure except that buffer replaced the IgG solution.

Immunoradiometric Assay. 100 ul of ^{125}I-anti-IgG containing 40,000 cpm was incubated for 30 min at 37°C with 100 ul of a series of dilutions of washed platelets suspended in VBS. One hundred microlitres of IgG-Sephasorb beads (10^6 beads) was added and incubated at 37°C for 30 min. Four ml of 5% normal sheep serum was added to the tubes and then 1 ml of Ficoll-Hypaque (6%:10%), was carefully layered below the mixture using a 20 guage needle and the beads separated from the platelets by centrifugation for 2 min at 260g. The supernatant was decanted,

the beads were washed once more in 5% normal sheep serum, and their radioactivity measured in a gamma counter (Amersham, Oakville, Ontario). A standard curve was prepared using a similar protocol except that increasing concentrations of human IgG standard were used. Maximal inhibition was determined using a 3 ug/tube IgG standard. Maximum binding (B_o) of radiolabelled proteins to the beads was determined by substituting buffer for the IgG standard.

The binding of the ^{125}I-anti-IgG to the IgG-beads was plotted against the logarithm platelet count (test curve) and against the logarithm of concentration of IgG (standard cruve). The amount of IgG which produced 50% of B_o binding was related to the number of test platelets that caused similar inhibition and in this way the amount of IgG per platelet was calculated.

Validation of the Technical Aspects of the Method. To determine the number of IgG-beads required to bind essentially all ^{125}I-anti-IgG, 100 ul of increasing concentrations of IgG-beads were incubated for 30 minutes at 37°C with a fixed dilution of ^{125}I-anti-IgG. The beads were separated and washed as before and the radioactivity counted.

The time required for the binding of the ^{125}I-anti-IgG to reach equilibrium with the washed platelets was determined by incubating platelets from ITP patients and from healthy controls with ^{125}I-anti-IgG for 30, 60, 90, 120 and 240 min. The PAIgG was then determined as described.

To measure the completeness of separation of the IgG-beads from the test platelets, platelets from healthy individuals were washed and 3×10^7 platelets were incubated with 100 uCi of ^{51}sodium chromate, and washed again. The IRMA assay was performed as described except that unlabelled anti-IgG was used in place of the ^{125}I-anti-IgG. The platelet contamination in the separated bead fraction was calculated using the specific activity of the platelets.

To validate the assay calibration that uses IgG in free solution, in contrast to the solid platelet-bound IgG, inverse Scatchard analysis was performed on the binding of antiserum to the IgG standard and to the test platelets (11).

The Binding of Radiolabelled Specific Antibody and Other Protein to Platelets. To measure the platelet binding of radiolabelled specific antibody and other proteins present in the antiserum, 100 ul of the five different radiolabelled antisera, diluted in PBS, were incubated with platelets. The dilution of each antiserum was adjusted so that 80 ng per 100 ul of IgG standard bound 50% of the anti-IgG. This dilution was incubated for 30, 60, 120, 240 and 360 minutes at 37°C with the washed platelets from a single healthy donor. After the incubation and centrifugation at 2000g for 10 minutes, the supernatants were removed and saved and the platelet pellets washed twice with PBS and their radioactivity measured. The supernatants containing ^{125}I-anti-IgG not bound to platelets were incubated with 100 ul

of IgG-beads (10^6) for 30 min at 37°C. The IgG-beads were washed twice and the amount of anti-IgG bound was measured.

To investigate whether radiolabelled proteins other than the specific anti-IgG had bound to platelets, washed platelets were incubated with ^{125}I-anti-IgG depleted antiserum. The anti-IgG was removed from the antiserum by incubation for 30 min at 37°C with 10^6 IgG-beads. The antiserum was separated from the IgG-beads by centrifugation at 260g x 2 min and 100 ul of the supernatant was incubated with 100 ul of a washed platelet suspension for 30 min at 37°C. As a control for the remaining anti-IgG, another 100 ul aliquot of the supernatant was incubated for 30 minutes at 37°C with 100 ul (10^6) IgG-beads. Both the platelets and the IgG-beads were washed twice in 5% normal sheep serum and the radioactivity counted.

Comparison of the Immunoradiometric Assay and the Antiglobulin Consumption Assay. Doubling dilutions of the ^{125}I-anti-IgG were incubated with either the IgG labelled sheep erythrocytes or the IgG-beads. The amount of anti-IgG bound to either target was measured as the radioactivity of the IgG-beads after washing or the amount of hemoglobin released by complement-mediated lysis of the sheep erythrocyte.

The precision of each assay was assessed by measuring the concentration of IgG standard required to produce 50% inhibition of binding (IRMA assay), or lysis (antiglobulin consumption assays) on determinations performed on 17 different days.

RESULTS

The Immunoradiometric Assay (IRMA) for Platelet-Associated IgG: Validation of Methodology. Less than 2% of the lymphocytes and less than 0.1% of the granulocytes in the whole blood specimen were recovered in the washed platelet sample. Non-specific adsorption of radiolabelled proteins to the beads was minimal and less than 2% of the ^{125}I-antibody preparation bound to control beads. Fewer than 2% of the platelets crossed the Ficoll-Hypaque gradient.

No measurable ^{125}I-anti-IgG remained in the aqueous phase after adsorption by 10^6 IgG-beads.

All measurable platelet-associated IgG (PAIgG) on intact platelets could be measured after 30 min incubation. Progressively increasing the length of incubation to six hours did not increase the amount of PAIgG that could be measured.

Inverse Scatchard analysis (11) was used to evaluate the appropriateness of calibration of the assay (Figure 2). When dilutions of aqueous IgG standard and dilutions of platelet suspension were incubated with fixed amounts of ^{125}I-anti-IgG, the dimensionless ratio of binding capacity to reciprocal equilibrium constant was identical for both sets of reactions. Since the concentration of antibody binding capacity is common to both sets of dilutions, the equilibrium constants of reaction of antibody with aqueous IgG and with platelet-bound IgG must be identical. Equality of equilibrium constants between standards

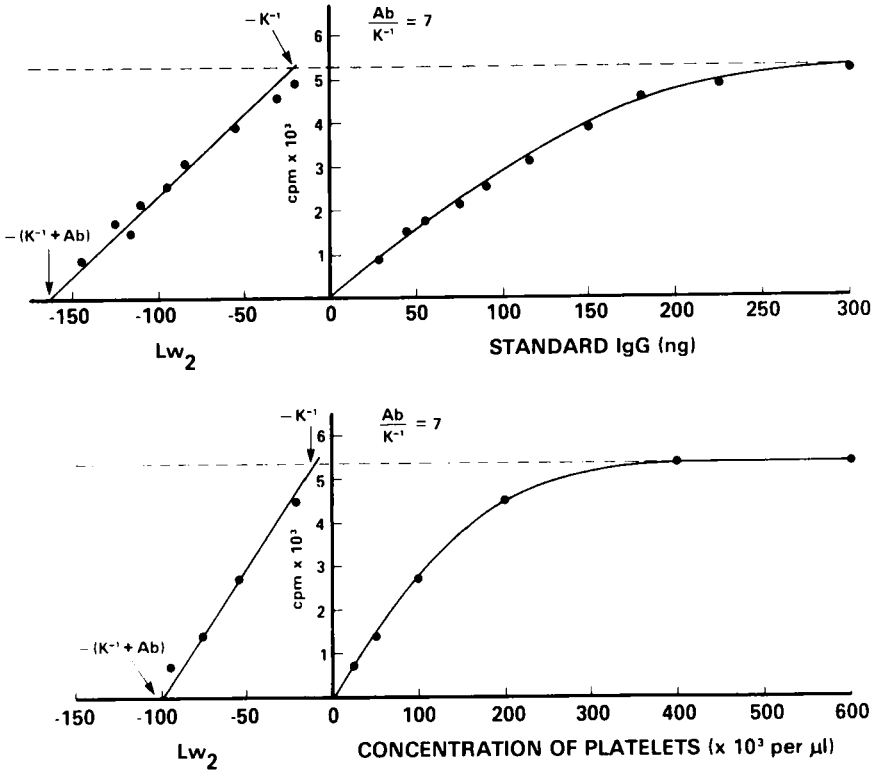

FIGURE 2. Inverse Scatchard analysis of the binding of the ^{125}I-anti-IgG to washed test platelets (upper figure 1) or to the IgG standard (lower figure). The dimensionless ratio of binding capacity (Ab) to the reciprocal equilibrium constant (K) was identical for both reactions. W = (1 - Ab/B) where Ab = maximum counts capable of being bound to beads and B = counts bound at varying concentrations of standards or platelets.

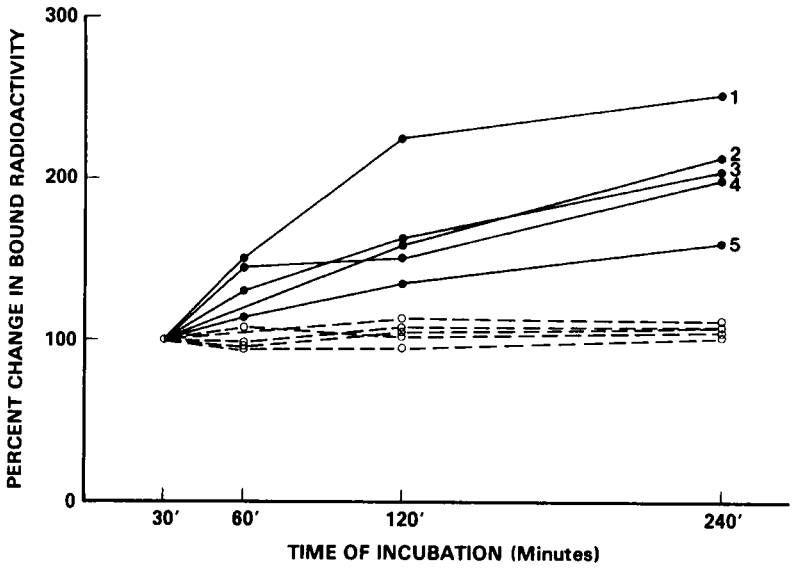

FIGURE 3. The relationship between the duration of incubation (abscissa), and the amount of platelet-bound radioactivity expressed as a percent of the radioactivity on the platelets after 30 minutes of incubation for five different anti-IgG reagents (o——o), (ordinate). The anti-IgG reagents used include: (1) Behring Diagnostics; (2) our own anti-IgG; (3) Meloy Laboratories; (4) Cedarlane Laboratories; (5) Atlantic Antibodies. The actual amount of anti-IgG not associated with IgG-beads is shown as (o---o). Although the platelet radioactivity continued to rise with progressively longer incubtions, the actual amount of anti-IgG bound did not change after the first 30 minutes of incubation.

and unknowns is a prerequisite for parallelism of calibration curves.

The Binding of Radiolabelled Anti-IgG and Other Non-Immune Proteins to the Platelets. The amount of platelet-bound radioactivity was proportional to the duration of incubation of the radiolabelled antiserum with the test platelets for each of the five different antisera (Figure 3). For certain antisera,

the platelet-bound radioactivity was three-fold higher at 4 hours of incubation compared to the radioactivity present at 30 min (Figure 3). This increase in radioactivity was due to the binding of radiolabelled proteins other than the anti-IgG since the residual ^{125}I-anti-IgG measured by the binding to the IgG-beads did not change after the first 30 min. The non-specific binding was related to the purity of the antiserum. It was lowest for an affinity purified anti-IgG from Atlantic Antibodies. In this preparation 30% of the total protein had anti-IgG activity. It was highest for the Behring Diagnostic preparation in which less than 1% of the protein had anti-IgG activity. The use of either non-radiolabelled normal sheep serum as antiserum diluent or the addition of a detergent (Triton X100) reduced but did not entirely eliminate the non-specific binding.

The propensity of the non-anti-IgG proteins to firmly bind to the platelet membrane was confirmed by the use of radiolabelled antiserum from which all anti-IgG had been removed by prior adsorption with IgG-beads. After 30 minutes of incubation of platelets with the anti-IgG depleted antiserum, the platelet-bound radioactivity was 40% of the radioactivity associated with the original antiserum. In contrast, binding of the anti-IgG depleted antisera to IgG-beads was less than 2% of the total radioactivity.

Comparison of the Immunoradiometric Assay and the Standard Antiglobulin Consumption Assay. The same antiserum was used in the immunoradiometric assay and the antiglobulin consumption

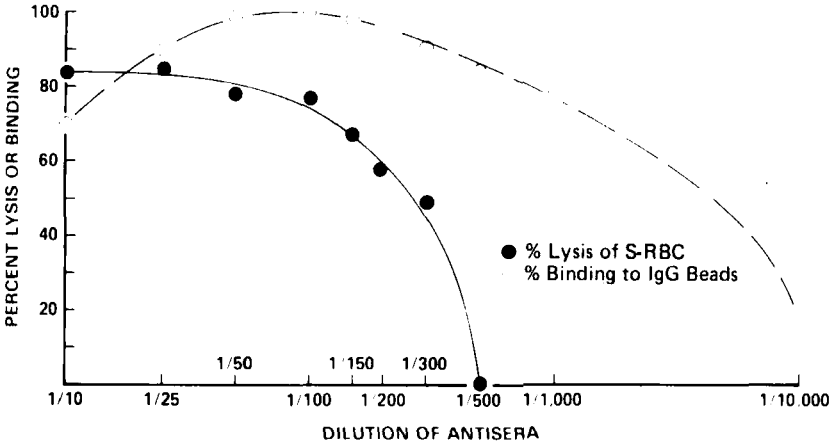

FIGURE 4. The relationship between the dilution of antiserum shown as a reciprocal titre along the abscissa and the endpoint sensitivity for two different assays used to measure PAIgG. The endpoint of the standard antiglobulin consumption assay (●——●) is lysis of sheep erythrocytes. The endpoint of the IRMA assay (o----o) is the binding of radiolabelled antisera to IgG-beads. The same antiserum was used in both experiments.

assay to determine the relative endpoint sensitivities of each procedure. Approximately a 20-fold greater dilution of antiserum could be detected using the IRMA assay than the antiglobulin consumption assay (Figure 4).

The between batch assay precision was determined by measuring the concentration of IgG standard required to produce 50% inhibition of binding (IRMA) or sheep cell lysis (antiglobulin consumption) and was 6.3% (n = 18) for the IRMA assay and 22% (n = 17) for the antiglobulin consumption assay.

Measurement of Platelet-Associated IgG on Platelets from Healthy Donors and Patients with ITP. The amount of IgG on platelets from healthy non-thrombytopenic individuals was

0.8 ± 0.5 (M ± SD) fg/IgG per platelet, n = 20. The amount of IgG progressively decreased as the platelets were washed up to three times. Further washes did not give lower results.

The mean IgG on platelets from patients with ITP was 8.6 fg IgG/platelet and ranged from 2 to 30 fg IgG/platelet.

DISCUSSION

It is likely that the amount of IgG on the surface of washed platelets from healthy individuals measured using the immunoradiometric assay (IRMA) represents a close approximation of the actual amount of IgG on these platelets. Technical artifacts were carefully excluded. Any significant contribution to the platelet IgG by contaminating cells such as lymphocytes or granulocytes was excluded. Methodological artifacts were also evaluated by inclusion of appropriate controls, and validation of the method of calibration using inverse Scatchard analysis. The same amount of IgG per platelet was determined when five different anti-IgG antisera reagents were used.

The mean amount of IgG on washed platelets from healthy, non-thrombcytopenic individuals was 0.8 fg of IgG per platelet or approximately 4000 molecules of IgG. Possible reasons for the considerable variation in previous estimates of the amount of PAIgG on washed normal platelets were also investigated. Previously, using the antiglobulin consumption assay, we have reported that washed platelets from healthy individuals had up to

5 fg IgG per platelet. The reason for the difference between this amount and our current result is uncertain but could, in part, be related to the increased precision of the IRMA assay compared to the antiglobulin consumption assay (C.V. of 7% and 22% respectively) as well as the greater endpoint sensitivity of the IRMA assay.

The studies described in this report also demonstrated that labelled proteins other than the specific anti-IgG invariably bind to the platelet surface (Figure 3). This potential artifact would occur when platelet-associated IgG is measured using direct binding techniques. Although the non-specific binding was lower when purified reagents were used, it was still significant and could influence the estimated amount of platelet-IgG. The use of the "two-stage" procedure described in this report overcomes this problem since only anti-IgG is measured in the second stage of the assay.

The IRMA method was used to measure the level of platelet-associated IgG on platelets from patients with idiopathic thrombocytopenic purpura. In keeping with previous observations from our own and other laboratories, we observed that washed platelets from these individuals had significantly higher levels of IgG on their surface compared to controls. Therefore, this assay can be used in the investigation of patients with suspected immune thrombocytopenia. However, one must recognize that the specificity of increased platelet bound

IgG for the diagnosis of idiopathic thrombocytopenia purpura is low (12,13).

These studies were supported by a grant provided by the Medical Research Council of Canada.

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