Reactions of Immunoglobulin G-binding Ligands with Platelets and Platelet-associated Immunoglobulin G

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Abstract. Immunoglobulin G (IgG) bound to platelets is usually detected by one of two general methods: binding of labeled anti-IgG or consumption of anti-IgG. The latter method gives, in general, values 5–10-fold greater than the former under the same conditions. To investigate these discrepancies, we have compared the detection of platelet-bound IgG by a labeled anti-IgG binding assay and by a quantitative antiglobulin consumption test using the same antibodies.

The interaction of ^{125}I-labeled monoclonal anti-IgG or polyclonal anti-IgG with washed and IgG-coated platelets was studied. The binding of these ligands to washed normal platelets was largely (50–80%) nonspecific; the binding was not saturable and was only partially inhibitable by excess unlabeled anti-IgG. The binding of anti-IgG to platelets coated with anti-Pl^{A1}, a platelet-specific IgG antibody, appeared to be saturable and inhibitable; the dissociation constant (K_D) of this IgG-anti-IgG reaction was 4.9×10^{-9} for monoclonal and 1.4×10^{-7} for polyclonal anti-IgG. The ratio of sites present on the membrane (determined by ^{131}I-labeled anti-Pl^{A1}) to the number of binding sites for anti-IgG determined by Scatchard analysis was 0.53 for monoclonal anti-IgG and 1.3 for polyclonal anti-IgG. The binding of monoclonal anti-IgG to platelet-bound immune complexes or IgG aggregates appeared to be complex.

^{131}I-Labeled IgG was affixed to platelets and was detected by three tests: direct binding of radiolabeled monoclonal anti-IgG and quantitative antiglobulin consumption (QAC) tests, which were quantitated either by measuring directly the amount of radiolabeled anti-IgG consumed from fluid phase (direct QAC), or indirectly by reference to a calibration curve relating the consumption of anti-IgG by known amounts of fluid-phase, non-immune IgG (indirect QAC). The amount of platelet-bound IgG detected by the direct binding of ^{125}I-labeled monoclonal anti-IgG and by the direct QAC approximated that known to be bound to the platelet. The results of the indirect QAC test were 10-fold greater. The discrepancy appears to be due to the fact that there is a difference between the IgG-anti-IgG interaction when IgG is bound to a platelet and when it is in solution or bound to plastic nonspecifically or specifically. This difference results in a falsely high value for platelet-bound IgG when fluid-phase or plastic-bound IgG is used to calibrate the antiglobulin consumption test.

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Introduction

For many years, the techniques available for the detection of antibody on platelets were either unreliable or difficult to perform. At present, there are two general methods in use for the detection of platelet-bound IgG: antiglobulin consumption tests or direct labeled anti-IgG binding tests. Antiglobulin consumption tests are usually based on the principle that IgG affixed to platelets will adsorb proportionate amounts of anti-IgG from solution. The stoichiometry is assumed to be the same for platelet-bound IgG as for fluid-phase IgG and the tests are therefore calibrated by assessing the consumption of anti-IgG by known amounts of fluid-phase IgG. By these techniques, 5,000–20,000 molecules of IgG are detected on the platelets of normal donors and values >300,000 molecules/platelet have been reported in patients with idiopathic thrombocytopenic purpura (1–6). In addition, increased amounts of platelet-bound IgG have been found by these techniques on the platelets of thrombocytopenic patients with systemic lupus erythematosus, myeloproliferative disorders, sepsis and other infections, drug-induced purpura, various hematologic disorders, and cancer (7–9).

Techniques that directly measure the binding of labeled anti-IgG to IgG affixed to platelets are theoretically simple to perform and to interpret (10–13). Radiolabeled ligands have been fixed to platelet-bound IgG, but when heterologous anti-IgG was used as whole serum or as the IgG fraction of serum of immunized animals, the amount of IgG bound to normal

platelets was often great (10). Furthermore, it has been difficult to establish the stoichiometry between the amount of IgG on the platelet and the amount of anti-IgG bound (11).

With the advent of hybridoma-derived monoclonal anti-IgG antibodies which react with a single epitope of the IgG molecule, many of the objections to previous ligand-binding assays may be overcome. Using such a test, we have found that there is little binding to normal platelets (500–1,000 molecules/platelet for monoclonal antibody, 2,000–3,000 for polyclonal antibody). The amount bound to the platelets of patients with idiopathic thrombocytopenic purpura is usually greater (2,000–50,000 molecules/platelet) but is very much less than that estimated by using a quantitative antiglobulin test with calibration curve (Rosse, W. F., and D. V. Devine, manuscript in preparation).

In the present study, we analyzed and detailed the interaction between radiolabeled monoclonal or polyclonal anti-IgG and IgG bound to platelets. We compared the results of such assays with the results of an antiglobulin consumption test by using the monoclonal antibody with and without a calibration curve using known amounts of IgG. When a calibration curve in which IgG in fluid phase or bound to plastic or surfaces other than the platelet membrane is used to estimate the amount of anti-IgG consumed, falsely high values for the amount of IgG bound to the platelet are derived. When the amount of anti-IgG consumed is estimated either directly (by loss of radioactivity) or by calibration with known amounts of IgG affixed to platelets, the binding of IgG is the same as that detected using direct anti-IgG binding. This suggests that the discrepancy noted between antiglobulin consumption tests and direct binding assays is due to differences in the binding of anti-IgG when bound to IgG on the platelet surface compared with its binding to IgG affixed to other surfaces or in fluid phase.

Methods

Ligands

Monoclonal antibody. Monoclonal mouse antibody known to be specific for the Fc portion of human IgG (Bethesda Research Laboratories, Gaithersburg, MD) was purified from ascites fluid by two methods. 1 and 1.5 ml of ascites fluid was either dialyzed against 0.02 M KH_2PO_4, pH 8.0, 0.02% sodium azide, then passed over a 10-ml column of DEAE AffiGel Blue (Bio-Rad Laboratories, Richmond, CA) equilibrated in the same buffer or passed over a 3-ml column of AffiGel Blue (Bio-Rad Laboratories) in phosphate-buffered saline (PBS), pH 7.4, without prior dialysis. The fall-through protein from either method was then concentrated and dialyzed against PBS, pH 7.4, containing 5 mM benzamidine (Sigma Chemical Co., St. Louis, MO), 5 mM epsilon aminocaproic acid (Sigma Chemical Co.), and 0.02% sodium azide (Sigma Chemical Co.) prior to chromatography on Sephadex G-200. Fractions were pooled and purity was determined by polyacrylamide gel electrophoresis (PAGE).[1] The purified antibody contained minor (<5%) contamination with other proteins by sodium dodecyl sulfate-PAGE. The antibody was preserved in frozen fractions which were thawed for use. $F(ab')_2$ fragments were prepared by pepsin digestion of purified monoclonal antibody (14). Fc fragments and undigested IgG were removed by protein A-Sepharose affinity chromatography.

Goat anti-human IgG (gamma chain-specific) which had been purified on an IgG immunoadsorption affinity column was obtained from Tago, Inc., Burlingame, CA.

Antibodies

Serum. Serum or plasma of patients with alloimmune or autoimmune thrombocytopenia was frozen in aliquots and thawed for use. It was heated to 56°C for 30 min before use to destroy complement. The IgG fraction from normal serum and the serum of patients with alloimmune or autoimmune thrombocytopenia was purified by either of two procedures: (*a*) the euglobulin fraction was precipitated by 45% saturated ammonium sulfate (NH_4SO_4), and the supernatant fluid was dialyzed against 5 mM phosphate buffer and placed on a DEAE Sephacel column; fall-through protein was concentrated and examined by PAGE. If not pure, it was chromatographed on Sephadex G-200. (*b*) Immunoglobulin was prepared as above and was dialyzed against 0.1 M phosphate buffer at pH 8.0; this was applied to a staphylococcal protein A-Sepharose CL-4B (Sigma Chemical Co.) affinity column. The bound IgG was eluted with a 0.1 M citrate buffer, pH 3.5; pH was adjusted to 7.4 before dialysis against PBS, after the method of Ey et al. (15). Samples were tested for aggregate formation by using the C1q-binding assay described below. Only those samples free of detectable aggregates were used.

Aggregates of IgG. Purified normal or monoclonal (myelomatous) IgG was aggregated with dimethylsuberimidate according to the method of Segal and Hurwitz (16) as modified by Kurlander and Batker (17). The molar ratio of IgG to dimethylsuberimidate was 15:1. The aggregates were purified by serial passage through Sephadex G-200 and ACA 22. "Heavy polymers" (polymers of four IgG molecules and greater) were used in the present studies.

Immune complex assay. Immune complexes in serum or fractions of serum were detected by a modification of the solid-phase C1q assay (18). ^{125}I-Staphylococcal protein A was used as the detector of C1q-bound IgG aggregates. ^{131}I-Labeled aggregates of IgG were used for calibration.

Radiolabeling of proteins

Iodination of proteins was carried out by using a modification of the method of Fraker and Speck (19). 1.5-ml-polypropylene microfuge tubes were coated with IodoGen (Pierce Chemical Co., Rockford, IL) dissolved in dichloromethane. Tubes were prepared such that the IodoGen:protein ratio was 1:10 wt/wt and the volume of IodoGen solution equaled the volume of protein to be labeled. The IodoGen was dried down under a stream of nitrogen and the dessicated tubes were stored at room temperature. 1-mg quantities of ligand were labeled with 1 mCi [^{125}I]sodium iodide (Amersham Corp., Arlington Heights, IL) by incubating protein and radiolabel in an appropriate IodoGen-coated tube for 15 min at room temperature with mixing. The sample was briefly microfuged to pellet any loose IodoGen flakes. To remove free iodine, the sample was passed over a Sephadex G-25 column equilibrated in PBS.

2-mg quantities of IgG from human sera were labeled with 1 mCi [^{131}I]sodium iodide (Amersham Corp.) using the same technique.

Preparation of platelets

Platelets were separated from blood collected in EDTA (0.15 ml of 10% EDTA, pH 7.4, per 10 ml of whole blood). The erythrocytes and leukocytes were carefully sedimented by centrifugation at 110 *g*. The platelets

1. *Abbreviations used in this paper:* PAGE, polyacrylamide gel electrophoresis.

were washed three times in veronal-buffered saline, pH 7.4, 0.1% gelatin, 0.015 M EDTA (GVB-EDTA), resuspended, and counted on an Ortho ELT 8 automated blood counter (Ortho Pharmaceutical, Raritan, NJ). The platelets were adjusted to a final concentration of ~500,000/μl whenever possible.

Assay for the binding of ligands to platelets and to IgG bound to platelets

Washed normal platelets were incubated with an equal volume of serum or serum fractions for 30 min at 37°C, washed three times with GVB-EDTA, and resuspended to a concentration of 300,000/mm^3. 100 μl of the suspension was then reacted with 100 μl of ^{125}I-labeled antibody at a prescribed concentration. Following a 45-min incubation at 37°C, three 50-μl volumes of the mixture were pipetted into separate 400-μl microfuge tubes containing a mixture of phthalate esters (1.5 parts *n*-butyl phthalate [Fisher Scientific Co., Pittsburgh, PA] to 1.0 part *bis* (2-ethylhexyl)phthalate [Eastman Kodak Inc., Rochester, NY]). After centrifugation in a Beckman microfuge (Beckman Instruments, Inc., Fullerton, CA), a clamp was applied to the tube in the middle of the oil layer and the tip of the tube was cut off; the amount of radioactivity on the platelets in the tip was assessed in a well-type gamma scintillation counter. For analysis by Klotz's or Scatchard's method, fluid-phase ligand concentration was determined by counting 10 μl of the supernatant fluid from each microfuge tube after centrifugation.

Solid-phase antiglobulin consumption assay

Polystyrene tubes, 12 × 75 cm, were coated with purified myelomatous IgG according to the buffer-coating method of Parsons (20). The IgG-binding capacity of tubes coated in this manner was highly reproducible. Antiglobulin was used in this system at a concentration well below excess for the amount of IgG bound to the tubes. Approximately 65% of the labeled anti-IgG was affixed to the IgG-coated tubes. All IgG reacting with anti-IgG, except that in fluid phase, was labeled with ^{131}I by using the method described above. ^{125}I-Labeled antiglobulin, either monoclonal or polyclonal, was incubated with a source of IgG, either fluid phase or anti-Pl^{A1}-sensitized platelets, or IgG bound to Covaspheres (Covalent Technologies Corp., Ann Arbor, MI), or anti-Lewis^a (Le^a) bound to Le^a substance attached to crystalline silica particles (Chembiomed, Ltd., Edmonton, Alberta). The amount of IgG absorbing anti-IgG was determined chemically (for fluid phase) or by measurement of radioactivity due to ^{131}I, converted to a quantity of IgG by knowledge of the specific activity. After a 30-min incubation at 37°C, a standard volume of the mixture was placed in an IgG-coated tube and incubated for 30 min at 37°C. After three washings to remove unbound antiglobulin, the tubes were counted in a gamma scintillation counter. The results were calculated as fractional inhibition of binding based on the maximum binding of that amount of antiglobulin to a coated tube in the absence of another source of IgG.

The consumption of antiglobulin was directly determined by estimating the difference in the amount of anti-IgG affixed to a coated tube when the anti-IgG had not been preabsorbed with a known number of platelets and the amount affixed after such preabsorption. This difference was converted to molecules of IgG per platelet by knowledge of the number of platelets, the specific activity of the antiglobulin reagent, and the percentage of the reagent available for binding.

The consumption of anti-IgG was measured indirectly by reference to a calibration curve of consumption of anti-IgG by IgG in fluid phase or affixed to Covaspheres or to anti-Le^a antibody affixed to antigen bound to such spheres. The anti-IgG was incubated with the source of IgG of known concentration and fractional inhibition of subsequent binding to coated tubes was calculated. The fractional inhibition generated by a known number of platelets was determined and the amount of IgG thought to be responsible for this inhibition was read directly from the graph.

Results

Specificity of the monoclonal anti-IgG

To test the subclass specificity of the radiolabeled monoclonal anti-IgG antibody, erythrocytes were coated with myeloma proteins of each subclass and each light chain class. (These proteins were kindly provided by Dr. William Yount of the University of North Carolina, Chapel Hill). Radiolabeled anti-IgG detected subclasses of IgG equally regardless of light chain class (data not shown).

Binding of ligands to IgG-coated platelets

To test the time required for the binding of anti-IgG to IgG on the platelets, normal platelets were coated with IgG from the serum of a patient with alloimmune anti-Pl^{A1} antibody. Equal volumes of IgG-coated platelets and radiolabeled ligand were mixed at 37°C. At intervals of time, samples were removed and the binding of the radiolabeled ligand to the platelets was assessed. For both, binding occurred rapidly and was complete by 40 min (data not shown).

To evaluate the effect of varying concentrations of ligand on the amount of binding of ligand to normal platelets and IgG-coated platelets, normal platelets were coated with IgG from serum or IgG fractions of serum containing anti-platelet antibodies; for some studies, the IgG fraction of serum was labeled with ^{131}I. After being washed, the platelets were incubated with equal volumes of graded concentrations of radiolabeled ligand and the binding of radiolabeled ligand was determined. When ^{131}I-labeled IgG was used to coat the platelets, the amount of IgG bound was determined. Washed platelets not sensitized were treated similarly as controls.

The data were analyzed in three ways:

(*a*) The quantity of ligand bound to the platelet was plotted against a concentration of ligand used in the mixture (Figs. 1 and 2, *A*). By this analysis, the amount of ligand bound to normal unsensitized platelets was directly proportional to the concentration of ligand used in the reaction mixture; the dose-response curves were nearly linear at all concentrations tested, particularly for monoclonal anti-IgG. The amount of polyclonal anti-IgG bound for a given concentration of ligand was greater than for monoclonal anti-IgG.

The net binding to IgG-coated platelets (the total amount of ligand bound less the amount bound to normal platelets) reached a maximum value when 50–75 μg/ml of each ligand was used and was, at higher concentrations, nearly the same as this maximum.

(*b*) To determine whether sufficient ligand was used to estimate maximal binding, the data were plotted according to the method of Klotz (21); the logarithm of the concentration of free ligand in the supernatant fluid following centrifugation was

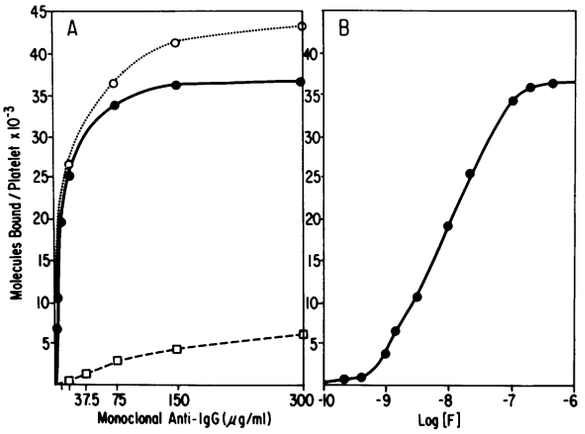

Figure 1. The binding of ^{131}I-labeled monoclonal anti-IgG to platelets coated with anti-PI^{A1}. (*A*) The binding of anti-IgG to washed normal platelets (– – □ – –) and to platelets which had been coated with anti-PI^{A1} (– – ○ – –); net binding to anti-PI^{A1}-coated platelets is shown in solid symbols. (*B*) The binding data expressed according to the method of Klotz (21); the log of the molar concentration in fluid phase is shown on the abscissa. F, free.

plotted against the number of molecules bound to each platelet (Figs. 1 and 2, *B*). With both monoclonal and polyclonal anti-IgG, a symmetric sigmoid curve was obtained, indicating that sufficient ligand was present to measure binding. The inflection point of this curve occurs at the point where half of the sites are bound.

(*c*) According to the method of Scatchard (22), the amount of radiolabeled ligand bound per platelet was plotted against the amount bound divided by the amount remaining in fluid phase at equilibrium expressed in moles per liter (Fig. 3). The line, when apparently straight, was analyzed by the least squares method. By such analysis, the intercept with the abscissa indicates the maximum number of binding sites available to the ligand;

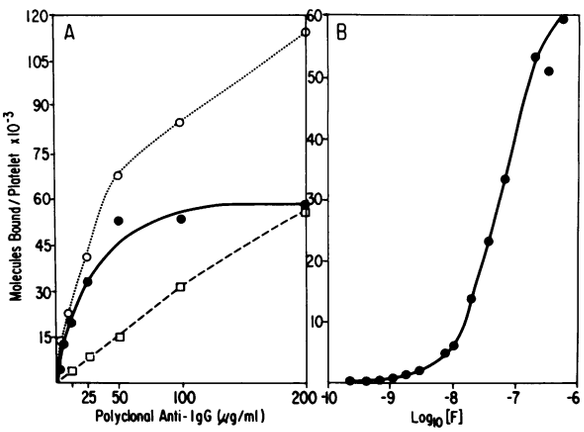

Figure 2. The binding of ^{131}I-labeled polyclonal anti-IgG to platelets coated with anti-PI^{A1}. See legend for Fig. 1.

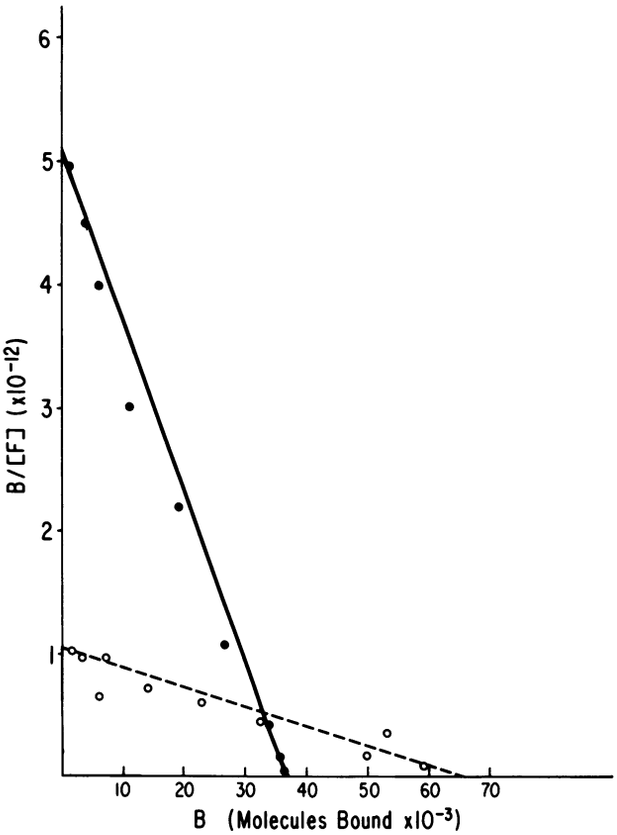

Figure 3. Analysis of the binding of the two ligands by anti-PI^{A1}-coated platelets by the method of Scatchard. The data for monoclonal anti-IgG are shown in solid circles and for polyclonal anti-IgG in open circles.

the ratio of predicted sites to sites measured by ^{131}I-IgG (varying between 10,000–50,000 molecules/cell) was calculated. The slope of the line was measured and the apparent dissociation constant (K_D) for the reaction was calculated as the reciprocal of the slope of the line (Table I).

In some instances, a line curvilinear to the origin was obtained. The assumption was made that the curvilinearity was due to heterogeneity of binding constants in the interaction and the simplest analysis was made; i.e., that reactions with two binding constants were being measured. The curves were then analyzed by a curve subtraction method.

Monoclonal anti-IgG. When monomeric IgG was used to sensitize platelets, monoclonal anti-IgG gave linear curves on Scatchard analysis. The affinity was high and the proportion of anti-IgG binding sites indicated by this analysis was consistently ~⅔ of that indicated when the ^{131}I-labeled IgG fraction from serum containing alloimmune anti-PI^{A1} or autoimmune antibody was used to sensitize the platelets (Table I). The proportion of sites detected remained constant over the fivefold difference in site number in these experiments. Assays performed in the

Table I. Analysis of the Binding of IgG-binding Ligands to IgG on Platelets

Ligand	K_D	Ratio of ligand-binding sites/^{131}I-IgG sites
Monoclonal Anti-IgG	4.9×10^{-9} (±1.6) (n = 8)	0.53±0.07 (n = 6)
Polyclonal Anti-IgG	1.4×10^{-7} (±1.9) (n = 5)	1.32±0.48 (n = 3)
Monoclonal* Anti-IgG	4.2×10^{-7} 3.4×10^{-9} (n = 4) (±1.4)	— —

* Aggregates or immune complexes attached to platelets.

absence of EDTA and gelatin gave entirely similar results (data not shown).

When artificially produced and chromatographically defined aggregates of IgG or serum or serum fractions containing immune complexes were used to coat the platelets and the binding was analyzed by the method of Scatchard, a curvilinear line resulted. These curves were analyzed as though the curvature resulted from the presence of binding sites with two different affinities; the affinity of the majority of the sites corresponded to the affinity of monomeric IgG with the monoclonal anti-IgG (Fig. 4).

Polyclonal anti-IgG. When the binding of the polyclonal, heterologous, affinity-purified anti-IgG was analyzed by the Scatchard method, a straight line was obtained (Fig. 3). The K_D was greater than for monoclonal and the ratio of sites predicted by the Scatchard analysis to the number determined by ^{131}I-labeled IgG was >1 (Table I). The curve obtained upon plotting according to the method of Klotz indicated that sufficient antibody was used to validate this extrapolation (Fig. 2 *B*).

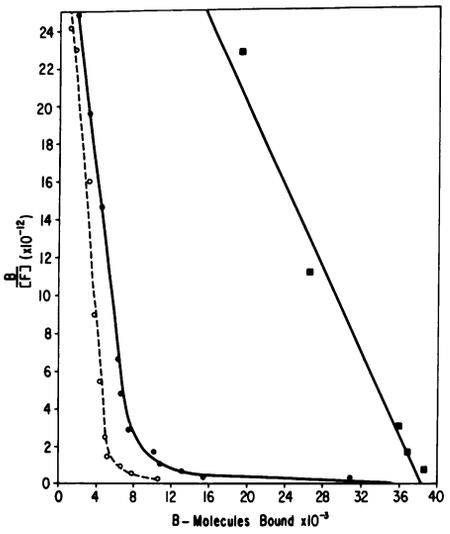

Figure 4. Scatchard analysis of the binding of monoclonal anti-IgG to platelets coated with complexes of IgG. The results of experiments with suberimidate-induced aggregates of IgG are shown in solid circles; those with immune complexes are shown in open circles. The results with monomeric anti-PI^{A1} antibody are shown for contrast (solid squares).

Inhibition of ligand binding by excess unlabeled ligand

Washed normal platelets were incubated with a given concentration of radiolabeled ligand and various amounts of unlabeled ligand to determine if an excess of unlabeled ligand competed for binding sites and inhibited the fixation of labeled ligand. At all concentrations of unlabeled ligand tested, there was relatively little inhibition of binding by ligand 100-fold greater than the concentration of labeled ligand (Table II).

When platelets sensitized with the IgG fraction of antiserum containing anti-PI^{A1} were substituted in the experiments de-

Table II. Inhibition of Binding of Radiolabeled Ligand by a 100 M Excess of Unlabeled Ligand

			Molecules/platelet-labeled ligand		
	Radiolabeled ligand	Unlabeled ligand	No unlabeled ligand	With unlabeled ligand	Percent inhibition of binding
Washed platelets	Monoclonal anti-IgG	Monoclonal	1,774	1,245	29.8
		Polyclonal	1,176	971	17.4
	Polyclonal anti-IgG	Polyclonal	3,650	2,589	29.1
		Monoclonal	3,500	1,927	44.1
Platelets sensitized with anti-PI^{A1}	Monoclonal anti-IgG	Monoclonal	14,174	1,165	91.8
		Polyclonal	14,855	2,575	82.7
	Polyclonal anti-IgG	Polyclonal	10,433	2,968	71.6
		Monoclonal	9,455	5,463	42.2

scribed above, the amount of ligand bound was very much greater than that bound to unsensitized platelets and the binding was markedly inhibited by unlabeled ligand (Table II).

To determine whether monoclonal and polyclonal anti-IgG reacted with the same part of the IgG molecule, the inhibition of binding of radiolabeled ligand to anti-Pl^{A1}-coated platelets by either the same or the other anti-IgG was tested. Unlabeled polyclonal anti-IgG nearly completely inhibited the binding of monoclonal anti-IgG, indicating that antibodies in that preparation bound to the same antigen that binds the monoclonal anti-IgG or the antigens nearby. The binding of polyclonal anti-IgG was only partially inhibited by monoclonal anti-IgG, indicating that antibodies to other epitopes are present in the polyclonal anti-IgG preparations (Table II).

Inhibition of the binding of radiolabeled ligand by fluid-phase IgG

Washed normal platelets were incubated with a given concentration of radiolabeled ligand and various amounts of human IgG, which was purified from a patient with multiple myeloma, to determine if reaction with IgG would inhibit the binding of anti-IgG to platelets. At the highest concentrations of fluid-phase IgG, the binding of polyclonal but not monoclonal anti-IgG was inhibited (Table III).

When sensitized platelets were used in such experiments, the binding of radiolabeled ligand was markedly reduced compared with that observed with unsensitized platelets.

Binding of $F(ab')_2$ fragments of monoclonal anti-IgG

To determine whether the "nonspecific" binding required the Fc portion of the monoclonal antibody molecules, $F(ab')_2$ fragments of the antibody were reacted with platelets sensitized with anti-Pl^{A1} serum and with autologous serum. Approximately the same number of $F(ab')_2$ fragments were affixed as were IgG antibody molecules (Table IV).

Detection of platelet-bound IgG by antiglobulin consumption

The reduction in the amount of monoclonal anti-IgG remaining after incubation with known amounts of IgG in fluid phase,

Table III. Inhibition of Binding of Radiolabeled Ligand by Fluid-phase IgG in 100 M Excess

		Molecules ligand per platelet		
	Ligand	No IgG	With IgG	Percent inhibition of binding
Washed platelets	Monoclonal anti-IgG	392	404	0
	Polyclonal	3,031	1,690	44.2
Platelets sensitized with anti-Pl^{A1}	Monoclonal Anti-IgG	11,242	607	94.6
	Polyclonal	38,505	2,508	93.5

Table IV. A Comparison of the Binding of ^{125}I-Monoclonal Anti-IgG and ^{125}I-$F(ab')_2$ of Monoclonal Anti-IgG to Anti-Pl_{A1}-sensitized Platelets

		Molecules bound per platelet	
Serum	Dilution	Anti-IgG	$F(ab')_2$ of anti-IgG
Anti-Pl^{A1}	1:1	22,300	15,600
	1:10	5,800	5,500
Autologous	1:1	3,500	2,000
None		5,700	3,300

bound to plastic or to specific antigens bound to plastic, or to platelets as alloantibody was estimated by using a solid-phase antiglobulin consumption assay. The fraction of anti-IgG adsorbed by a given quantity of those forms of IgG not bound to platelets was approximately equal in all cases. However, when IgG was bound to platelets, only $\frac{1}{5}$–$\frac{1}{15}$ as much IgG was required to inhibit a given amount of anti-IgG as was required when IgG was in any other form (Fig. 5). This indicates that the stoichiometry of binding of anti-IgG and IgG is different when IgG is bound to the platelet than when IgG is in fluid phase or bound to plastic either nonspecifically or as antibody.

IgG was attached to platelets and the amount measured by three techniques: (*a*) the direct ligand-binding assay measuring the amount of monoclonal anti-IgG binding to the IgG-coated

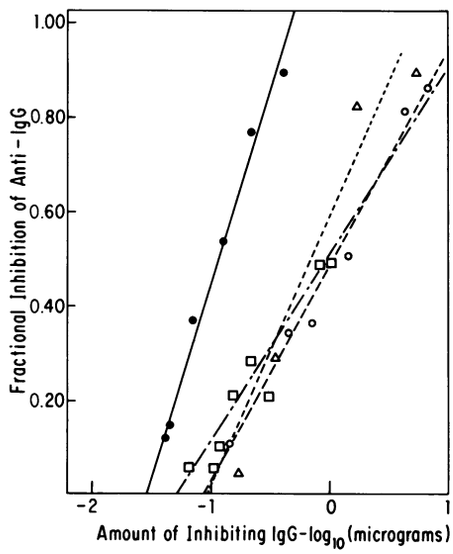

Figure 5. Inhibition of the attachment of anti-IgG to IgG-coated tubes by preabsorption with IgG in various forms. The amount of IgG in fluid phase (– – ○ – –) or ^{131}I-labeled IgG attached to platelets (– – ● – –), to Covaspheres (– – △ – –), or attached as specific anti-Le^a antibody attached to Le^a substance bound to crystalline silica spheres (– – □ – –) is shown on the abscissa on a logarithmic scale. The fraction of inhibition of maximal absorption to coated tubes is shown on the ordinate.

platelets, (*b*) the direct antiglobulin consumption test in which the number of molecules of anti-IgG consumed was estimated by difference, and (*c*) the indirect antiglobulin consumption test in which the amount of anti-IgG consumed was estimated by reference to a calibration curve given by fluid-phase IgG. (This latter method most nearly resembles the antiglobulin consumption techniques in general use). The results of assays using these techniques on sensitized platelets as well as on patient samples are shown in Table V. The first two techniques give similar results; the last technique gives results which are one to two orders of magnitude greater and are similar to results given by antiglobulin consumption tests currently in use (1).

Discussion

In order for a radiolabeled ligand to be used in the detection of platelet-bound IgG, the characteristics of the reaction of the ligand with the "receptor" (platelet-bound IgG) and with the platelet itself must be carefully analyzed. The stoichiometry of the specific reaction can be determined from such studies of the nature of the binding.

When radiolabeled ligands are reacted with washed normal platelets, ligand is bound. The amount varies with the ligand and is greater with polyclonal anti-IgG and less with monoclonal anti-IgG. The binding could be to IgG bound to the platelet but not removed on washing (specific binding) or to the platelet directly (nonspecific binding); previous studies have suggested that IgG is present in small amounts on the surface of the normal, washed platelet (23).

The present studies suggest that much of the binding of anti-IgG to washed normal platelets is nonspecific since the interaction between each of the radiolabeled ligands examined and the washed platelets does not have the characteristics of ligand-receptor interaction. The amount of ligand bound was directly proportional to the concentration of ligand reacted with the platelet and did not appear to achieve saturation within the range studied in the present studies (~0.5–300 μg/ml). Furthermore, the binding of radiolabeled ligand is only partially inhibited by either excess unlabeled ligand or fluid-phase IgG.

Table V. Platelet-bound IgG Determined by Three Methods

	Antiglobulin consumption		Direct ligand binding assay
	Indirect method	Direct method	
	(molecules/ platelet)	*(molecules/ platelet)*	*(molecules/ platelet)*
Anti-PI^{A1} serum dilution			
1:1	662,900	20,500	18,900
1:3	135,000	13,400	19,000
1:9	27,800	5,000	6,200
0	9,000	300	900
Patient's platelets			
Si	70,000	4,300	4,200
Sn	336,200	14,800	14,800
We	50,100	3,100	3,200
Sc	22,200	1,250	1,700
Ha	77,200	4,700	6,300
De	64,700	3,500	8,900

The present studies show that when the IgG is attached to the platelet either as specific antibody or as the result of nonspecific passive adsorption, the interaction between the ligands studied and the platelet-bound IgG has the characteristics of ligand-receptor interactions. Ligand binding reaches saturation and is inhibited by excess unlabeled ligand or by unlabeled fluid-phase IgG. The binding is reversible; although in the case of monoclonal anti-IgG, the affinity is so great as to render this difficult to measure.

Of the two ligands examined, monoclonal anti-IgG appeared to have the most easily analyzed characteristics by Scatchard analysis. The K_D was very low, indicating a high affinity. This ligand appeared to detect only about 60% of the molecules of IgG present on the membrane. The reason for the discrepancy between the number of sites as determined by Scatchard analysis is not apparent. This may have to do with a relative inaccessibility of some IgG associated with the platelet membrane. However, the fact that the proportion of IgG detected by the ligand was the same when various amounts of IgG were affixed to the membrane permits the use of the ligand in assessing the number of molecules of IgG adsorbed to the platelet surface.

The polyclonal antibody used in these studies also appeared to react with platelet-bound IgG. Despite the fact that there is probably heterogeneity in the binding constants of different antibody molecules, the Scatchard plot was linear. The number of sites estimated by the Scatchard analysis was somewhat greater than the number estimated by measuring the ^{131}I when radiolabeled IgG was affixed to the platelet. Whether this indicates that all IgG molecules are accessible, in contrast to the binding of monoclonal anti-IgG, or whether the same proportion is accessible but more than one molecule of polyclonal anti-IgG may be adsorbed to each IgG molecule is not clear. The latter condition may obtain because antibodies to several epitopes of the IgG molecule are present in the anti-IgG preparation. The availability of this ligand may make it a more practical one for the routine detection of platelet-bound IgG, even though the amount of anti-IgG bound nonspecifically to washed normal platelets tended to be greater than for monoclonal antibody.

When aggregated or complexed IgG adherent to platelets was reacted with monoclonal anti-IgG, the line derived by Scatchard analysis was curvilinear. The K_D of the reactions of higher affinity was much like that of the reaction with monomeric IgG, suggesting that the aberrant reactions are of lower affinity. This may be due to relative inaccessibility of some IgG molecules in the complex. This heterogeneity of interaction suggests that the results of the interaction of monoclonal anti-IgG with immune complexes must be interpreted with caution.

The number of molecules detected by the monoclonal anti-

IgG assay on normal platelets and on the platelets of patients with immune thrombocytopenia was considerably less than the amount detected by quantitative antiglobulin consumption assays. In part, this may be due to nonspecific adsorption of the antiglobulin in the antiglobulin consumption assay; it is difficult to correct for this in such assays. Furthermore, it has been suggested that in antiglobulin consumption assays, platelet-related debris which contains IgG may be simultaneously analyzed (24); such debris would not be detected in the ligand-binding assays as performed in the studies as the platelets are separated by centrifugation through an immiscible fluid. However, the present studies suggest that the major reason for the observed discrepancy lies in the fact that the assumption made in the calibration of antiglobulin consumption assays, which is that the interaction between antiglobulin and IgG on the platelet is the same as between antiglobulin and IgG in fluid phase, is not correct.

In the present studies, we have shown that the same amount of anti-IgG was consumed by much less IgG when it was bound to the platelet surface than when it was in fluid phase or when it was bound nonspecifically to other surfaces such as plastic or specifically to antigens immobilized on particles. The reason for the difference is not apparent. However, if one attempts to use a calibration curve which measures the consumption of antiglobulin by fluid-phase IgG or IgG attached to surfaces other than platelets, a falsely high value is given for the amount of IgG present on the platelets. If one measures antiglobulin consumption using the calibration curve in which known amounts of IgG are attached to platelets or in which the amount of antiglobulin consumed is measured directly without reference to a calibration curve, very much less IgG is measured; the amount detected in these assays was similar to the amount detected by direct radioligand assays. Thus, from these studies, it is apparent that the radiolabeled monoclonal anti-IgG assay most accurately measured the amount of IgG on the platelet surface. A direct antiglobulin consumption assay which measured the consumption of radiolabeled ligand without reference to a calibration curve was equally accurate. However, any assay which relies upon a calibration curve to measure the consumption of antiglobulin in which the IgG used to calibrate the assay is not attached to platelets will vastly overestimate the amount of IgG present on the platelet surface.

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