Evaluation by Quantitative Acid Elution and Radioimmunoassay of Multiple Classes of Immunoglobulins and Serum Albumin Associated With Platelets in Idiopathic Thrombocytopenic Purpura

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Immunoglobulins (Igs) and serum albumin were eluted from normal platelets and platelets from patients with idiopathic thrombocytopenic purpura (ITP) with a quantitative acid elution procedure followed by solid-phase radioimmunoassay (SPRIA). Acid elution was shown to release a reproducible fraction of platelet-associated Igs, and the amounts released per platelet were independent of the platelet concentration over a wide range of concentrations. This procedure is suitable for sensitive, reproducible, and specific quantitation of large numbers of samples. Washed platelets from 13 normal donors contained the following components (expressed in femtograms per platelet, mean ± 2 SEM): IgG, 1.40 ± 0.26; IgA, 0.72 ± 0.36; IgM 0.078 ± 0.036; albumin 7.7 ± 1.5. Immunoglobulins and albumin eluted from the platelets of ten ITP patients (two in remission), expressed as femtograms per platelet, mean (range), were: IgG 104 (0.3 to 750); IgA 90 (0.3 to 715); IgM 162 (1.2 to 1,300); and albumin 34 (6.8 to 198). All platelet-associated Igs from thrombocytopenic ITP patients were found to be elevated twofold to 2,300-fold with one Ig class occasionally elevated 50-fold to 100-fold higher than the others. A similar group of ten thrombocytopenic ITP patients was found to have twofold to 26-fold elevations of platelet-associated albumin. This demonstration of increases in multiple classes of Igs as well as serum albumin associated with platelets from ITP patients suggests that some nonimmune process may be contributing to the phenomenon of increased platelet-associated proteins in ITP. © 1986 by Grune & Stratton, Inc.

ITP FACTOR capable of inducing thrombocytopenia by passive transfer has been shown to be present in the purified IgG fraction from the plasma of patients with idiopathic thrombocytopenic purpura (ITP). Measurement of platelet-associated IgG (PAIgG) has become a frequently used diagnostic test in ITP. Numerous reports have shown that PAIgG is elevated in most cases of ITP and that the degree of elevation is inversely related to the platelet count (for review, see ref. 3). Recent reports, however, have described some ITP patients who have elevated platelet-associated IgM9; other studies have shown increased platelet-associated C39'3'25 and C49 in thrombocytopenic ITP to IgG; furthermore, normal platelets also possess measurable albumin are also elevated in most instances. These results suggest that the increased levels of immunoglobulins in platelet preparations from ITP patients may be due partly to mechanisms unrelated to antigen–antibody binding; the same mechanisms may also account for the attachment of albumin and possibly other serum proteins.

MATERIALS AND METHODS

Platelet preparation. Blood was drawn by the twosyringe technique and immediately added to a 10% solution of dipotassium EDTA 17 μ/mL of whole blood. The platelet-rich plasma (PRP)

was made by centrifuging whole blood at 400 g for four minutes. PRP was centrifuged at 1,500 g for 15 minutes and the platelet pellet was suspended in 1.0% ammonium oxalate for 10 minutes. After being pelleted, the platelets were washed three times in 0.15 mol/L of NaCl (saline) and suspended in saline. Platelets were either eluted immediately or were stored at 4 °C with 0.02% sodium azide.

Quantitative acid elution. All subsequent steps were carried out at 4 °C unless otherwise noted. The general elution procedure was to suspend the platelets in unbuffered saline and add some volume of acid buffer with bovine serum albumin (BSA) (Miles, Naperville, Ill, Fraction V) to achieve a final acid concentration of ~50 mmol/L and a final BSA concentration of 0.5%; for example, a 200-μL aliquot of the final saline suspension containing from 0.1 x 108 to 109 platelets was acidified by the addition of 100 μL of 150 mmol/L of H2PO4, 100 mmol/L of NaCl, 1.5% BSA, pH 2.8. Acetic acid (150 mmol/L), titrated to pH 2.8 with HCl, can be used in place of H2PO4 with identical results. After ten minutes, the suspension was centrifuged in a Beckman Microfuge 12 at maximum speed (~10,000 g) for two minutes. A predetermined amount of 2.5 mol/L of Tris base was added to 250 μL of the supernatant to raise the pH to 8.0 to 8.1, and sodium azide was added to 0.02%. This platelet extract could be stored at 4 °C for periods up to two months without adsorption of the Igs to polyethylene tubes or loss of immunologic reactivity.

Ig quantitation. Igs were quantitated by a solid-phase radioimmunoassay (SPRIA) technique using flexible polyvinyl chloride plates (Dynatech, Alexandria, Va) coated with heavy chain-specific, affinity-purified, goat anti-human IgG, IgA, or IgM (Kirkegaard & Perry Labs, Rockville, Md). The plates were coated by adding 80 μL of 10 mmol/L of NaPO4, pH 8.0, with 0.02% NaN3 to each well of the 96-well microtiter plate followed by 20 μL per well of the heavy chain-specific antibody, at 25 ng/μL, in the same phosphate buffer. To avoid adsorption of the anti-Ig to the test tube, all procedures were done at 4 °C, and only enough of the antibody was diluted from a 1 mg/mL solution in 150 mmol/L of NaCl, 10 mmol/L PO4-P, pH 7.4 (PBS) for preparation of one plate. The plates were incubated overnight at 4 °C in a humidified airtight container and were then washed three times by flooding with phosphate-buffered saline (PBS), 0.5% BSA, 0.02% NaN3 (BSA/PBS) and incubated with BSA/PBS for at least 30 minutes prior to use to block residual protein binding sites. Usually, 10 to 12 plates were prepared at one time; these could be stored full of the final wash solution at 4 °C in a humidified chamber for as long as three months.

The same heavy chain-specific antibody used to coat the plates

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was used as the labeled second antibody; $^{125}$I was coupled to these antibodies by the chloramine-T method. Na$^{125}$I [16.6 mCi/ g iodine (Amersham, Arlington Heights, Ill, #IMS300)] was added to the antibody in the ratio of 2 µCi/ mg of protein in 100 mmol/L (final) of phosphate buffer, pH 7.5. The reaction was initiated by the addition of chloramine-T (0.5 µg/µg of protein), incubated with gentle mixing for 90 seconds and terminated by the addition of a saturated tyrosine solution (0.5 mL/µg of protein). After 60 seconds, the mixture was applied to a Dowex AG1-X8 (200 to 400 mesh) column (7 x 25mm) which was preequilibrated with borate (345 mmol/L)-buffered BSA (0.1%) solution (BBSA, pH 8.55); the column was washed with successive 500-µL aliquots of BBSA. The major portion of radioactivity eluted in the second and third column fractions. More than 95% of the radioactivity in this preparation was eluted in the void volume of a Sephadex G25 column (Pharmacia PG-10). Therefore, the eluate from the Dowex column was used without further purification.

Between 10 and 40 µL of the platelet acid eluates were added to antibody-coated microtiter wells that had been sucked dry of the BSA/PBS solution and filled with 100 µL of radioimmunoassay (RIA) buffer (100 mmol/L of Tris, pH 8.1, 50 mmol/L of NaCl, 0.5% BSA, 0.02% NaN3). Chromatographically purified IgG (Cappel), secretory IgA (Calbiochem, Malvern, Pa), or IgM (Calbiochem, San Diego, Calif) standards from 0.1 to 500 ng per well, in the same volume as the eluates, were added to other wells for the standard curves, in a buffer that reproduced the neutralized acid eluate. The plates were either incubated at room temperature for two hours or overnight at 4°C. The plate was then placed on an ice bed for at least ten min, the unbound material was thrown off, and the plate was washed three times by flooding with ice-cold BSA/PBS.

The wells were aspirated dry, and 100 µL of the mixture containing 60,000 cpm was added to the Ig assays. Crystalline HSA (Miles, Naperville, Ill) was labeled, as described above, with $^{125}$I, 4 µCi/µg of protein; the labeled serum albumin was mixed with the platelet eluate or an albumin standard, and 100 µL of the mixture containing 60,000 cpm was added to the antiserum-coated wells, incubated for two hours at 21°C or overnight at 4°C, washed three times as with the Ig assays, dried, and counted. The maximum binding was typically 10,000 cpm, which could be maximally displaced by 500 ng of HSA per well; the useful range of the assay was typically from 2 to 200 ng per well.

This assay system could therefore measure platelet-associated albumin (PAA) given as little as 5 µL of whole blood from a normal donor or 40 µL of whole blood from a severely thrombocytopenic patient (a platelet count of 10,000/µL).

Patients with ITP. All patients had thrombocytopenia with an increased number of megakaryocytes in the bone marrow and no associated abnormality or disease process that might account for thrombocytopenia. The patients' tests for antinuclear antibodies and rheumatoid factor were all negative. The one pediatric patient studied had acute ITP, and all others had chronic ITP. Platelet counts of the patients are tabulated in the text.

RESULTS

Sensitivity of these assays was of primary importance in evaluating samples with scarce material from severely thrombocytopenic patients. Figure 1 shows that this solid-phase sandwich system is accurate to <1 ng/40 µL. When necessary, the sensitivity of these assays could be increased at least tenfold by using a smaller volume of the diluting RIA buffer and/or by concentrating the platelets in the acid elution procedure. Dilution of sample in the RIA buffer was performed to prevent minor pH variations between empirically neutralized samples. The use of polyclonal antibodies gives a wide standard curve which spans nearly two logs of concentration. The logit transformation of the standard curve, plotted by linear regression analysis, is convenient for measuring unknowns.

The ability of the assay system to distinguish between the classes of immunoglobulin was particularly critical for this study. The heavy chain-specific antibodies show insignificant cross-reactivity with other classes for the purposes of this study (Fig 1). The three other combinations for testing...
cross-reactivity (anti-IgG with IgM, anti-IgA with IgM, and anti-IgM with IgA) show similar results.

The effect of pH on the elution of various platelet-associated proteins is shown in Fig 2. IgG and IgM show a sharp increase in elution below pH 4, IgA shows a more gradual increase, and albumin shows a progressive elution over a wide range of decreasing pH values. A pH between 2.7 and 2.8 was suitable for eluting a high percentage of Igs at minimal risk of denaturation. Treatment of IgG, IgM, IgA, and HSA standard proteins under conditions of acid elution produced no detectable change of antigenicity as judged by comparing assays before and after treatment. Moreover, when 5 to 240 ng/mL of IgG, IgM, IgA, or HSA was added to 2 × 10⁸ platelets in 200 μL before acid elution was performed, essentially 100% of the added proteins was recovered, as measured by the solid-phase RIA (SPRIA) technique. Thus, acid treatment per se and the presence of soluble platelet materials released by acid treatment did not interfere with the specificity or sensitivity of the SPRIA.

The time course of elution of IgG from washed platelets is shown in Fig 3. The amount of IgG eluted approached an asymptotic value after eight minutes, and the rate of elution was only slightly increased at 24 °C as compared to 4 °C. A convenient elution condition was ten minutes at 4 °C.

A linear relationship should exist between platelet concentration and Ig yield. Because high concentrations of platelets could buffer the acid and alter Ig yield, the effect of platelet concentration on the linearity of the Ig elution was evaluated. Figure 4 shows a linear relationship between IgG, IgA, and IgM and the number of platelets up to 2 × 10⁸ platelets/700 μL of acidified platelet suspension.

A single elution was used for most experiments. However, this did not appear to release 100% of the Igs from the platelets (Fig 5). As judged by summation of four sequential elutions, the first elution released ~70% of platelet-associated IgG and IgA from both control and ITP platelets. IgM release in the first elution of control platelets was ~43% whereas that of ITP platelets was ~90% in the first elution. The variation in the yield of Igs from the first elution is ±10%. Because IgM levels were quite small and near the limits of sensitivity of the assay, there was some added uncertainty in the percentages of total IgM in elutions of control platelets. Platelets were also disrupted by sonication of freeze-thawing and were then acid-eluted and centrifuged at 20,000 g for 30 minutes; this increased the single elution yield of IgG, IgA, and IgM between 50% and 100%.

The amounts of Igs associated with normal platelets and ITP platelets are shown in Fig 6. Average levels of IgG, IgA, and IgM, from a sample of 13 normal donors are 1.4 ± 0.13, 0.72 ± 0.18, and 0.078 ± 0.018 μg per platelet, respectively (mean ± SEM). The ratio of normal value of PAIgA to PAIgG (0.5) appears to be higher than the ratio of average plasma levels (0.17), whereas the ratio of normal value of PAIgM to PAIgG (0.056) is close to that seen in plasma (0.074). Similarly, the ratio of PAIgG to platelet albumin (0.25) is not different from the ratio normally found in serum (0.26).

The mean platelet-associated Ig levels were elevated for all three Ig classes in ITP patients. In this series of eight patients (two were tested twice with at least one month between samples), IgG was elevated 70-fold; IgA was elevated 125-fold, and IgM was elevated 2,080-fold. The platelet counts for the ITP patients were, from bottom to top in the IgG
ELUTED IMMUNOGLOBIN AND ALBUMIN IN ITP

The nature of the antibody preparation has significant effects on the SPRIA technique described. Microtiter plates coated with affinity-purified antibodies have a much higher capacity and potentially greater selectivity than does the equivalent antisera or even purified IgG fraction. Monoclonal antibodies, because of their heterogeneity, generally give binding curves which reliably span up to 2 logs of antigen concentration, whereas monoclonal antibodies have much steeper antigen binding curves. The former test measures only surface proteins on platelets, in which case the concentration of the antigen of interest must be relatively high for efficient quantitation because an antibody-antigen-antibody procedure to assay soluble and eluted platelet-associated proteins permits efficient binding of the antigen of interest to the microtiter plate regardless of the concentration of other proteins in the sample. This is in contrast to procedures in which the antigen is bound directly to the microtiter plate, in which case the concentration of the antigen of interest must be relatively high for efficient quantitation because all proteins in cell extracts and eluates or serum samples compete for binding to a degree approximately proportional to their concentration. The SPRIA described is therefore more sensitive than previously described assays for measuring specific proteins in mixture with other proteins. Another advantage of the acid elution SPRIA procedure, in contrast to measurement using monoclonal anti-IgG or protein A binding to intact platelets, is the ability, with no ambiguity, to relate the eluted, soluble Ig to a purified, soluble reference Ig by a standard SPRIA curve. This eliminates assumptions about the availability, affinity, and stoichiometry of binding sites of platelet membrane-associated IgG molecules for assay reagents. Reports using procedures in which such assumptions are used in final calculations gave normal PAIgG values 15 to 30 times lower than that determined in this study and most others (see ref. 3, p 315).

DISCUSSION

Others have used SPRIA or enzyme-linked procedures for the measurement of platelet-associated IgG. The present modification, using an antibody-antigen-antibody procedure to assay soluble and eluted platelet-associated proteins permits efficient binding of the antigen of interest to the microtiter plate regardless of the concentration of other proteins in the sample. This is in contrast to procedures in which the antigen is bound directly to the microtiter plate, in which case the concentration of the antigen of interest must be relatively high for efficient quantitation because all proteins in cell extracts and eluates or serum samples compete for binding to a degree approximately proportional to their concentration. The SPRIA described is therefore more sensitive than previously described assays for measuring specific proteins in mixture with other proteins. Another advantage of the acid elution SPRIA procedure, in contrast to measurement using monoclonal anti-IgG or protein A binding to intact platelets, is the ability, with no ambiguity, to relate the eluted, soluble Ig to a purified, soluble reference Ig by a standard SPRIA curve. This eliminates assumptions about the availability, affinity, and stoichiometry of binding sites of platelet membrane-associated IgG molecules for assay reagents. Reports using procedures in which such assumptions are used in final calculations gave normal PAIgG values 15 to 30 times lower than that determined in this study and most others (see ref. 3, p 315).

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Selectivity of the antibodies used in this report is also a significant point. Because at least one report specifically denied the presence of IgA and IgM on normal platelets, it was necessary to demonstrate that the antibodies used in this procedure were sufficiently specific to avoid spurious results. The cross-reactivity of each Ig assay system for other Ig classes was negligible.

The acid elution of platelet-associated proteins is a convenient non denaturing procedure that is much less disruptive of platelets and their membranes than either detergent or freeze-thaw sonication. Platelets treated with detergent become nonrecognizable as cells, and remaining insoluble material appears as debris. Although sonication treatment is difficult to compare among laboratories, this treatment usually results in total mechanical disruption of platelet membranes and solubilization of a substantial amount of intracellular material. Acid elution at pH 2.8 leaves platelets morphologically distinguishable by electron microscopy and does not produce the fragmentation and debris formation that is characteristic of the more disruptive procedures. The percentage of recovery of total platelet-associated proteins from the acid elution is reproducible and appears to be identical for normal and ITP platelets with respect to IgG and IgA. Although the pattern of recovery for IgM appears to be different for normal and ITP platelets, this could reflect the small quantities of IgM released from control platelets relative to the sensitivity of the assay, particularly in eluates subsequent to the first. Whether or not the SPRIA we used measures only surface proteins on platelets is open to question. We did measure PAIgG of 20 normal controls using the antiglobulin consumption assay with the same antiglobulin reagents and standard IgG used in the SPRIA. The mean value for normal PAIgG was 1.9 ± 1.5 (2 SD) fg per platelet as compared with the mean SPRIA value of 1.4 fg per platelet. Because the antiglobulin consumption assay and the SPRIA measure similar amounts of PAIgG, the assumption by some workers that the former test measures only surface IgG may also apply to the SPRIA, even though the true value for surface PAIgG remains controversial. However, we do not claim to measure only surface proteins by the SPRIA but have shown that the proteins measured are soluble, antigenically intact, and reproducibly measurable by an unambiguous quantitative assay.

This investigation demonstrates two main points. First, it has been shown that there are measurable levels of IgA, IgM, and albumin, as well as of IgG, on normal platelets. Second, all three immunoglobulin classes associated with platelets and even platelet-associated albumin were elevated in most of the ITP patients studied.

PAA measurements in ITP patients on whom PAA levels were determined were, from bottom to top in the ALB column (x 103/μL: 73, 50, 30, 18, 80, 54, 10, 21, 27, and 4). There were two examples of normal PAIgG with elevated PAIgA or PAIgM and one instance of normal PAIgA and elevated PAIgG and PAIgM. Linear regression analysis was made to determine if there was a correlation between the levels of different Ig classes. There was no significant correlation between IgG and IgA (r = 0.47, n = 10) or IgG and IgM (r = 0.24, n = 9); however, there was a significant correlation between IgA and IgM (r = 0.95, n = 9, P < .01). This latter correlation disappears if the patient with the highest Ig levels, a pediatric ITP patient, is removed from the group (r = 0.31, n = 8). Finally, there is high variability in the patient groups: (mean ± SEM) IgG 104 ± 74; IgA 90 ± 70; and IgM 162 ± 142 fg per platelet.

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This investigation demonstrates two main points. First, it has been shown that there are measurable levels of IgA, IgM, and albumin, as well as of IgG, on normal platelets. Second, all three immunoglobulin classes associated with platelets and even platelet-associated albumin were elevated in most of the ITP patients studied.
Measurement of PAIgG is a frequently used diagnostic test in ITP. Elevations of PAIgG are often interpreted as the amounts of specific antiplatelet antibody bound to platelets and responsible for their destruction. However, we and others have questioned this interpretation for several reasons. The increased quantities of PAIgG involved in ITP are higher by one to several orders of magnitude than cellular antibody levels causing platelet destruction in well-defined disorders of immunologic origin involving isoantibodies or drug antibodies, and are often more than tenfold the amounts of these antibodies that saturate platelets in vitro. If the high levels of immunoglobulin associated with platelets in ITP are responsible for platelet destruction, the mechanism of destruction must be significantly different from that caused by high-affinity antiplatelet isoantibodies or drug antibodies. Moreover, highly elevated PAIgG levels similar to those found in ITP have also been associated with thrombocytopenias of apparent nonimmune origin. Other major problems of interpretation are raised by the lack of correlation between direct PAIgG measurements in ITP and indirect serologic assays for antiplatelet antibodies, even when such assays are performed in the same test system used for measuring the direct PAIgG. Finally, the finding of elevated albumin levels associated with platelets may destroy platelets at the same low levels per platelet as do isoantibodies or drug antibodies that are not detectable by direct assays on circulating platelets. Platelet destruction may then lead to high elevations of platelet-associated proteins by a secondary nonspecific phenomenon.

One postulated mechanism for elevating PAIgG is promotion of accessibility of platelet membrane Fc receptors by injuring specific ITP antibody, leading to nonspecific immunoglobulin binding. Certain aspects of nonspecific binding of IgG, and presumably other Ig classes, however, remain unclear. Although a platelet Fc receptor of IgG has been characterized and quantitated, it has not been proven that this type of binding can account for the relatively large amount of IgG associated with extensively washed normal platelets (~100-fold that for a normal red cell) or the many-fold increases in PAIgG that may accompany ITP since the IgG “specifically” bound by Fc receptor was reported to be removed by a single PBS wash. Moreover, Fc binding does not account for elevated platelet-associated albumin.

We have proposed an alternate mechanism for elevation of platelet-associated immunoglobulin and albumin that appears to be as reasonable as other theories to explain the observed phenomena; namely, that entrapment of plasma components in the platelet canalicular system or in resealed cell membrane fragments may account for the large amounts of plasma proteins associated with platelets normally and in pathological states when fragmentation is apparently augmented by platelet destruction. Growing evidence shows that a component of the increase in immunoglobulins associated with platelets in ITP involves a nonspecific process whether by this postulated “trapping” mechanism or other mechanisms. The size of the nonspecific component and the exact mechanism of the phenomenon await further studies.

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