CONCISE REPORT

The Amount of Platelet-Bound Albumin Parallels the Amount of IgG on Washed Platelets From Patients With Immune Thrombocytopenia

By John G. Kelton and Karen Steeves

The biologic relevance of the increased platelet-associated IgG (PAIgG) on platelets from patients with idiopathic thrombocytopenic purpura (ITP) is unclear. Platelets from ITP patients are often larger than normal, and it is possible that the increased IgG is not specific but passively related to platelet size. The measurement of platelet-bound albumin could provide information concerning the specificity of the platelet-bound IgG, since albumin, like IgG, is a plasma protein, but unlike IgG, is not an active participant in immunologic reactions. Albumin is also a normal constituent of platelet membrane, and increased platelet albumin could indicate an increased platelet mass. Platelet-bound albumin, IgG, and total platelet protein were measured on both intact and disrupted platelets from healthy individuals (n = 25) and patients with ITP (n = 21). Platelet IgG and albumin were measured in an immunoradiometric assay using intact antisera and Fab\(^1\)\(_2\) fragments prepared from the same antisera. There was no relationship between platelet-bound IgG or albumin, and platelet size measured by either platelet protein or platelet volume, (r < 0.3 for all interactions). In contrast, there was a significant correlation between platelet-bound albumin and platelet-bound IgG (r = 0.7, n = 21, p < 0.001). Those patients with elevated platelet PAIgG also had elevated platelet albumin, and this relationship was irrespective of the total platelet protein content or mean platelet volume. It is possible that the increased platelet-bound IgG in ITP reflects an increase in platelet surface area or contaminating platelet fragments that are not manifested as an increase in platelet volume or total platelet protein. Alternatively, a platelet membrane abnormality may occur in ITP that results in the uptake of significant amounts of plasma proteins. Either possibility implies that not all of the IgG on platelets from patients with ITP is pathologic IgG.

A NUMBER OF INVESTIGATORS have shown that IgG is quantitatively increased on washed platelets from thrombocytopenic patients with idiopathic thrombocytopenic purpura (ITP). However, the biologic relevance of the increased platelet-associated IgG (PAIgG) is unclear. All assays that measure PAIgG relate the amount of IgG to a unit number of platelets. Therefore, it is possible that the elevated PAIgG could represent IgG adsorbed to megathrombocytes present in increased numbers in immune thrombocytopenia. Previously, we did not observe a relationship between the amount of platelet-bound IgG and the platelet size in patients with immune thrombocytopenia. However, others have reported that the amount of platelet-associated IgG is proportional to the total platelet protein in certain patients with immune thrombocytopenia. It is possible that neither platelet size, nor total platelet protein levels, are directly related to platelet surface area. To investigate this more directly, we measured both platelet-bound IgG and platelet-bound albumin on intact and solubilized platelets from healthy individuals and patients with ITP, and related these results to the total platelet protein. We measured platelet-bound albumin, since albumin, like IgG, is a plasma protein, but unlike IgG, albumin does not participate actively in immunologic reactions. Furthermore, albumin is a normal constituent of platelet membrane, and an increased amount of platelet albumin could indicate an increased platelet mass within the washed platelet preparation. Hence, the ratio of platelet-albumin to platelet-IgG could provide information about the specificity of the finding of increased PAIgG in ITP.

MATERIALS AND METHODS

Patients and Controls

Platelets were obtained from healthy adult volunteers who were not taking any medications. Twenty-seven different unselected ITP patients were investigated over a 6-month period. Whole blood was collected into acid-citrate-dextrose (ACD) pH 4.5 (1:7, v:v). The platelet-rich plasma was isolated by centrifugation and washed 3 times prior to measurement of PAIgG. A separately collected EDTA anticoagulated sample was used to determine the platelet count and mean platelet volume using a Coulter S-Plus (Coulter Electronics, Hialeah, FL).

Measurement of Total Platelet Proteins, Platelet-Associated IgG, and Albumin on Platelets

Platelet-associated IgG, albumin, and total protein were measured on intact and solubilized platelets using an immunoradiomet-
ric assay. The washed platelet samples were resuspended to a final concentration of 50-200 × 10^4/ml in 0.15 M phosphate-buffered saline, pH 7.4 (PBS). One hundred microliters of varying dilutions of platelet suspension was added to 100 µl of the intact or F(ab')2, fragments of ^{125}I-anti-IgG or ^{125}I-anti-albumin (Atlantic Antibodies, Scarborough, ME). The anti-human IgG was raised in sheep antiserum was labeled with ^{125}I-sorb beads (Pharmacia Fine Chemicals, Piscataway, NJ). Each IgG or albumin that was covalently linked to Sephasorb beads was assessed using the opposite antiserum.

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The beads were incubated with the platelet-antiserum mixture (30 min at 37°C), and the unbanded ^{125}I-anti-IgG or ^{125}I-anti-albumin was measured by the addition of 100 µl of 10^4 IgG-beads or 3 × 10^4 albumin-beads. The beads were produced by covalently binding human IgG (Sigma Chemicals, St. Louis, MO) or human albumin (Cutter Laboratories, Calgary, Canada) to Sephasorb HP-ultrafine beads (Pharmacia Fine Chemicals) using carbonyl-di-imidazole.

The beads were incubated with the platelet-antiserum mixture (30 min at 37°C), and the beads were separated from the platelet fraction by centrifugation across a Ficoll-Hypaque gradient (6%: 10%). Less than 2% of the platelets crossed the barrier.

The concentration of platelets that inhibited 50% of the binding of the antiserum to the appropriate beads was related to the concentration of IgG or albumin standard that produced equal inhibition of binding. Platelet-associated IgG and albumin were also measured following solubilization of the platelets with 10 µl of 10% Triton X-100 (Sigma Chemicals). An equal amount of Triton X was added to each standard curve.

Total platelet protein was measured using the Bio-rad protein assay (BIO-RAD Laboratories, Richmond, CA). A standard curve was performed with each measurement.

Platelet IgG and albumin were also measured using the IRMA F(ab')2, fragments prepared from the same antiserum. The anti-IgG and anti-albumin were digested with pepsin (2%), dialyzed, and passed through a Sephadex S-200 column. The F(ab')2 were further purified by passage across Protein-A-Sepharose column. The F(ab')2 showed a single band on analytical polyacrylamide gel electrophoresis (5%) in sodium dodecyl sulphate (SDS).

RESULTS

The amount of platelet-associated IgG (PAIgG) on intact and solubilized platelets from healthy individuals was 1.4 ± 0.7 and 3.6 ± 1.2 fg IgG/platelet (mean ± SD), n = 25. The amount of albumin on intact and solubilized platelets from the healthy controls was 5.8 ± 1.8 and 11.8 ± 2.8 fg IgG/platelet, n = 25. The total platelet protein was 2.6 ± 0.6 pg protein/platelet, and the mean platelet volume was 8.1 ± 0.9 fl.

The amount of IgG on intact and solubilized platelets from the patients with ITP measured using the intact antiserum was 6.9 ± 6.6 (mean ± SD) and 16.0 ± 10.6, n = 21, and the amount of albumin was 14.6 ± 11.1 and 29.2 ± 20.9, respectively. For 15 of the 21 ITP patients, sufficient numbers of platelets could be recovered to measure total protein, and it was 3.9 ± 1.3 pg protein/platelet, and the mean platelet volume was 10.1 ± 3.2. The platelet IgG, albumin, total protein, and mean platelet volume were all significantly higher on platelets from the ITP patients compared to the control (p < 0.005, unpaired t test).

The serum albumin and IgG for all individuals was within the normal range.

There was no relationship between the IgG or albumin on intact or solubilized platelets and the total platelet protein (Fig. 1), r < 0.3 for all interactions. In contrast, there was a close relationship between the platelet-bound albumin and IgG measured either on the surface of intact platelets (r = 0.7) or after solubilization of platelets from patients with ITP (r = 0.7, n = 21). Those patients with ITP having elevated platelet-bound IgG also had elevated platelet-bound albumin (Fig. 2). This relationship was independent of the total platelet protein or median platelet volume.

To evaluate if the increased albumin and IgG associated with platelets from ITP patients was an artifact caused by the binding of the antiserum to the platelet Fc receptors, the binding of F(ab')2, fragments was measured. The values of platelet-bound IgG and albumin on solubilized platelets were virtually identical when measured using either intact or F(ab')2, antisera: for the controls, the IgG and albumin was 4.9 ± 1.8 and 11.0 ± 2.8 (mean ± SD), respectively, n = 6; for the patients, sufficient numbers of platelets could be recovered to measure total protein, and it was 3.9 ± 1.3 pg protein/platelet, and the mean platelet volume was 10.1 ± 3.2. The platelet IgG, albumin, total protein, and mean platelet volume were all significantly higher on platelets from the ITP patients compared to the control (p < 0.005, unpaired t test).

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Patients with idiopathic thrombocytopenic purpura (ITP) have larger than average platelets and increased levels of platelet-associated IgG, and it is possible that the amount of IgG per unit surface area is not increased. In this study, we correlated platelet-associated IgG measured on intact and solubilized normal and ITP platelets with the platelet volume and protein content. There was no correlation between the platelet-bound IgG measured and the total platelet protein or platelet size for either group.

Our demonstration that there was no correlation between platelet-bound IgG and total platelet protein is somewhat different from the results of another group of workers. These investigators reported that a subgroup of patients with immune thrombocytopenia demonstrated a relationship between platelet-bound IgG and total platelet protein. However, our current results are consistent with our previous studies using density-separated platelets from healthy controls or washed platelets from patients with ITP. It is pertinent, therefore, to comment on reasons for the apparent differences between the studies of Pfueller and coworkers and ourselves. These investigators performed an initial division of their patient results into two groups prior to correlation analysis. Group I was restricted to those patients in whom the ratio of PAIgG to platelet protein was within the range of the normal controls. Group II included any patients with a value above this ratio. Such an analytical procedure increases the likelihood of showing an apparently significant correlation in group I.

It is also important to note that patients with immune thrombocytopenia have an inverse relationship between the level of PAIgG and the platelet count. A similar relationship also exists between the platelet size and the severity of the thrombocytopenia. Thus, subgroup analysis of two variables that might indirectly be related to each other through a third mechanism will incorrectly suggest a causal relationship.

The observation that the increased platelet-bound IgG in patients with ITP was unrelated to platelet size or platelet protein content is consistent with the hypothesis that it represents "pathologic" antibody. Unexpectedly, we observed a linear relationship between platelet-IgG and platelet albumin on platelets from patients with ITP. Elevated levels of platelet-bound IgG were associated with increased platelet-bound albumin, and these proteins were increased on both normal and larger than normal sized ITP platelets measured by either total protein or mean platelet volume. To exclude any possible artifact caused by the binding of the radiolabeled antiserum to the platelet Fc receptor, F(ab')2 fragments were prepared from each antiserum. Identical results were obtained using either the intact or digested antibody.

There are several possible explanations of our results. The increased platelet-bound IgG and albumin could be passively related to an increased mass of platelet membrane in the washed platelet preparation. Albumin is a normal constituent of platelet membranes, and the increased platelet albumin associated with the ITP platelets might indicate that the platelet test samples contained increased amounts of membrane or platelet fragments that were not detectable by measurements of the platelet volume. Against this possibility was the observation that the platelet albumin was not correlated with total platelet protein. Had the increased platelet IgG or albumin been secondary to contaminating membranes or protein debris, one would have expected that those platelet samples having the highest amount of albumin would also have been associated with the largest amount of measurable protein. No such relationship existed.

Our results are also consistent with the possibility that the platelet membrane is abnormal in ITP, and consequently, there is increased uptake of plasma proteins. The current studies do not prove this latter hypothesis, since the increased platelet-bound albumin could either have represented platelet membrane albu-
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min or plasma albumin absorbed to altered membranes. However, each hypothesis indicates that at least some of the increased IgG on platelets from patients with ITP is not "pathologic." This conclusion is also indirectly supported by the observation that the relative proportion of each subclass of IgG on platelets from patients with ITP exactly parallels the subclass distribution in the plasma.

ACKNOWLEDGMENT

The authors thank G. Denomme and A. Santos for their technical assistance and Michele Bérubé for typing the manuscript.

REFERENCES

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