Platelet-associated IgG (PAIgG) is elevated in idiopathic thrombocytopenic purpura (ITP), but it also is elevated in other thrombocytopenic disorders traditionally considered to be nonimmune. Consequently, it is possible that elevated PAIgG is a nonspecific finding secondary to thrombocytopenia. To study this issue we developed a rabbit model of immune and nonimmune mediated thrombocytopenia. The mechanism of the thrombocytopenia was validated by platelet survival studies. Immune thrombocytopenia was produced by injection of antirabbit platelet serum that was raised in guinea pigs. Nonimmune aregenerative thrombocytopenia was produced by irradiation of the animals; nonimmune consumptive thrombocytopenia was produced by injection of adenosine diphosphate (ADP). PAIgG was measured in a direct binding assay using 125I-labeled staphylococcal protein A (SpA). Washed platelets from normal, nonthrombocytopenic rabbits bound an average of 81 molecules of SpA per platelet (81 ± 168, mean ± 2 SD, n = 39). Infusion of the antplatelet antiserum produced thrombocytopenia with a rise in PAIgG that was closely correlated with the level of PAIgG (r = 0.86, n = 12). The thrombocytopenia was consumptive, as shown by a very short platelet life span using 111In-labeled platelets. In contrast, both nonimmune thrombocytopenic states resulted in an equal or greater drop in the platelet count but no change in the level of PAIgG. The animals with aregenerative thrombocytopenia had normal or only moderately reduced platelet life spans; however, in every animal the level of PAIgG was not different from the nonthrombocytopenic controls, irrespective of the platelet count. Similarly, the level of PAIgG was unchanged in those rabbits with nonimmune consumptive thrombocytopenia following infusion of ADP (82 ± 55 molecules of SpA per platelet, mean ± 2 SD, n = 6). These studies indicate that elevated PAIgG is a specific finding of immune thrombocytopenia and is not secondary to thrombocytopenia itself. Indirectly, these results support our hypothesis that immune mechanisms contribute to more thrombocytopenic disorders than was once thought likely.

MATERIALS AND METHODS

Animal Studies

New Zealand white rabbits of either sex weighing 2 to 4 kg were used in all experiments. The animals were anesthetized with 30 mg/kg sodium pentobarbitol (MTC Pharmaceuticals, Hamilton, Ontario) administered intravenously (IV) through a marginal ear vein.

Preparation of Antiplatelet Serum

Antiserum against rabbit platelets was prepared in female guinea pigs. Whole blood from rabbits was collected into acid citrate dextrose (ACD, 6:1, vol:vol, pH 4.5), and the platelets were isolated by centrifugation, washed, and suspended in sterile saline. The guinea pigs were sensitized with an intraperitoneal (IP) injection of 107 platelets. Two further injections were given at one month intervals, and blood was collected, by cardiac puncture, at six days following the last injection. The serum was pooled and absorbed once (37°C for 60 minutes) with packed rabbit red cells (1:1, vol:vol) and once with washed rabbit lymphocytes. The serum was aliquoted and stored at −70°C until used.

The antiplatelet activity of the guinea pig serum was tested in vitro by two methods: enzyme-linked immunosorbent assay (ELISA) and radioimmunoprecipitation. For the ELISA, rabbit platelets were isolated, washed three times, and resuspended to a concentration of 60,000/μL in 0.15 mol/L phosphate-buffered saline (PBS), pH 7.4. Fifty microliters of the platelet suspension was added to Immulon II (Dynatech, Alexan-
DRY, VA) wells and centrifuged at 560 g for 13 minutes. The wells were incubated with a blocking buffer (0.2% gelatin, 0.1% sodium azide, 0.05% Tween, 0.15 mol/L PBS) overnight at 4°C and washed. Fifty microliters of test serum was added, and the wells were incubated at 37°C for one hour. After washing, 100 μL of staphylococcal protein A alkaline phosphatase conjugate (Boehringer Mannheim Canada Ltd, Dorval, Quebec) was added and the wells were incubated at 22°C for 45 minutes. After four washes, 200 μL P-nitrophenyl phosphate disodium (Sigma Chemical Co, St. Louis) at 1.5 mg/mL in diethanolamine buffer (Sigma) was added and after a 30-minute incubation at 37°C, the reaction was stopped by the addition of 50 μL of 1 N sodium hydroxide. The optical density (OD) of the solution was read at 405 nm using a Microplate Autoreader (Bio-tec Instruments, Burlington, VT). A result was defined as positive if the OD was greater than two times the background OD.

For the radioimmunoprecipitation assay, rabbit platelets were isolated from platelet-rich plasma (PRP) by centrifugation at 2,200 g for ten minutes and washed twice in 10 mmol/L Tris, 10 mmol/L EDTA, 150 mmol/L NaCl, pH 7.4. The platelets were resuspended at 10⁶ per mL in the same buffer and labeled with 1 μCi Na125I per mL using IOODO-GEN (Pierce Chemical Co, Rockford, IL). The labeled platelets were washed and then lysed in 1% Triton X-100 (Biorad, Mississauga, Ontario), 10 mmol/L Tris, 10 mmol/L EDTA, 150 mmol/L NaCl, pH 7.4 containing 100 μg/mL leupeptin (Sigma) and 0.1 mmol/L phenylmethylsulfonylfluoride (Sigma). Radiolabeled platelet lysate plus 50 μL of the guinea pig antplatelet serum was incubated at 4°C for two hours and then added to 50 μL of protein A Sepharose CL-4B beads (Pharmacia, Dorval, Quebec) for 30 minutes. The beads were washed in lysing buffer and the bound proteins eluted by boiling for three minutes in 60 mmol/L Tris-HCl, 2% sodium dodecyl sulphate (SDS), pH 6.8. The supernatant was added to the stacking gel of a 5% to 20% gradient SDS-polyacrylamide gel and run at 10 mA for 20 hours. The gel was stained with Coomassie blue and dried for autoradiography. Labeled proteins were detected with Kodak XAR-1 film after 24 hours of exposure.

Animal Models of Thrombocytopenia

Consumptive immune thrombocytopenia. Animals were anesthetized and infused with increasing amounts of antiplatelet serum through the marginal ear vein. At 15-minute intervals, whole blood was sampled through a carotid cannula into EDTA tubes (Becton Dickinson, Mississauga, Ontario), and platelet counts were performed. At 90 minutes blood was collected into acid-citrate-dextrose (ACD) for determination of PAIgG. Control animals were infused with either normal (nonimmunized) guinea pig serum (NGPS) or 0.9% sodium chloride. All platelet counts were performed on a model ZBI Coulter electronic counter (Coulter Electronics, Hialeah, FL).

Consumptive nonimmune thrombocytopenia. Consumptive thrombocytopenia was produced by the infusion of 100 mg/kg adenosine diphosphate (ADP) (Sigma) through the marginal ear vein. Blood for platelet counts and PAIgG determination was collected through a carotid cannula at ten minutes after infusion. This time was selected as preliminary studies demonstrated that the platelet nadir had occurred by ten minutes. Because the platelet nadir occurred within ten minutes, the effects of multiple ADP infusions were also tested. ADP (80 mg/kg) was infused once daily for four days, and the platelets were then tested for PAIgG following the last infusion.

Regenerative thrombocytopenia. Regenerative thrombocytopenia was produced by irradiation of the rabbits with a 137Cesium source (γ-radiation) for 30 minutes. This exposure to 930 rad produced thrombocytopenia at five to seven days. Blood was collected as previously described.

Measurement of platelet-associated IgG. Platelet-associated IgG was measured in a direct binding assay using staphylococcal protein A (SpA). SpA binds with the same relative affinity to both rabbit and guinea pig IgG. Whole blood was collected into ACD, the platelets were isolated, washed three times in 0.15 mol/L PBS, 0.013 mol/L EDTA, 0.2% bovine serum albumin (BSA), and the platelet count was determined. Duplicate samples of 100 μL of platelet suspension were incubated with 25 μL of increasing concentrations (0.125 μg/mL to 10 μg/mL) of 125I-labeled SpA (Amersham, Oakville, Ontario). After incubation for one hour at 22°C with agitation, the platelets were carefully layered onto silicon oil (Dow Corning, New Bedford, MA) of specific gravity 1.022 and centrifuged at 12,000 g for three minutes. The radioactivity of the platelet pellet was measured. To distinguish specific binding from nonspecific binding, the same binding study was repeated with a 50-times excess of unlabeled SpA (Sigma).

The number of molecules of SpA bound per platelet was calculated as follows: specific binding was defined as the saturable binding of 125I-SpA that could be displaced by the 50-times excess unlabeled SpA. The radioactivity at saturation was converted to molecules of SpA using the total number of platelets and the specific activity of the SpA.

Indium-labeled platelet survivals. One hundred milliliters of rabbit blood was collected into ACD. The platelet pellet was obtained by centrifugation of the PRP, and the pellet surface was gently washed five times with calcium-free Tyrode's buffer, pH 6.2. The platelets were resuspended in calcium-free Tyrode's buffer plus ethylene glycol tetra-acetic acid (EGTA) and labeled with 180 μCi 111Indium oxine (Amersham). The labeled platelets were washed twice and resuspended in 1 to 2 mL platelet-poor plasma. The radioactivity associated with platelets and any contaminating red blood cells was determined as follows: one hundred microliters of the final labeled suspension was added to each of 2 mL of 1% ammonium oxalate and to 2 mL of 0.9% sodium chloride, and the suspensions were centrifuged. The platelet-bound radioactivity was calculated from the ammonium oxalate pellet, and the total cell-bound radioactivity was determined from the sodium chloride pellet.

The labeled platelets were infused IV through the marginal ear vein of recipient rabbits, and blood samples (3 mL) were collected into ACD at 30, 60, and 120 minutes postinfusion and once daily for five to seven days. To estimate the platelet life span, the radioactivity of whole blood samples was measured and the data was analysed by computer best fit using gamma function analysis.

To perform platelet survivals in the immune thrombocytopenic animals, labeled platelets were infused as described, but at 15 hours postinfusion antiplatelet antiserum was injected, and blood samples were taken at 30, 60, 180, and 300 minutes after the serum injection.

Statistical analysis. The means of platelet counts in the animal models were compared using an unpaired Student’s t test at the 0.05 significance level.

RESULTS

Measurement of Platelet-Associated IgG on Rabbit Platelets

The percent recovery of platelets through the silicon oil was validated using 51Cr-labeled platelets. At the specific gravity of 1.022 used, greater than 95% of the platelets crossed the gradient. Over a platelet concentration of 60,000 to 1,000,000 platelets/μL, recovery was consistently greater than 95%.
Since ADP infusion may cause a decrease in the mean platelet density of circulating platelets, the passage of these platelets through oil was validated in the same way. Platelets from ADP-treated animals were labeled with $^{3}C$Chromium and passed through silicon oil (specific gravity, 1.022). Over a platelet concentration of 300,000 to 1,000,000 platelets/μL, recovery was equal to or greater than 99%.

Sizing experiments performed on ADP-treated platelets using a particle sizer and stained blood film showed them to be of normal size with no fragments.

Normal, nonthrombocytopenic rabbits bound $81 \pm 168$ molecules of SpA per platelet at saturation (mean ± 2 SD; n = 39 different rabbits). A representative binding curve is shown in Fig 1.

Platelet Survival Studies

The in vivo recovery of the radiolabeled platelets was defined as the percent radiolabeled platelets in the circulation at two hours postinfusion and was always greater than 75%. The mean platelet-associated radioactivity was 82%, and on average there was 4% red cell contamination. The average platelet life span of nonthrombocytopenic rabbits as determined by gamma function analysis was 68 ± 23 hours (mean ± SD; n = 15) (Table 1).

Consumptive Thrombocytopenia (Immune)

The antiplatelet activity of the serum was confirmed by the ELISA and the radioimmunoprecipitation assay. Normal (nonimmunized) guinea pig serum did not bind to rabbit platelets and gave a mean OD of 0.158 in the ELISA (n = 3). However, the immunized guinea pig serum reacted strongly with rabbit platelets, producing an OD of greater than 1.50 neat and an OD of 0.809 at a 1:128 dilution in buffer (n = 3).

The antisera was further tested using a radioimmunoprecipitation assay. The immune serum contained IgG anti-platelet antibodies that reacted with a large number of rabbit platelet glycoproteins as shown in Fig 2.

Platelet counts and PAIgG determinations were performed on sham-treated (0.9% sodium chloride) rabbits and rabbits injected with normal guinea pig serum. In these animals the mean post-treatment platelet count was not significantly different from the mean platelet count of normal rabbits, and the PAIgG was within the normal range at a mean of 45 molecules of SpA per platelet (range 9 to 136 molecules SpA per platelet, n = 12).

Infusion of the antiplatelet antiserum produced an immediate consumptive thrombocytopenia. The mean platelet count for these animals differed significantly from that of normal, nonthrombocytopenic animals ($P < 0.05$), (Fig 3B). In every rabbit given the antiserum antiserum, the level of PAIgG rose to a mean of 2,400 molecules SpA per platelet (range 815 to 4,330 molecules SpA per platelet, n = 12), (Fig 3A). The rise in PAIgG and the fall in the platelet count were both strongly correlated with the amount of antiserum administered. The severity of the thrombocytopenia correlated significantly with the level of PAIgG ($r = 0.86$, n = 12), as shown in Fig 4A. Similarly, the level of PAIgG
was directly correlated with the amount of antiserum injected \((r = 0.94, n = 12)\).

Platelet survival studies were performed on the antiserum-injected animals. Following infusion of the antplatelet antiserum, approximately 80% to 90% of the platelets were lost from the circulation within 30 minutes (Table 1).

**Consumptive Thrombocytopenia (Nonimmune)**

Infusion of ADP resulted in a significant fall in the mean platelet count \((P < 0.005)\) in comparison with nontreated animals (Fig 3B). However, in contrast to the animals with immune thrombocytopenia, all of the rabbits with nonimmune consumptive thrombocytopenia caused by ADP infusion had levels of PAIgG that were within the normal range at 82 ± 55 molecules of SpA per platelet (mean ± SD, \(n = 6\)). In these animals there was no correlation between the platelet count and the level of PAIgG (Fig 3A). In these animals there was no correlation between the platelet count and the level of PAIgG (Fig 3A). In these animals there was no correlation between the platelet count and the level of PAIgG (Fig 3A). In these animals there was no correlation between the platelet count and the level of PAIgG (Fig 3A).

**Aegenerative Thrombocytopenia**

Irradiation of the rabbits resulted in platelet counts that were significantly lower than the mean for normals \((P < 0.0005)\) (Fig 3B). In every one of these animals the PAIgG level remained within the normal range at 109 ± 84 molecules SpA per platelet (mean ± SD; \(n = 20\)), as shown in Fig 3A. In these animals there was no correlation between the platelet count and the level of PAIgG (Fig 4B).

The radiation-treated animals had a platelet life span that was normal or marginally reduced (Table 1).

**DISCUSSION**

Techniques for detecting IgG on platelets have been available for a number of years and have been used to investigate many different thrombocytopenic disorders. There is now general agreement that the level of platelet-associated IgG (PAIgG) is increased in about 80% to 90% of thrombocytopenic patients with idiopathic thrombocytopenic purpura. The recent demonstration that antplatelet antibodies in the serum of patients with ITP bind to platelet-specific antigens via the F(ab) terminus of the antibody molecule indicate that at least some of the IgG on the platelet surface represents true autoantibody. However, a number of studies from our laboratory have raised several issues that are as yet unresolved. For example, our demonstration that the increase of platelet-associated IgG is paralleled by an
increase in platelet-associated albumin suggests that much of the increased platelet-associated IgG is not true autoantibody. In addition, platelet-associated IgG is elevated not only in typical "immune" thrombocytopenic disorders but also in many disorders traditionally not considered to be caused by immune mechanisms. Taken together these observations have several possible interpretations: first, elevated levels of platelet-associated IgG might in part be due to artifacts of the test system, for example, contaminating platelet fragments. Recent studies suggest that this is an unlikely explanation. Another possible reason for the elevated levels of platelet-associated IgG in nonimmune thrombocytopenic disorders is that the thrombocytopenia by itself could lead to elevated levels of platelet-associated IgG (the opposite of what is currently postulated). The studies described in this report were designed to address this issue. We studied animal models of thrombocytopenia because it is difficult to study "pure" thrombocytopenic disorders in humans. Typically, patients with one type of thrombocytopenia, for example, aplastic anemia, have thrombocytopenia that is complicated by other conditions, such as sepsis, which makes it difficult to isolate a single cause for the thrombocytopenia.

To investigate the relationship between the platelet count and the level of PAIgG, thrombocytopenia was produced in rabbits using several different techniques. The general mechanism responsible for the thrombocytopenia was validated with 111In-labeled platelet survival studies. The life span of 111In-labeled platelets in normal, nonthrombocytopenic rabbits was 68 hours (gamma function analysis), a finding similar to that reported by others. Independently, the level of PAIgG was measured with a direct binding assay. The "positive control" was an animal model of immune thrombocytopenia. IgG antirabbit platelet antibody was raised in guinea pigs and was demonstrated by both ELISA and radioimmunoprecipitation (Fig 2) to react in vitro against a number of different glycoproteins. Following infusion of the antiserum into rabbits, a rapid destructive thrombocytopenia resulted, and greater than 80% of the 111In-labeled platelets were cleared from the circulation within 30 minutes after infusion. The fall in the platelet count was strongly correlated (r = 0.89) with the amount of serum injected. Thus we were able to conclude that the binding of the antibody to the rabbit platelets was responsible for the thrombocytopenia. The amount of PAIgG on the rabbit platelets was measured with a direct binding assay using radiolabeled staphylococcal protein A (SpA) as the ligand. Staphylococcal protein A binds equally well to both rabbit and guinea pig IgG. To measure PAIgG on washed rabbit platelets, the specific binding of the 125I-SPa to the washed platelets was measured in the standard fashion with subtraction of background nonspecific binding from total binding. The amount of PAIgG was expressed as molecules of staphylococcal protein A per platelet because the binding ratio of the SpA to platelet-bound IgG was not determined. There was a relatively wide range in the level of PAIgG in the control rabbits (81 ± 168 molecules of SpA per platelet [mean ± 2 SD, n = 39; range of 10 to 361]). The range is similar to the range of PAIgG in healthy humans using a similar assay. The large range may reflect the difficulty in precisely measuring very low levels of PAIgG plus the large sample size (n = 39).

The infusion of the antiplatelet antiserum into the rabbits resulted in a significant fall in platelet count (P < 0.05) that was inversely correlated with the rise in the level of PAIgG. The level of PAIgG increased from an average of 81 molecules of IgG per platelet (normal rabbits) to a mean of 2,400 molecules of SpA per platelet (Fig 3A). The level of PAIgG was significantly correlated with the amount of antiserum injected (r = 0.94) and more importantly, with the severity of the thrombocytopenia (r = 0.86) as shown in Fig 4A. Sham-treated animals and animals administered nonimmunized guinea pig serum did not have a change in the platelet count or level of PAIgG.

The level of PAIgG was also measured in the animals with nonimmune destructive and nonimmune regenerative thrombocytopenia. Nonimmune destructive thrombocytopenia was produced by the infusion of adenosine diphosphate (ADP) into rabbits. Other investigators have shown that ADP infusions result in a dramatic fall in the platelet count, presumably due to platelet sequestration within the lungs and microcirculation. When the rabbits were administered ADP, the platelet count immediately fell to similar levels caused by the antiplatelet antibody. Yet, when the level of PAIgG was measured on the platelets from these rabbits, it remained within normal limits (Fig 3A), and there was no correlation between the severity of the thrombocytopenia and the level of PAIgG (Fig 4B). To exclude the possibility that nonimmune thrombocytopenia of a longer duration might produce a rise in PAIgG, ADP was infused daily into rabbits, and the platelet-bound IgG was measured on day 4. The level of PAIgG remained within normal limits.

Aregenerative thrombocytopenia was produced by irradiation of the rabbits. This treatment results in destruction of stem cells and megakaryocytes, and the platelet count fell to an average of 50% of the pretreatment platelet count at five to eight days following irradiation. There was considerable variation in the life span of platelets in these animals ranging from normal (63 hours) to moderately shortened (11 hours) with the overall mean being 30 hours (Table 1). The reason for the shortened platelet life span in irradiated animals is uncertain but could be due to "fixed" platelet requirements, as has been postulated by one group of investigators. Alternatively, infection could have caused some degree of platelet destruction; however, this hypothesis is unlikely, as the animals appeared healthy and blood cultures in four animals were negative for pathogens. Regardless of the precise cause for the slightly shortened platelet life span in these animals, the severity of the thrombocytopenia was equal to or more severe than the thrombocytopenia in the animals given the antiplatelet serum, yet in no irradiated animal did the level of PAIgG rise.

In none of our studies were we able to demonstrate any correlation between the severity of the thrombocytopenia and the level of PAIgG except in those animals whose thrombocytopenia was caused by immune mechanisms (Fig 4). These studies indicate that thrombocytopenia by itself is no associated with elevation in the level of PAIgG. However,
it should be noted that we were only able to produce moderate thrombocytopenia in the animals, and it is possible that if the animals became extremely thrombocytopenic, slight elevations in the level of PAIgG might have been observed. Against this hypothesis is the demonstration that the fall in the platelet count in the nonimmune thrombocytopenic animals was equal to or greater than that achieved with the immune thrombocytopenic animals. Yet in contrast to the markedly elevated levels of PAIgG in the immune thrombocytopenic animals, there was absolutely no change in the level of PAIgG in the nonimmune thrombocytopenic animals. Looking for further evidence against the possibility that more severe thrombocytopenia might have "unmasked" a slight elevation in PAIgG, we carefully examined the level of PAIgG in those animals with the most severe nonimmune thrombocytopenia (Fig 3). In none of these animals was there even a trend toward increased levels of PAIgG.

In summary, these studies indicate that elevated levels of PAIgG are specific findings of immune thrombocytopenia and are not observed in animals with well-characterized nonimmune thrombocytopenia. Taken in conjunction with previous studies in humans, the current investigations support our hypothesis that immune mechanisms contribute to more thrombocytopenic disorders in humans than was once thought likely.

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Measurement of platelet-associated IgG in animal models of immune and nonimmune thrombocytopenia

J Arnott, P Horsewood and JG Kelton