Quinidine Purpura: Evidence That Glycoprotein V Is a Target Platelet Antigen
By Raphael B. Stricker and Marc A. Shuman

Quinidine-induced thrombocytopenia has been associated with both immune complex and autoantibody binding to platelets. In the present study, serum antibody from six of six patients with quinidine purpura was shown by immunoblotting to bind to a single platelet membrane protein of mol wt 80,000. This target protein was absent from Bernard-Soulier (BSS) platelets. F(ab)2 prepared from one patient's serum also bound to this protein, indicating autoantibody rather than immune complex binding to the target antigen. Antibody binding to the 80-kd protein was preserved after treatment of platelets with concentrations of trypsin or chymotrypsin that completely removed glycoprotein Ib (GPIb). Preincubation of platelet proteins with one patient's serum blocked binding of a polyclonal rabbit antibody against glycoprotein V (GPV), indicating that these antibodies recognize the same antigen. By wheat germ affinity chromatography, GPV was shown to copurify with GPIb. Quinidine-induced antibody bound to the wheat germ-purified GPV but not to GPIb. We conclude that quinidine purpura is associated with autoantibody directed against platelet GPV.

Quinidine PURPURA is an immune-mediated thrombocytopenia induced by quinidine or its metabolites. Although previous studies have postulated an immune complex mechanism of platelet destruction in this disease, more recent work indicates that autoantibody binding to platelets may be the causative mechanism. The target platelet antigen in quinidine purpura has been reported to be glycoprotein Ib (GPIb), based on immunoprecipitation studies in one patient and binding inhibition studies using alloantibody against GPIb to block platelet lysis by drug-dependent antibody in another patient. However, a direct relationship between GPIb and quinidine-dependent antibody has not been established.

We previously used immunoblotting with patient serum to define target platelet antigens in homosexual men with immune thrombocytopenic purpura and in a patient with acquired Bernard-Soulier syndrome (BSS). In the present study, we used immunoblotting of platelet proteins to determine the nature of target platelet antigen(s) in quinidine-induced thrombocytopenia.

MATERIALS AND METHODS

Serum samples and F(ab)2 preparation. Blood was obtained from six patients with quinidine-induced thrombocytopenia. All patients were taking quinidine sulfate, and all were thrombocytopenic at the time that blood was drawn. The platelet counts returned to normal in all six patients following discontinuation of the drug. Three of the patient samples had serum quinidine levels of 0.6, 0.7, and 1.7 mg/dL, whereas the other three samples had undetectable levels (<0.2 mg/dL) at the time that blood was drawn. Sera from three patients with thrombocytopenia due to phenytoin or trimethoprim/sulfamethoxazole and from six quinidine-treated patients with normal platelet counts were used as controls. The quinidine-treated controls had serum drug levels ranging from 0.9 to 5.1 mg/dL. Drug-induced thrombocytopenia was documented by an indirect platelet antibody assay showing binding of serum antibody to control platelets in the presence of the specific drug. Rabbit antisera against GPV and GPIb were generously provided by Dr David R. Phillips and Dr Joan Fox of the Gladstone Institute Foundation, San Francisco. F(ab)2 fragments were prepared as previously described from the staphylococcal protein A-purified IgG fraction of blood from one patient with quinidine-induced thrombocytopenia. The IgG was used at a concentration of 2 mg/mL and the F(ab)2 was used at a concentration of 3 mg/mL for immunoblotting.

Platelet and GPV preparation. Control and BSS platelets were isolated as described. The BSS platelets were generously provided by Dr Margaret Johnson of the Medical Center of Delaware, Newark. Trypsin-treated platelets were prepared according to the method of Phillips. Chymotrypsin-treated platelets were prepared by the method of Greenberg et al using 10 U/mL of a-chymotrypsin (Sigma, St Louis). GPV was partially purified and concentrated by a modification of the method of Berndt and Phillips; freshly prepared platelets (4 x 10^8/mL) were incubated with elution buffer (0.01 mol/L of Hepes, 1 mmol/L of EDTA, 0.3 mol/L of NaCl, pH 7.6) at 37°C for 14 hours. The resultant platelet eluate was dialyzed and concentrated overnight at 4°C against phosphate-buffered saline (PBS) containing 5 mmol/L of EDTA and 10% polyethylene glycol. The eluate was then suspended in PBS-EDTA at a protein concentration of 1.1 mg/mL. Protein measurements were made using a modification of the Lowry technique. The GPV preparation was then solubilized in 2% sodium dodecyl sulfate (SDS) sample buffer. It was subsequently shown to be free of GPIb by immunoblotting with rabbit anti-GPIb antibody (data not shown).

Wheat germ affinity chromatography was performed according to the method of Nachman et al. Normal platelets (4 x 10^8/mL) were solubilized in 1% SDS. The SDS was then diluted to 0.05% in a solution containing 5 mmol/L of EDTA and 1% (vol/vol) aprotinin, and the solubilized platelets were layered over a column of wheat germ coupled to agarose. Elution was performed using 0.2 mol/L of N-acetylglucosamine. The nonadherent fraction (WGA I) and adherent (N-acetylglucosamine-eluted fraction (WGA II) were dialyzed against PBS-EDTA -1% aprotinin and solubilized in 2% SDS at equivalent protein concentrations (3.5 mg/mL).

Immunoblotting. Fifty-microliter aliquots of platelet samples, partially purified GPV, WGA I, or WGA II, were electrophoresed in SDS-8% polyacrylamide gels or SDS-5% polyacrylamide gels under nonreducing conditions. The samples were transferred to nitrocellulose and blotted as previously described. Patient serum samples were diluted 1:100 in Tris-buffered saline (TBS) containing 1% gelatin. Rabbit serum was diluted 1:500 in the same buffer.

Blocking experiments were performed as follows: nitrocellulose strips were incubated with patient or control serum for one hour,
followed by washing in TBS and incubation with rabbit anti-GPV for two hours. Binding of rabbit antibody to GPV was detected with biotin-conjugated goat F(ab)\textsubscript{2}, anti-rabbit IgG (Tago, Burlingame, Calif) and avidin-peroxidase (Cappel, Malvern, Pa). The anti-rabbit antibody did not react with human IgG.

RESULTS

Figure 1 shows the results of immunoblotting using serum incubated with platelet proteins that have been transferred to nitrocellulose after electrophoresis in SDS-8% polyacrylamide gels. In contrast to control serum (lane A), antibody binding to a platelet protein with a mol wt of 80,000 was detected using patient serum (lane B). Identical results were obtained with all six sera from patients with quinidine-induced thrombocytopenia. Sera from the six quinidine-treated controls and from the three patients with other drug-induced thrombocytopenias showed no antibody binding to this antigen (data not shown). Binding did not occur when the patient's serum was incubated with BSS platelets (lane C), indicating absence of the target antigen on these platelets. Incubation of patient serum with partially purified GPV revealed binding to a protein of identical mol wt (lane D). F(ab)\textsubscript{2} (3 mg/mL) prepared from serum of the only patient tested also bound to this target protein (lane E). No antibody binding was seen in the region of GP IX (mol wt 22,000, not shown).

Figure 2 shows the results of immunoblotting with platelet proteins that have been transferred to nitrocellulose after electrophoresis in SDS-5% polyacrylamide gels. In lane A, patient serum again shows antibody binding to an 80-kd protein at the bottom of the gel. The band at 150 kD corresponds to IgG in the platelet preparation. This band is also detected when BSS platelets are incubated with patient serum, but the 80-kd band is not seen in these platelets (lane B). The 80-kd band corresponds to concentrated GPV, seen in lane C after incubation with patient serum. In lane D, incubation of platelet proteins with rabbit anti-GP\textsubscript{1b} yields a band at 170 kD. This band is not seen in lane A, indicating that the patient's antibody does not bind to GP\textsubscript{1b}.

Figure 3 shows binding and blocking experiments using rabbit anti-GPV antibody. In contrast to control rabbit serum (lane A), the anti-GPV antiserum bound to an 80-kd protein detected in control platelets (lane B) and in the partially purified GPV preparation (lane C). In the blocking experiment, control platelet protein (lane E) or purified GPV
(lane F) was first incubated with patient serum, followed by rabbit anti-GPV. Binding of the rabbit antiserum was abolished by preincubation with patient serum, indicating that both antibodies recognize the same binding site. Preincubation with either normal human serum or serum from a patient with phenytoin-induced thrombocytopenia failed to block binding of the rabbit antibody to GPV (data not shown).

Figure 4 shows binding of patient serum and rabbit anti-GPV to platelet proteins eluted from a wheat germ affinity column. The patient's serum and anti-GPV antibody bound to an 80-kd antigen in the WGA II (adherent) fraction (lanes B and D) but not in the WGA I (nonadherent) fraction (lanes A and C). The WGA II fraction also contained a 170-kd protein that reacted with rabbit anti-GP Ib (data not shown). Thus, GPV copurifies with GP Ib when both are subjected to wheat germ affinity chromatography. Binding of rabbit antibody to GPV was blocked by preincubation with patient serum (lane E), again showing that both antibodies bind to GPV. Preincubation with control human serum did not block rabbit antibody binding to GPV (data not shown). For comparison, binding of patient IgG (2 mg/mL) to GPV in platelets is shown in lane F.

Figure 5 shows the results of immunoblotting using trypsin-treated and chymotrypsin-treated platelets. In contrast to control platelets (lane A), rabbit anti-GP Ib antibody failed to bind to trypsin-treated platelets (lane B) or to chymotrypsin-treated platelets (lane C). However, patient serum still bound to GPV in both of these platelet preparations (lanes E and F), indicating that GPV is not removed by exposure to trypsin or chymotrypsin. The band at 150 kd in lanes D through F again corresponds to IgG in the platelet preparations. Thus, enzyme-treated platelets that are deficient in GP Ib retain the ability to bind anti-GPV antibody.

**DISCUSSION**

Although quinidine-induced thrombocytopenia is a well-described entity, the target platelet antigen and the mechanism of platelet destruction in this disease remain controversial. Previous reports have suggested that a drug-antibody immune complex binds to the platelet F(c) receptor, resulting in destruction of platelets as “innocent bystanders.” However, recent findings are inconsistent with the innocent bystander hypothesis. Kunicki et al. showed that binding of heat-aggregated human IgG to the F(c) receptor on platelets failed to inhibit binding of quinidine-dependent antibody. Conversely, formalin fixation of platelets under conditions that do not inhibit binding of immune complexes abolished binding of drug-induced antibody. Furthermore, Christie et al. demonstrated that anti-F(ab) antibody inhibited binding of quinidine-dependent antibody to platelets, suggesting the presence of autoantibody rather than immune complex binding to the target antigen. Similar observations were made by Smith et al. using a different technique.

Initial studies showed that BSS platelets failed to bind antibody from patients with quinidine purpura, suggesting that the binding site is GP Ib, known to be deficient in these platelets. In addition, Degos et al. demonstrated immunoprecipitation of radiolabeled GP Ib by an IgG alloantibody isolated from the serum of a BSS patient. This alloantibody blocked binding of quinidine-dependent antibody to normal platelets in the only patient studied, further implicating GP Ib as the antibody binding site. Because GP Ib was reported to be an immune complex receptor on platelets, it was postulated that the mechanism of platelet destruction in quinidine purpura involved binding of drug–antibody complex.
plex to this protein. However, when platelets were treated with concentrations of trypsin or chymotrypsin that completely removed GPIb, they retained 80% of the receptor activity for quinidine-dependent antibody. Based on this observation, Kunicki et al suggested that a membrane protein other than GPIb may be responsible for drug-induced antibody binding in quinidine purpura.

Our results suggest that GPV is a target platelet antigen recognized by quinine-associated antibody. GPV has three characteristics that have been attributed to the receptor for this antibody. First, the glycoprotein is missing from BSS platelets, explaining the absence of antibody binding to these platelets. Second, we have found that removal of GPIb using trypsin or chymotrypsin fails to remove GPV from normal platelets. These GPIb-deficient platelets retain the ability to bind quinidine-induced anti-GPV antibody. Third, our results using wheat germ affinity chromatography demonstrate for the first time that GPV copurifies with GPIb in the wheat germ-adherent fraction. Kunicki et al previously showed that 80% of antigenic activity for quinine-dependent antibody can be isolated in the wheat germ-adherent platelet membrane fraction. Our findings suggest that at least in some of the patients we have studied, this antigenic activity is due to the presence of GPV rather than GPIb in the adherent fraction. However, we have not excluded the possibility that quinine induces antibody formation against GPIb as well as GPV.

Previous studies have shown that quinine-dependent antibody binds to whole platelets or to Triton-solubilized platelet membranes only in the presence of quinine. Our results using platelet membranes fixed in nitrocellulose show that binding of the anti-GPV antibody occurs with serum, purified IgG, and F(ab)2 in the apparent absence of the drug. Addition of exogenous quinine (1 mmol/L final concentration) to the incubation mixture did not increase antibody binding to GPV on the nitrocellulose membranes. A possible explanation for antibody binding in the absence of drug is that platelet membrane fixation in nitrocellulose may render the target antigen more accessible to the antibody, even in the absence of quinine. An attractive hypothesis based on our findings is that quinine causes platelet destruction in two steps: (1) it induces autoantibody production against GPV, and (b) it directly interacts with the platelet membrane to make GPV more accessible to the antibody. Although antibody binding to intact platelets or to soluble membranes requires both steps, binding to membranes fixed in nitrocellulose does not require the presence of drug. This hypothesis is also consistent with the "neoantigen" model proposed by Christie et al. The putative neoantigen responds to GPV exposed by quinidine. Further testing of purified antibody and soluble GPV in the presence or absence of the drug may resolve this issue.

Another possibility is that quinidine purpura involves binding of both drug-dependent and non-drug-dependent antibodies to platelets, as suggested in two recent reports. Drug-dependent antibody binding may not be detectable by the immunoblot technique. Whether non-drug-dependent anti-GPV antibody plays a role in the pathogenesis of thrombocytopenia is unclear. We believe that this antibody is significant, however, since it was present in all six patients with quinidine purpura but in none of the six quinidine-treated patients with normal platelet counts. The relationship of the anti-GPV antibody to the development of thrombocytopenia must still be determined.

Our results are consistent with F(ab)2 binding to a target platelet antigen in at least one patient. How quinidine induces autoantibody formation against platelets must still be determined. We have found serum antibody against GPV in two patients with acute leukemia refractory de novo to platelet transfusions, as well as in two children with ITP (R.B. Stricker and M.A. Shuman, unpublished observations, 1985). In addition, Beardsley et al recently demonstrated antibodies against GPV in three children with varicella-associated thrombocytopenia. Thus, antibody production against GPV may result from an alteration in immune surveillance induced by a specific drug or underlying disease. This type of immune dysfunction has been invoked to explain the lupuslike syndrome associated with quinidine and other drugs. The mechanism of autoantibody induction by quinidine requires further study.

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Quinidine purpura: evidence that glycoprotein V is a target platelet antigen

RB Stricker and MA Shuman