Quinidine-Induced Thrombocytopenia and Leukopenia: Demonstration and Characterization of Distinct Antiplatelet and Antileukocyte Antibodies

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A patient with the rare syndrome of simultaneous quinidine-induced thrombocytopenia and leukopenia was studied. A quinidine-dependent antiplatelet antibody was detected in her serum by platelet aggregometry and by indirect platelet-suspension immunofluorescence. A drug-dependent antileukocyte antibody was demonstrated by leukoagglutination and by granulocyte immunofluorescence. Both antibodies were found to belong to the lgG class by immunofluorescence using monospecific antisera. There appeared to be two distinct antibodies, since the antibody eluted off sensitized platelets reacted only with platelets and that eluted off sensitized granulocytes reacted only with granulocytes. In addition, the patient’s serum and quinidine gave a negative reaction on immunofluorescence with platelets from a patient with Bernard-Soulier syndrome (BSS, which lack glycoprotein Ib [GPIb] complex, but gave a positive reaction with BSS granulocytes. The quinidine-dependent antiplatelet antibody immunoprecipitated GPIb and a glycoprotein of molecular weight 22,000 (GPIb complex) from Triton-solubilized, periodate-labeled platelets. Similar attempts to identify the granulocyte surface receptor/antigen were unsuccessful. Factor VIII/von Willebrand factor was not required for binding of the drug-dependent antibody to platelets.

THROMBOCYTOPENIA, leukopenia, and immune hemolytic anemia are recognized complications of quinidine therapy. Simultaneous occurrence of two or more of these drug-induced complications in the same patient is extremely uncommon.1,2 These complications are now known to be caused by clearance of opsonized cells mediated by drug-dependent antibodies. Two mechanisms have been proposed. Ackroyd suggested that the drug becomes bound to blood cells and the drug–cell-membrane complex acts as an antigen for antibody production.3 An alternative mechanism was postulated by Meischer, who suggested that the antibody reacts with the drug, and the drug–antibody complexes are adsorbed onto the cells, resulting in their destruction as “innocent bystanders.”4 Distinction between these two possibilities remains unclear. Kunicki et al.5,6 have demonstrated that Bernard-Soulier syndrome platelets fail to react with the drug-dependent antibody, suggesting that one or more of the absent membrane glycoproteins in this disorder (GPIb, GPV, and glycoproteins of molecular weight 100,000 and 22,000)7,8 may be the receptor that interacts with the drug-dependent antibody. Partial purification of this component using wheat germ agglutinin affinity chromatography revealed two major proteins of molecular weight 150,000 and 210,000 (GPIb and its “putative analogue”).9 The absence of receptor for drug-dependent antibody on Bernard-Soulier syndrome platelets, however, has been recently disputed.10 In drug-induced thrombocytopenia, Pfeufler et al.11 reported that a plasma factor (factor VIII/von Willebrand factor) was also requisite for formation of the immunogenic complex. In this article, we report a rare case of simultaneous occurrence of quinidine-induced thrombocytopenia and leukopenia. Evidence is presented that the quinidine-dependent antibodies are distinct and cell specific. The granulocyte membrane component recognized by the granulocyte-specific antibody was not identified. The drug-dependent antiplatelet antibody immunoprecipitated a complex consisting of glycoprotein Ib and a glycoprotein of molecular weight 22,000. In this patient, factor VIII/von Willebrand factor did not appear to be required for binding of the antibody to platelets.

MATERIALS AND METHODS

Materials

Bovine serum albumin (fraction V), quinidine sulfate, and leupeptin were purchased from Sigma, St. Louis, MO; sodium periodate (Univar) from Ajax Chemicals, Sydney, Australia; Pansorbin and diisopropylfluorophosphate (DFP) from Calbiochem, La Jolla, CA; Triton X-100 from BDH, Poole, England; 'H-sodium borohydride from Amersham, Sydney, Australia; DEAE-Affigel Blue from Bio-Rad, Richmond, CA; Ficoll-Paque from Pharmacia, Uppsala, Sweden. Fluorescein-labeled rabbit anti-human immunoglobulin and complement reagents (polyspecific anti-Ig, monospecific anti-IgG, anti-IgM and anti-C3) were obtained from Dako-immunoglobulins (a/s) Copenhagen, Denmark. An anti-PIII antiplatelet antibody was obtained from a patient with posttransfusion purpura. Factor VIII/von Willebrand factor was purified from fresh frozen plasma, essentially as previously described.12,13
Patient

A 49-yr-old woman was admitted with a history of vaginal bleeding for 6 wk, easy bruising, and recurrent epistaxis for 2 wk. She had been taking quinidine sulfate irregularly for 10 mo and regularly for 3 wk for supraventricular tachycardia. Physical examination revealed widespread purpura and bruises and mild hepatosplenomegaly. Her hemoglobin was 8.1 g/dl, white cell count was 600/µl with 18% neutrophils, 80% lymphocytes, and 2% monocytes, and her platelet count was 6,000/µl. Blood smear showed marked hypochromasia and microcytosis of red cells, but no spherocytes. Her reticulocyte count was 1.1%. A bone marrow aspirate revealed erythroid hyperplasia, myeloid hyperplasia with marked left shift, increased megakaryocytes, and absent iron stores. Direct and indirect (with quinidine) antiglobulin tests, performed according to Zeiger et al., were negative. Other studies, including serum bilirubin, serum haptoglobin, and urinary hemosiderin, gave normal results. Because of the patient’s clinical condition, she was transfused with 5 U of packed cells and 10 U of platelet concentrate before blood was taken for serum iron studies. At this stage, her serum iron was 8.4 µmole/liter (normal for female, 8.2–30 µmole/liter), total iron binding capacity 73.0 µmole/liter (normal, 48–67 µmole/liter) and percent saturation 11.5% (normal 15.7%–50.9%). Her quinidine was ceased and she was started on oral ferrous sulfate. Her hemoglobin, white cell and platelet count returned rapidly to normal. Her liver and spleen had decreased in size on her discharge a week later.

Platelet, Serum, and IgG Preparation

Blood was collected into either 5% EDTA or 3.18% sodium citrate (9 volumes blood to 1 volume of either anticoagulant). Platelet-rich plasma (PRP), platelet-poor plasma (PPP), and serum were prepared as previously described, except for PRP from a patient with Bernard-Soulier syndrome (BSS) which was prepared by centrifugation at 1,000 g for 10 min. IgG was purified from patient and normal serum by performing two 0%–40% ammonium sulfate fractionations, followed by chromatography on DEAE-Affigel Blue. The purified IgG was dialyzed against 0.01 M Tris, 0.15 M NaCl, pH 7.4.

Detection of Drug-Dependent Antiplatelet Antibody

Quinidine-dependent antiplatelet antibody was assayed by platelet aggregometry as described by Deykin and Hellerstein and by the indirect platelet suspension immunofluorescence test (PSIFT). PSIFT was performed as described by Borne et al., with minor modification. Platelets prepared from EDTA blood washed in EDTA-PBS (0.009 M EDTA, 0.0264 M Na2HPO4, 0.14 M NaCl, pH 7.0) were fixed in 3 ml of 1% (w/v) paraformaldehyde for 2 min at 20°C. After fixation, the platelets were washed twice and resuspended to a concentration of 4–8 × 107/ml in EDTA-PBS containing 0.2% (w/v) bovine serum albumin (BSA) with or without 1 mM quinidine sulfate. Serum (0.1 ml) and 0.1 ml of platelet suspension were mixed and equilibrated for 30 min at 37°C. The platelets were then washed 3 times with EDTA-PBS and mixed with 0.1 ml of optimally diluted fluorescein-isothiocyanate (FITC) tagged monospecific or polyspecific rabbit anti-human immunoglobulin or anti-complement reagents for 30 min at 20°C. After 2 more washings with EDTA-PBS, the platelets were resuspended in glycerol-PBS (3:1, v/v), mounted on a slide, covered, and examined under the fluorescence microscope. Normal AB serum served as a negative control. Serum from a patient with an anti-PIII antibody was used as a positive control. Fluorescence was scored between +...+ and ++++ where normal AB sera was always negative and the immunofluorescence with the anti-PIII antibody always gave +...+ or ++++.

Detection of Drug-Dependent Antileukocyte Antibody

Quinidine-dependent antileukocyte antibody was assayed by drug-related leukoagglutination according to Eisner et al. and by the indirect granulocyte immunofluorescence test (GIFT). GIFT was performed with minor modification after the method described by Verheugt et al. Granulocytes were prepared from EDTA blood by dextran sedimentation of red cells, followed by Ficoll-Paque gradient centrifugation of the leukocyte-rich supernatant. The granulocytes were washed twice in phosphate-buffered saline (PBS), fixed for 5 min at 20°C with 1% (w/v) paraformaldehyde in PBS, and washed twice in PBS-BSA (0.2% w/v in BSA). The granulocytes were resuspended in PBS-BSA with and without quinidine (1 mM) to a concentration of 107/ml. Serum (0.1 ml) and 0.1 ml of granulocyte suspension were mixed and equilibrated for 30 min at 37°C. After 3 washings with PBS-BSA, the granulocytes were mixed with 0.1 ml of optimally diluted FITC-labeled monospecific or polyspecific antiimmunoglobulin or anti-complement reagents for 30 min at 20°C. After 2 more washings, the granulocytes were suspended in glycerol-PBS (3:1, v/v) and examined by fluorescence microscopy. Normal AB serum was used as the negative control. Fluorescence was scored as described above.

In experiments in which complement bound to platelets and granulocytes was studied, 0.1 ml fresh normal AB serum was added to the incubation mixture of test serum and platelet/granulocyte suspension as a source of complement. Platelets were washed and suspended in PBS instead of EDTA-PBS.

In some experiments, BSS platelets or granulocytes were used instead of normal granulocytes. The identity of the cells was always determined by phase-contrast microscopy before fluorescence of the cells was evaluated.

Preparation of Platelet and Granulocyte Eluates

Platelet and granulocyte eluates were prepared by mixing 1 ml of normal platelets (6 × 109/ml) suspended in EDTA-PBS (1 mM in quinidine) or 1 ml normal granulocytes (107/ml) suspended in PBS (1 mM in quinidine) with either 1 ml of patient serum or normal AB serum and equilibrating at 37°C for 45 min. After 3 washings with PBS or PBS-EDTA, the adsorbed antibodies were eluted off the sensitized platelets or granulocytes using the ether elution method of Rubin, as modified by Borne et al. Platelet and granulocyte eluates were then tested for antibody specificity using indirect immunofluorescence with normal platelets and granulocytes.

Requirement of Factor VIII/von Willebrand Factor (FVIII/vWF) for Antibody Binding to Platelets

To investigate if FVIII/vWF was required for binding of the quinidine-dependent antibody to platelets, the indirect immunofluorescence tests were repeated using washed paraformaldehyde-fixed platelets from a normal individual and a patient with severe von Willebrand’s disease, patient or normal purified IgG, and quinidine (1 mM), with or without purified FVIII/vWF (10 µg/ml). The severe von Willebrand disease patient had <2% of the normal levels of plasma and platelet FVIII/vWF as determined by radioimmunossay.

Immune precipitation

Immune precipitation was performed to determine the identity of the platelet and granulocyte membrane components involved in the
formation of the antigenic complex. Washed platelets were surface labeled by the periodate-¹H-sodium borohydride procedure, as previously described, except that the last washing buffer was made 200 µg/ml in leupeptin to block proteolysis due to endogenous calcium-dependent protease. Granulocytes (3 x 10⁷/ml) were also periodate-labeled using the same procedure. After the final centrifugation, the labeled platelets were resuspended to 10⁹/ml in 0.01 M HEPES, 0.15 M sodium chloride, 0.005 M EDTA, pH 7.4. The suspension was made 200 µg/ml in leupeptin and 0.5 mM in DFP and solubilized with 1% (v/v) Triton X-100 (final concentration). The solubilized platelets were centrifuged at 8,370 g for 5 min in a Beckman microfuge (Beckman Instruments, Inc., Spinc Division, Palo Alto, CA) to remove the insoluble platelet cytoskeletons. The granulocyte lysate was similarly prepared except that the final Triton X-100 concentration was 2% (v/v). To 200 µl of the solubilized platelets or granulocytes was added 50 µl of either patient’s serum or patient’s purified IgG (6.5 mg/ml), followed by 65 µl of saline or quinidine in saline (0.4 mM, final concentration), and finally 10 µl of buffer or purified factor VIII/von Willebrand factor (7 µg). After 2 hr at 4°C, 1 ml of a 1.5% suspension of fixed protein-A-coated S. aureus cells in 0.01 M sodium phosphate [0.15 M in sodium chloride, 100 µg/ml in leupeptin, 0.5 mM in DFP, 1 mM in EDTA, 1% (v/v) in Triton X-100], pH 7.4, was added and the mixture equilibrated a further 30 min at 4°C. The bacteria were isolated by centrifugation at 1,500 g for 15 min at room temperature, washed twice in 1 ml of the above buffer containing 2% (w/v) BSA and once in the same buffer diluted 1:10 with 0.01 M sodium phosphate, 0.001 M EDTA, pH 7.4. The pellet was suspended in 2% (w/v) SDS, with or without 5% (v/v) 2-mercaptoethanol, heated for 10 min at 100°C to solubilize bound antibody–antigen complex, and centrifuged at 1,500 g for 15 min. Protein was electrophoresed through slab gels according to the method of Laemmli, using a 5%–15% exponential gradient of acrylamide in the resolving gel and 3% acrylamide in the stacking gel. Protein was stained with Coomassie brilliant blue as previously described. The tritium distribution in the dried gels was visualized by fluorography according to the method of Bonner and Laskey.

RESULTS

Detection of Quinidine-Dependent Antiplatelet and Antileukocyte Antibodies

Patient serum and quinidine gave a positive reaction (+++++) with normal platelets and granulocytes. Controls using patient serum alone or normal serum with the drug were negative. Platelet aggregation and leukoagglutination were observed when patient serum and quinidine were incubated with normal PRP and leukocyte suspension, respectively (data not shown). However, patient serum alone or normal serum and quinidine had no effect on either normal PRP or the leukocyte suspension.

Immunohemical Characteristics of the Antibodies

The immunochemical characteristics of the quinidine-dependent antiplatelet and antileukocyte antibodies were studied by performing indirect 

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Fig. 1. Indirect immunofluorescence tests on (A) normal platelets, (B) Bernard-Soulier syndrome platelets, (C) normal granulocytes, and (D) Bernard-Soulier syndrome granulocytes, with patient serum and quinidine (1 mM).
immunofluorescence on normal platelets and granulocytes using patient serum, quinidine, and monospecific anti-human immunoglobulin and complement reagents. There was a positive reaction with polyvalent Ig and with monospecific anti-IgG; IgM was not detected. C3 was present on platelets, but was not detectable on granulocytes.

**Specificity of Platelet and Granulocyte Antibodies**

To investigate whether the quinidine-dependent antiplatelet and antileukocyte antibodies were identical or cell specific, indirect immunofluorescence was performed on normal platelets and granulocytes using antibody eluted off either platelets or granulocytes. In the presence of quinidine, the platelet eluate reacted positively \((++++)\) only with platelets, and the granulocyte eluate reacted positively \((++++)\) only with granulocytes. With normal serum, both eluates gave negative reactions with platelets and granulocytes. This result strongly suggests that there are two distinct antibodies with separate cell specificity. To confirm that there are two separate antibodies, indirect immunofluorescence was performed using Bernard-Soulier syndrome (BSS) platelets and granulocytes, since BSS platelets have been reported to be nonreactive with quinidine-dependent antibodies.\(^5^,^6^,^26^,^27\) With patient serum and quinidine, indirect immunofluorescence with BSS platelets was negative, but BSS granulocytes, normal platelets, and granulocytes were positive (Fig. 1). BSS platelets gave a positive reaction on indirect immunofluorescence with serum containing anti-PI\(^{\text{A1}}\) antibody (not shown).

**Identification of the Platelet Membrane Glycoprotein Interacting With the Quinidine-Dependent Platelet Antibody**

Both patient serum and purified patient immunoglobulin gave quinidine-dependent immunoprecipitation of a glycoprotein with the molecular weight characteristics of glycoprotein Ib (Fig. 2, most intensely labeled glycoprotein with periodate-labeling, nonreduced mol wt = 170,000, reduced mol wt = 145,000).\(^28\) Prolonged fluorography of the reduced gel gave a faint band with mol wt = 25,000, consistent with the \(\beta\)-subunit of glycoprotein Ib, and a faint band of molecular weight 22,000, which was also evident on prolonged fluorography of the nonreduced gel. Control experiments with normal serum plus or minus quinidine were negative. Glycoprotein Ib and the glycoprotein of molecular weight 22,000 are also both immunoprecipitated by monoclonal antibodies against glycoprotein Ib.\(^8^,^29\) The amount of glycoprotein Ib immunoprecipitated by the patient drug-dependent antibody was independent of exogenously added factor VIII/von Willebrand factor (FVIII/vWF) (lane 7 versus lane 6). The platelet lysate would, however, have contained \(\approx 0.3\) U/ml of endogenous platelet FVIII/vWF.\(^30\) Similar attempts to identify the granulocyte membrane component that interacts with the drug-dependent antibody were negative under all conditions examined.

**Requirement of Factor VIII/von Willebrand Factor (FVIII/vWF) for Antibody Binding to Platelets**

To investigate the requirement of FVIII/vWF for the binding of the quinidine-dependent antibody to platelets.
The occurrence of simultaneous thrombocytopenia and leukopenia secondary to quinidine is very rare and has not been well documented. Although our patient developed pancytopenia while receiving quinidine, her anemia was found, upon laboratory investigation, to be due to iron deficiency and not drug-induced hemolytic anemia. Her thrombocytopenia and leukopenia were, however, quinidine-induced. A quinidine-dependent antiplatelet antibody was demonstrated by platelet aggregometry and by indirect platelet immunofluorescence, and a drug-dependent antileukocyte antibody was found by leukoagglutination and indirect granulocyte immunofluorescence. There are two possible mechanisms for the simultaneous occurrence of quinidine-induced thrombocytopenia and leukopenia. One possibility is that there is an antibody that cross-reacts with both platelets and leukocytes. An alternative mechanism is that there are two antibodies with separate cell specificity. Although we found that both the antiplatelet and the antileukocyte antibodies were IgG class, they appeared to be distinct. The antibody eluted off sensitized platelets reacted with platelets and not with granulocytes, and the eluted granulocyte antibody similarly reacted only with granulocytes. Furthermore, in agreement with previous studies, the drug-dependent antibody did not react with Bernard-Soulier syndrome (BSS) platelets, but gave a positive reaction with normal platelets and normal and BSS granulocytes.

BSS platelets are deficient in several membrane glycoproteins (GPIb, GPV, and glycoproteins of molecular weight 100,000 and 22,000), and any of these glycoproteins could conceivably be that recognized by the quinidine-dependent platelet antibody. Kunicki et al. have recently isolated a mixture of two glycoproteins with molecular weights of 210,000 and 150,000 (possibly GPIb) purified from Triton X-100 solubilized platelet membranes by wheat germ agglutinin affinity chromatography. These two proteins inhibited the quinine- and quinidine-dependent antibody-induced release of $^{51}$Cr from labeled platelets, suggesting that one or both of these glycoproteins may be the membrane component that is recognized by the drug-dependent antibody. Our results using an immunoprecipitation technique considerably extend these observations. Using Triton X-100 solubilized, peridate-labeled platelets, both patient sera and purified IgG gave quinidine-dependent immunoprecipitation of a glycoprotein with the molecular weight characteristics of GPIb (mol wt = 170,000 nonreduced; mol wt = 145,000 and 25,000, reduced; most intensely labeled band by the sialic-acid-specific periodate labeling technique). Prolonged exposures of the fluorograph revealed an additional glycoprotein band of molecular weight 22,000 (termed GPIX). Recent evidence from several laboratories suggests that GPIb and GPIX are tightly complexed in the platelet membrane, since both glycoproteins are genetically absent in BSS and both are coimmunoprecipitated by antiglycoprotein Ibi monoclonal antibodies. The present data suggest that the patient antibody also recognizes this same glycoprotein complex. Our attempts to similarly identify the granulocyte component that reacted with the patient antibody were unsuccessful. Nevertheless, the available evidence suggests that the granulocyte component is distinct from GPIb. First, the patient antibody recognized Bernard-Soulier granulocytes but not Bernard-Soulier platelets. Second, both anti-GPIb monoclones, AN 51 and FMC 25 (M. C. Berndt and B. H. Chong, unpublished observation), are negative by immunofluorescence against leukocytes and granulocytes, respectively.

In the immunoprecipitation experiment, the amount of glycoprotein Ibi "complex" precipitated by the quinidine-dependent antibody was independent of added factor VIII/von Willebrand factor (FVIII/vWF) (Fig. 2). Since the platelet lysate contained significant levels of intraplatelet-derived FVIII/vWF, no definitive conclusion could be made from this experiment on the requirement of FVIII/vWF for antibody–platelet recognition. However, the results of indirect immunofluorescence with washed, fixed von Willebrand platelets, patient purified IgG, and quinidine exclude the involvement of FVIII/vWF in the antigenic complex, since a positive reaction was obtained regardless of the presence of FVIII/vWF. This finding is in agreement with Christie and Aster, who found that platelet binding of drug-dependent antibody was independent of FVIII/vWF. Pfuel et al. reported that FVIII/vWF was requisite for drug-dependent antibody-induced platelet release, aggregation, and platelet factor 3 availability, as well as for the interaction between platelet and drug that produces an antigen able to transform sensitized patient lymphocytes. It is interesting to speculate that, although FVIII/vWF may not be required for the initial antibody–platelet binding, the quinidine-dependent antibody/glycopro-
tein Ib complex may allow binding of FVIII/vWF. There is good evidence that glycoprotein Ib is the von Willebrand factor receptor for ristocetin-induced platelet agglutination and for platelet adhesion to exposed vascular subendothelium. Resolution of the precise mechanism of reaction of quinidine-dependent antibodies with platelets awaits the results of current studies with the purified glycoprotein Ib complex."

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REFERENCES

Quinidine-induced thrombocytopenia and leukopenia: demonstration and characterization of distinct antiplatelet and antileukocyte antibodies

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