Characteristics of Quinine- and Quinidine-Induced Antibodies Specific for Platelet Glycoproteins IIb and IIIa

By Gian P. Visentin, Peter J. Newman, and Richard H. Aster

Drug-induced, immunologic thrombocytopenia (DITP) is a severe, sometimes life-threatening disorder affecting patients who are sensitive to certain medications. More than 100 drugs have been implicated in its pathogenesis. Quinidine and quinine appear to cause the condition most often, with the probable exception of heparin, which may act by a different mechanism. The antibodies responsible for platelet destruction in DITP appear to be tissue-specific and are unusual in that, in general, they bind to platelets only in the presence of drug. It was formerly thought that this binding occurred in the form of immune complexes produced by the interaction of drug and antibody in solution; recent studies have shown that binding of drug-dependent antibodies (DDAb) to platelets is Fab-mediated. On the basis of these findings and other observations, we hypothesized that drug binds noncovalently to platelet membrane constituents to produce reversible conformational changes for which DDAb are specific. Early studies suggested that DDAb induced by quinine and quinidine react primarily with platelet membrane glycoprotein (GP) GP Ib or the GP Ib/IIIa complex. Recent work in our laboratory and others, using newer methods for detecting Ig-platelet interaction, have shown that certain DDAb are specific for the GP Ib/IIIa complex.

The relative frequency with which DDAb recognize GP Ib/IIIa versus GP IIb/IIIa has not been well defined. Whether binding of DDAb to multiple targets is mediated by more than one Ig has not been determined, and little is known about the epitopes on platelet GPs recognized by these antibodies. We have performed a series of studies to address these questions, with emphasis on the characterization of DDAb reactive with GP IIb/IIIa.

MATERIALS AND METHODS

Reagents. Quinine monohydrochloride and quinine sulfate were purchased from Aldrich Chemicals Company, Inc (Milwaukee, WI); sodium dodecyl sulfate (SDS), acrylamide, N,N'-methylene-bisacrylamide and alkaline phosphatase (BCIP/NBT) color development solution from Bio-Rad Laboratories (Richmond, CA); affinity-purified alkaline phosphatase goat antimouse IgG (y-chain specific), antihuman IgG (y-chain specific), antirabbit IgG (H + L-chain specific), and p-nitrophenyl phosphate (PNPP) from Zymed Laboratories, Inc (South San Francisco, CA); chymotrypsin A (from bovine pancreas) and endo-β-N-acetylglucosaminidase H (endo-H; from a recombinant Escherichia coli strain) from Boeringer Mannheim; Na3PO4 from Amersham; Sephadex G25 and Sepharyl S-300 from Pharmacia; Protein A-Sepharose CL-4B, 3-cyclohexylamino-1-propanesulfonic acid (CAPS), bovine serum albumin fraction V, Nonidet P-40, phenylmethylsulfonyl fluoride (PMSF), leupeptin, and aprotinin from Sigma Chemical Co (St Louis, MO); Immobilon-P (PVDF Transfer Membrane) from Millipore Co (Bedford, MA); fetal bovine serum from HyClone Laboratories Inc (Logan, UT). All other chemicals were reagent grade.

Antibodies. Murine monoclonal antibodies (MoAbs) specific for human GP IIb/IIIa were obtained from the following sources: 10E5 and AP-2, reactive with the GP IIb/IIIa complex from Drs Barry Coller, SUNY at Stony Brook, NY, and Thomas Kunicki of our institution, respectively; Fab, specific for GPIb, from Dr Rodger McEver, University of Texas Health Science Center, San Antonio, TX; AP1, specific for GPIIa, from Dr Robert Montgomery, Milwaukee, WI; and AP3 specific for GPIIIa from Dr Kunicki.

AP3, produced by one of the authors (P.J.N.), is specific for GPIIb/IIIa complex.
GPIIIa. All of the monoclonals used are mouse IgG1 subclass except for 10E5 (IgG2a) and Tab (IgG2b).

Polyclonal rabbit antibodies specific for GPIIb and GPIIIa were produced by immunization of rabbits with unreduced and reduced, purified GPIIb or GPIIIa electrosed from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. A human alloantibody (Kro) reactive with the platelet alloantigen PIIb was obtained from a patient during the acute phase of posttransfusion purpura. Thirteen drug-dependent, platelet-reactive antisera were obtained from patients during the acute phase of DITP. Five of these (WAG, GLE, RAD, MUR, STA) were induced by quinidine, and eight (SHE, DUN, WJL, SCH, NAR, ADS, BAR, SZA) were induced by quinidine.

**Purification and radiolabeling of GPIIb/IIa.** The GPIIb/IIa complex was purified as described by Fitzgerald et al. Platelets were solubilized in 1% Triton X-100 containing 10 mM leupeptin and 1 mM N-ethylmaleimide, and eluted with 100 mM L-a-methyl-D-mannoside. The eluted material was absorbed on heparin Sepharose, and the flow-through was posttransfusion purpura. Thirteen drug-dependent, platelet- and 1 mM CaCl2, absorbed on Concanavalin A-Sepharose, and eluted with 100 mM L-a-methyl-D-mannoside. The eluted material was absorbed on heparin Sepharose, and the flow-through was posttransfusion purpura. Thirteen drug-dependent, platelet-reactive antisera were obtained from patients during the acute phase of DITP. Five of these (WAG, GLE, RAD, MUR, STA) were induced by quinidine, and eight (SHE, DUN, WJL, SCH, NAR, ADS, BAR, SZA) were induced by quinidine.

**Enzymatic deglycosylation of GPIIb/IIa.** Enzymatic deglycosylation of GPIIb/IIa was performed according to Newman et al. by adding 150,000 units of endo-β-N-acetylglucosaminidase (endo-H) digest of pure GPIIb/IIa in 0.1 mM phosphate-buffered saline (PBS), pH 5.9 and SDS 0.09%, and incubating the reaction mixture for 18 hours at 37°C. To prevent proteolysis, PMSF, leupeptin, and aprotinin were added to the reaction mixture at final concentrations of 0.4, 4, and 15 mM, respectively, immediately before endo-H.

**Immunoprecipitation.** The labeled GPIIb/IIa, untreated or digested with chymotrypsin, was diluted to a protein concentration of 0.1 mg/mL in phosphate-buffered saline (PBS), pH 7.4 and incubated in CAPS (pH 7.4) buffer, pH 7.4 (TBST) with 0.5 mM CaCl2, and 15 mM MgCl2, respectively, immediately before endo-H.

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tions using 1.5 mg IgG for 1.0 mL of beads. The washed beads were stored in Tris-glycine buffer, pH 8.8 until used. Platelets were isolated from blood collected in acidified citrate-dextrose anticoagulant and were washed in Ringer's-citrate-dextrose buffer, pH 6.5, containing prostaglandin E, (PGE) 20 ng/mL. A suspension of 5 x 10⁹ platelets was solubilized in Tris-glycine buffer, pH 8.8 containing 1.0% Triton X-100 and 10 µg/mL leupeptin. One hundred microliters of beads coated with AP1 or AP2 were incubated with 0.5 mL of platelet lysate overnight at 4°C with agitation. The beads were then washed twice in Tris-glycine buffer containing 0.1% Triton X-100 and used for absorption. Plasma samples containing DDAb were diluted to a point at which they still gave strong reactions against GPIb/IX and GPIIb/IIIa in MACE. Four hundred microliters of the diluted plasma was incubated with 100 µL of coated beads in presence of 1.0 mmol/L drug, incubated for 2 hours, and pelleted. Following dialysis to remove residual drug, reactions of the absorbed sera with GPIb/IX and GPIIb/IIIa were measured by MACE.

RESULTS

Sera from most patients with DITP induced by quinidine or quinine contain antibodies that recognize GPIb/IX and GPIIb/IIIa. Reactions of 10 sera with immobilized GPIb/IX and GPIIb/IIIa using ACE are summarized in Fig 1. Seven reacted with both GPIb/IX and GPIIb/IIIa to varying degrees. Two of the sera, one induced by quinidine and one by quinine, gave detectable reactions only with GPIb/IX and one, induced by quinine, reacted only with GPIIb/IIIa. Three other sera (SZA, MUR, STA) were found to react with both GPIb/IX and GPIIb/IIIa by MACE (not shown).

Reactions of DITP sera with GPIb/IX and GPIIb/IIIa are mediated by at least two different antibodies. Five sera reactive with both GPIb/IX and GPIIb/IIIa (WAG, GLE, SZA, MUR, STA) were absorbed in the presence and absence of drug with Sepharose beads coated with GPIb/IX and GPIIb/IIIa. As shown in Fig 2 for serum WAG, absorption with GPIb/IX-coated beads in the presence of drug removed all Ig specific for GPIb/IX but not that specific for GPIIb/IIIa. Conversely, serum absorbed with GPIIb/IIIa-coated beads reacted only with the GPIb/IX complex. No activity was lost when serum was absorbed with GP-coated beads in the absence of drug. Similar results were obtained with the other four sera (not shown).

DDAb specific for GPIIb/IIIa recognize multiple epitopes. Each of the eight GPIIb/IIIa-specific DDAb identified by ACE (Fig 1) also reacted in the presence, but not in the absence, of drug with purified GPIIb/IIIa complex fixed directly to microtiter wells without the aid of MoAb. When the GPIIb/IIIa was dissociated by incubation with EDTA at 37°C before being immobilized, binding of WJL, KEM, and GLE was completely lost, and binding of WAG was reduced in strength by about 40% (Table 1). The remaining sera (NAR, BAR, SCH, and ADS) reacted as well with dissociated GPIIb/IIIa as with the intact complex.

Binding of the eight drug-dependent antisera to GPIIb/
the presence (second two bars), but not in the absence, of drug (first two bars). Following absorption with Sepharose 4B coated with GP complex (not shown). The reverse pattern was seen following absorption with GP-coated Sepharose (fourth pair of bars). Absorbed serum failed to react in the absence of drug with either GP complex (not shown). The same patterns were obtained with four other sera (GLE, GPlb/lX, the serum gave drug-dependent reactions against GPllb/llla (third pair of bars). WAG was almost completely inhibited by 10E5, specific for GPIIb/IIIa was inhibited to varying degrees by pretreating the immobilized GP with selected MoAbs (Table 1). Serum WAG was almost completely inhibited by 10E5, specific for the GPIIb/IIIa complex, but not by a different GPIIb/IIIa-specific monoclonal, AP2. NAR was completely blocked by the GPIIb-specific monoclonal Tab, but reactions of the other sera were unaffected by Tab. Sera BAR, SCH, and ADS were markedly inhibited by monoclonal AP3, specific for GPIIIa, and WAG was partially inhibited by this antibody. The same four sera were inhibited by a polyclonal, GPIIIa-specific rabbit antibody.

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On the basis of these findings, the eight sera were grouped into four classes (Table 1). Sera WJL, KEM, and GLE (class 1) appear to recognize only the intact GPIIb/IIIa complex. Serum NAR (class 2) appears to be specific for GPIIb because it was inhibited by Tab and reacted with dissociated GP. Sera BAR, SCH, and ADS (class 3) appear to be specific for GPIIIa because they were inhibited by both the anti-GPIIIa MoAb AP3 and a rabbit polyclonal anti-GPIIIa antibody. Consistent with this classification, class 3 DDAb (BAR and SCH) gave immunoprecipitation reactions similar to those of ADS (not shown).

To localize the epitopes recognized by class 3 DDAb more precisely, they were incubated with a chymotryptic digest of radiolabeled GPIIb/IIIa. As shown in Fig 5, each DDAb precipitated a major band of about 61 Kd and minor bands of molecular weights (MW) of about 43 Kd and 32 Kd in the presence, but not in the absence, of drug. The 32-Kd band was present as a minor contaminant of the untreated GPIIb/IIIa preparation and appears not to be a product of chymotrypsin digestion. The GPIIIa-specific MoAb AP3 bound primarily to the 61-Kd and 32-Kd bands of chymotryptic fragments of GPIIIa. Table 1 shows the percentage of optical density (OD) obtained with untreated GPIIb/IIIa complex.

Table 1. Classification of Drug-Dependent Antisera Reacting With GPIIb and GPIIia

<table>
<thead>
<tr>
<th>Class Sera</th>
<th>WJL, KEM, GLE</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>WAG</th>
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<tbody>
<tr>
<td>% Binding in presence of:</td>
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<tr>
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<td>0</td>
<td>123</td>
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<td>59</td>
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<td>100</td>
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</tr>
<tr>
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<td>35</td>
<td>74</td>
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</table>

Values shown represent the average of three different ELISA assays, each performed in triplicate, expressed as percentage of optical density obtained with untreated GPIIb/IIIa complex.

80% by monoclonal 10E5 (GPIIb/IIIa-complex specific) and in being partially inhibited by preincubation with anti-GPIIIa and by dissociation of the GPIIIa complex. It is likely that these reactions are mediated by two different DDAb in the WAG serum, one specific for GPIIIa and the other specific for a GPIIb/IIIa-complex epitope close to the 10E5 binding site.

Class 3 DDAb specifically precipitate GPIIIa and its major chymotryptic digestion products. Immunoprecipitation studies performed with the class 3 DDAb (BAR, ADS, and SCH) and other selected antibodies using radiolabeled intact GPIIb/IIIa complex are shown in Fig 3. Monoclonal AP3, rabbit anti-GPIIIa, and anti-PIA1 alloantibody each precipitated bands corresponding to GPIIb and GPIIIa. Serums BAR, ADS, and SCH each precipitated the same bands, but only in the presence of drug (Fig 3).

To determine whether the antibodies could react with individual subunits of the 1b/IIIa complex, GPIIb/IIIa was dissociated with EDTA at 37°C. As shown in Fig 4, following treatment of GPIIb/IIIa with EDTA, serum ADS precipitated primarily a band corresponding to GPIIIa in the presence, but not in the absence, of drug. The same band was precipitated by the GPIIIa-specific monoclonal AP3. Monoclonal AP2, specific for the GPIIb/IIIa complex, failed to precipitate significant amounts of protein from the EDTA-treated preparation. The other two class 3 DDAb (BAR and SCH) gave immunoprecipitation reactions similar to those of ADS (not shown).

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Fig 3. Immunoprecipitation of radiolabeled GPIIb/IIa complex by selected drug-dependent and non-drug-dependent antibodies (nonreduced SDS-PAGE). Bands corresponding to GPIIb and GPIIa were precipitated by rabbit anti-GPIIa (lane 1) and anti-PI\textsuperscript{\textsc{b}} (lane 2). The same bands were precipitated by the GPIIla-specific DDAb BAR, ADS, and SCH in the presence (lanes 4, 6, and 8), but not in the absence (lanes 3, 5, and 7), of drug. No precipitation occurred with normal serum in the presence or absence of drug.

The mobility of GPIIb was unaffected by endo-H treatment (lane D3), confirming that this protein contains few N-linked high mannose oligosaccharide residues.\textsuperscript{16,19}

Following reduction with 2-mercaptoethanol, the 61-Kd band migrated at about 66 Kd (not shown) as determined by binding of rabbit anti-GPIIa. None of the class 3 DDAb

Fig 4. Immunoprecipitation of intact and dissociated radiolabeled GPIIb/IIa by selected antibodies. With the intact complex, the DDAb ADS precipitated bands corresponding to GPIIb and GPIIa in the presence (lane 2), but not in the absence (lane 1), of drug. The same bands were precipitated by MoAbs AP3 (lane 3) and AP2 (lane 4). Following dissociation of the GPIIb/IIa complex with EDTA at 37°C, serum ADS precipitated only GPIIa in the presence (lane 6), but not in the absence (lane 5), of drug. The same major band was precipitated by AP3 (lane 7) but not by the complex-specific monoclonal AP2 (lane 8).
Fig 5. Immunoprecipitation of chymotrypsin-treated, radiolabeled GPIIb/IIIa by selected drug-dependent and non-drug-dependent antibodies (nonreduced SDS-PAGE). The starting preparation before and after chymotrypsin digestion is shown in lanes 1 and 2, respectively. The GPIIb-specific DDAb ADS, BAR, and SCH each precipitated bands of apparent MW 61 Kd, 43 Kd, and 32 Kd in the presence (lanes 7, 9, and 11), but not in the absence (lanes 6, 8, and 10), of drug. The GPIIIa-specific monoclonals AP3 (lane 3) and APS (lane 4) precipitated the 61-Kd and 32-Kd bands and the 61-Kd and 43-Kd bands, respectively. No precipitate was obtained with the GPIIb-specific monoclonal Tab (lane 5).

DISCUSSION

Immunologically mediated tissue damage is a relatively common side effect of various medications. In the case of solid tissues, evidence for involvement of DDAb in tissue destruction is largely indirect. However, drug-dependent binding of IgG to erythrocytes, platelets, and neutrophils has been convincingly demonstrated in patients manifesting drug-associated destruction of these peripheral blood cells. A few reports have suggested that hematopoietic precursor cells can also be targets for DDAb. One characteristic of drug-induced antibodies that cause cytopenia is that they are highly tissue specific. In rare instances in

Fig 6. Immunoblot of nonreduced GPIIb/IIIa (lanes 1) and of GPIIb/IIIa treated with chymotrypsin (lanes 2) or endo-H (lanes 3). Antibodies used for blotting were (A) monoclonal AP-3 (anti-GPIIb); (B) anti-Pi alloantibody (Kro); (C) quinine-induced DDAb (ADS); and (D) rabbit anti-GPIIb. No binding was obtained in lanes C 1 through 3 in the absence of quinine and no binding was obtained in lanes A 1 through 3, B 1 through 3, or C 1 through 3 when gels were run under reducing conditions (not shown).

recognized GPIIIa or any of its digestion products when they were electrophoresed under reducing conditions (not shown).
which two different cell types were affected, e.g., red cells and platelets or platelets and neutrophils, distinct Igs, each reactive with a different cell type, have been identified.

A few drugs, such as penicillin, appear to trigger drug-dependent Igs by binding covalently to membrane proteins of erythrocytes and, possibly, platelets where they act as haptons. Such antibodies can be detected by their reactions with target cells previously treated with drug and then washed. With most other medications, however, the drug must be present in solution for antibody to bind to the target cell. How this type of antibody is induced and the mechanism by which antibody, once formed, interacts with drug and target molecules in the membranes of platelets and other tissues is uncertain.

Platelets are perhaps affected by drug-induced antibodies more often than any other cell type, and more than 100 compounds have been implicated as causes of DITP. Our current studies demonstrate that most quinidine- and quinine-dependent antibodies react with GPIIb/IIIa as well as with GPIb/IX, in confirmation of earlier studies with smaller numbers of DDAb. DUN appears to be the second example of a DDAb that recognizes GPIIb/IIIa, but fails to react with GPIb/IX. The only previous report of DDAb of this type is that of PfueIler et al., who used Western blotting for antibody detection. Because only a small amount of this serum was available, we were unable to rule out an alternative explanation for the failure of DUN to react with GPIb/IX, i.e., that it might recognize the same site on GPIb as the MoAb AP-1 used to immobilize the GPIb/IX complex (Fig 1). Our absorption studies performed with immobilized GPIb/IX and IIb/IIa (Fig 2) showed that each of five drug-dependent antisera reactive with GPIb/IX and GPIIb/IIIa contained at least two different antibodies, each reactive with only one of the GP complexes in the presence of drug. Similar findings were made with a single DDAb by Christie et al.

As shown in Table 1, the eight DDAb reactive with GPIIb/IIIa could be subclassified on the basis of their reactions with dissociated GPIIb and GPIIa and inhibition of their binding by different MoAbs. From these studies, it appears that three of the sera contain antibodies specific for GPIIb/IIIa in complex form only, one recognizes GPIIb only, and three recognize GPIIla only. Reactions of the final serum (WAG) suggest that it contains two antibodies, one specific for the GPIIb/IIIa complex and one specific for GPIIla alone. Inhibition of WAG by monoclonal 10E5 indicates that it recognizes an epitope on GPIIb/IIIa different from the one recognized by class 1 antibodies. Thus, the eight DDAb studied appear to react with at least four different epitopes on the GPIIb/IIIa complex.

We sought to further localize the binding site of the three GPIIla-specific (class 3) DDAb by examining their interaction with GPIIb/IIIa digestion products. Reactions of these DDAb with endo-H-treated GPIIa (Fig 6, lane C3) indicates that high-mannose residues, the major oligosaccharides of GPIIa, are not required for binding of these antibodies. Each of the three class 3 DDAb precipitated chymotryptic fragments of GPIIa with MW approximately 61 Kd and 43 Kd in the presence, but not in the absence, of drug (Fig 5). However, the same three DDAb bound only to the 61-Kd fragment in immunoblots (Fig 6, lane C3). The 61-Kd fragment migrated with an apparent MW of about 66 Kd after reduction. On the basis of its mobility reduced and nonreduced together with the finding that in nonreduced form it carries the PI alloantigen (Fig 6), this fragment is probably identical to the 62.4-Kd fragment obtained by Niewiarowski et al., and the 66-Kd fragment obtained by Beer and Coller after prolonged chymotryptic digestion of GPIIb/IIIa on intact platelets. The nature of the 43-Kd band precipitated by class 3 DDAb remains to be determined. This fragment was not observed in other studies, but could result from the action of chymotrypsin on solubilized as opposed to membrane-associated GPIIb/IIIa. Failure of the 43-Kd fragment to bind class 3 DDAb in immunoblots (Fig 6, lane C2) is consistent with the possibility that conversion of the 61-Kd fragment to the 43-Kd fragment results in loss of the binding site(s) for class 3 DDAb. In that case, presence of the 43-Kd band in immunoprecipitates obtained with class 3 DDAb (Fig 5) may reflect nonspecific co-precipitation of this fragment with the 61-Kd band.

Our studies of the digestion products of GPIIb/IIIa were performed in the hope that small fragments of GPIIla reactive with class 3 DDAb might be identified that could be isolated and used for studies of the molecular basis of drug-peptide-DDAb interaction. Recent studies indicate that the major chymotryptic digestion product of GPIIIa
represents the protein remaining after enzymatic removal of a large loop of GPIIIa extending from amino acids 130 to 347 from the N terminus. This chymotrypsin-resistant fragment consists of two disulfide-linked peptides comprising approximately the 130 N-terminal and the 414 C-terminal amino acids. The larger chain appears to be maintained in a compact configuration by multiple intrachain disulfide bonding. Therefore, it is likely that the determinants recognized by these antibodies are dependent on intrachain disulfide bonding. Therefore, it may not be possible to identify linear peptide sequences that react with these antibodies in vitro in the presence of drug.

Quinidine and quinine consist of linked quinoline and quinuclidine ring structures and are lipophilic molecules. Such drugs are known to accumulate at amphiphilic surfaces. It seems possible that quinidine and quinine concentrate preferentially in hydrophobic pockets within the chymotryptic-resistant portion of the GPIIIa molecule (Fig 7) where they might induce structural changes (neoantigens) that are immunogenic in certain individuals. Antibody bound to such determinants could stabilize the drug-GP complex so that the drug (quinidine or quinine) is not readily dissociated from the molecule by washing. The finding of Christie et al that titrated quinidine is stabilized in the platelet membrane by the binding of quinidine-dependent DDAb is consistent with this possibility.

In summary, our studies provide evidence that many, probably most, DDAb induced by quinidine and quinine recognize GPIIb/IIIa, in addition to GPIb/IX glycoprotein complexes. One of the antibodies we studied (DUN) appears to be the second example of a DDAb recognizing only GPIIb/IIIa. DDAb that react with GPIIIa/IIa are distinct from those that bind to GPIb/IX and are capable of reacting with four or more epitopes on GPIIb/IIIa in the presence of drug. Certain DDAb (class 3) react with isolated GPIIIa and with its major chymotryptic digestion product. Chong et al have studied the reactions of quinidine and quinine-induced DDAb with GPIb/IX and, in a recent preliminary report, provided evidence that such antibodies recognize at least three different sites on the GPIb/IX complex. Their findings, together with ours, indicate that patients who develop DITP following ingestion of quinidine or quinine produce multiple antibodies reactive with various sites on platelet glycoproteins GPIb/IX and GPIIIa/IIa in the presence of drug. In DITP, it is distinctly unusual for any tissue other than platelets to be affected, with the possible exception of endothelial cells, known to express the von Willebrand factor heavy chain identical to platelet GPIIb. Why platelets are so often targeted for destruction by multiple DDAb in persons sensitized to quinidine and quinine is an intriguing, but unanswered question.

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