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Drug-Dependent and Non–Drug-Dependent Antiplatelet Antibody in Drug-Induced Immunologic Thrombocytopenic Purpura

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The mechanism of drug-dependent immunologic thrombocytopenic purpura (DITP) was investigated by studying the sera of four patients with classic DITP (two with quinidine-, one with acetaminophen-, and one with phenazopyridine-dependent antiplatelet antibody) using a solid-phase radioimmunoassay with ^{125}I-staphylococcal protein A. Two forms of antiplatelet antibody could be demonstrated: one that required drug to bind to platelets and one that bound to platelets in the absence of drug. Drug-dependent antiplatelet antibody required the simultaneous addition of drug and the Fc domain of the drug-dependent IgG molecule for binding to platelets. It did not require serum complement or factor VIII-related antigen for binding to platelets. Drug-dependent binding of antibody to platelets was saturation-dependent. Non–drug-dependent antiplatelet antibody of two patients (one with quinidine-induced thrombocytopenia and the other with acetaminophen-induced thrombocytopenia) reacted with autologous platelets as well as with homologous platelets, indicating that they were autoantibodies. Both autoantibodies had disappeared when their sera were tested 23 and 138 days, respectively, after withdrawal of their initial positive sera. Non–drug-dependent antiplatelet antibody binding could be demonstrated with the F(ab')$_2$ fragment of the purified IgG of the serum of the second patient with quinidine DITP, who did not have detectable alloantibodies against HLA. None of the four patients with non–drug-dependent antiplatelet antibody had a past or present history of autoimmune thrombocytopenic purpura.
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IT IS GENERALLY ASSUMED that drug-dependent antiplatelet antibody binds to platelets by way of drug-Ab immune complexes binding to platelet Fc receptors. But this has not been proven. Ackroyd[1-3] first demonstrated the requirement of the drug sedormid for the agglutination of human platelets by serum obtained from a patient recovering from sedormid-induced thrombocytopenia. With the addition of complement, platelets underwent lysis. He postulated that the drug bound to the platelet surface, rendering it autoantigenic for the production of anti-drug antibody. This concept was challenged by the work of Miescher and coworkers[4-7] and Shulman.[8,9] Miescher demonstrated that experimentally produced immune complexes agglutinated platelets in vitro and that in vivo injection of immune complexes into rabbits resulted in thrombocytopenia. Shulman demonstrated a low affinity of either drug (quinidine) or patient's serum for platelets but a high affinity for the combination of drug and serum. Both Shulman and Miescher postulated that the platelet acted as an innocent bystander for the deposition of drug-Ab immune complexes.

Other studies are not completely in accord with this hypothesis. For example, Karpatkin et al,[10] using the platelet factor 3 immunoinjury technique (an indirect method for measuring antiplatelet antibody), provided data suggesting the presence of antiplatelet antibody directed against the platelet in the absence of drug, which was enhanced by the addition of drug to the reaction mixture. Kelton et al[11] have recently reported observations on four patients with documented drug-induced immunologic purpura, in whom thrombocytopenia persisted for one month or more after cessation of drug ingestion. This was associated with in vitro detection of drug-dependent antiplatelet antibody. Christie and Aster[12] have recently presented evidence that ^{3}H-quinine can bind to platelets in the absence of drug-dependent antiplatelet antibody. Drug-dependent antiplatelet antibody did not bind to these platelets unless additional soluble quinine was added to the system.

In this report, we offer evidence for the presence of both drug-dependent antiplatelet antibody and non–drug-dependent antiplatelet antibody in four patients with drug-induced thrombocytopenic purpura, using a direct, highly sensitive immunoassay. Quinidine-dependent antiplatelet antibody did not bind to platelets when F(ab')$_2$ fragments were used.

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MATERIALS AND METHODS

Preparation of platelets. Platelets from random donors were prepared as described previously.[13] A manual platelet count was obtained using phase optics and the platelet suspension adjusted to 2.5×10^8/mL in human Ringer solution, pH 7.1,[14] containing 2 mmol/L EDTA, 10 mmol/L benzamidine, and 100 μg/mL soybean trypsin inhibitor.

Antisera. Sera from four patients with good clinical histories for drug-dependent immunologic purpura and high drug-dependent titers of 1:1,280 and 1:128 for quinidine sulfate, >1:512 for acetaminophen, and >1:384 for phenazopyridine were obtained and frozen in aliquots at −20 °C. Sera were collected after the drugs had been withdrawn from the patient. There was no past history of autoimmune thrombocytopenia or easy bruising. All four patients recovered rapidly after the withdrawal of drug. Sera from healthy laboratory personnel were similarly obtained, treated in the same manner, and used as controls. Heat-inactivated sera were prepared by incubation at 56 °C for 30 minutes.

Globulin fractions. Globulin fractions were prepared by 50% saturated ammonium sulfate precipitation and dissolved in phosphate-buffered saline, (PBS), 0.01 mol/L, pH 7.4, as described previously.[10]

Purified IgG fractions. The above globulin fractions were extensively dialyzed against 0.01 mol/L phosphate buffer, pH 8.0, and applied to a DEAE-52 chromatography column pre-equilibrated with the dialysis buffer (Whatman Chemicals, W and R Balston, Maidstone, Kent, England).

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F(ab')₂ fragments. The purified IgG was first dialyzed against 0.1 mol/L sodium acetate, pH 4.0, and then digested with pepsin (Sigma Chemical Co, St Louis) 1:50 ratio of enzyme to IgG, according to the method of Nisonoff et al.[15] The digested IgG fragments were then dialyzed against PBS. Trace amounts of undigested IgG or pFc' fragments were removed by passage twice through a column of protein A-Sepharose 4B (Sigma). The completeness of the digestion was confirmed by molecular weight determination on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) by the method of Weber and Osborn.[16] Protein was determined by the method of Lowry et al.[17]

Solid-phase radioimmunoassay. Forty microliters of platelet suspension containing 10^7 platelets was applied to various wells of a plastic microtiter plate (96-well, U-shaped flexible microtiter plates, Scientific Products, McGraw Park, Md). The platelets were allowed to incubate in the wells for one hour at room temperature. The number of adherent cells was determined with ^{51}Cr-labeled platelets as described previously.[13] The microtiter plate was then either used or stored at 4 °C after being wrapped tightly with parafilm (American Can Co, Greenwich, Conn). These could be stored for eight weeks.[13] The wells were then rinsed three times with 1% bovine serum albumin (BSA) in PBS and inverted to drain. Test or control sera or fractions, with or without drug, were applied as 40-μL serial dilutions in 1% BSA-PBS, with or without drug, to maintain a constant final drug concentration. After incubation for one hour at room temperature, the wells were washed three times with 1% BSA-PBS. Forty microliters of ^{125}I-staphylococcal protein A (Amersham, Arlington Heights, Ill, 55 mCi/mg) diluted in 1% BSA-PBS was then added to each well (150,000 cpm per well). After incubation at room temperature for one hour the wells were again washed three times and drained.

An optional step was used in those experiments wherein $F(ab')_2$ fragments and purified IgG were simultaneously measured in the same microtiter plate. Forty microliters of rabbit antihuman IgG (gamma heavy chain specific, Miles Research Prods, Elkhart, Ind) at a dilution of 1:640 in 1% BSA-PBS was added to each well. After incubation for one hour the wells were washed three times with 1% BSA-PBS and ^{125}I protein A added. Each dry well was then separated from the plastic plate with a custom-made cutting machine, as described previously[18] and placed in 12- × 75-mm plastic tubes for assay of radioactivity in a Beckman Biogamma II counter (Beckman Instruments, Irvine, Calif). Background counts were 50 to 100 cpm. Each dilution, with or without drug, was performed in duplicate and the results averaged. A difference between experimental and control samples of 1.3-fold or greater (>2 SD) was considered abnormal.[13]

RESULTS

Quinidine-, acetaminophen-, and phenazopyridine-dependent antiplatelet antibodies. Figure 1A demonstrates quinidine-dependent binding of antiplatelet antibody to platelets from a partially purified globulin fraction of serum to a titer of 1:1,280 (ratio of patient's globulin fraction plus quinidine to patient's globulin fraction >1.3 [2 SD]) with 1 mmol/L quinidine sulfate. Quinidine had no effect on the binding of control serum to platelets. Of note is the enhanced binding to platelets of the patient's globulin fraction in the absence of quinidine compared with a control globulin fraction in the absence of quinidine (see later). Similar results were obtained with the same patient's globulin fraction in five other experiments using five control globulin fractions, as well as with DEAE-purified IgG and 0.25 mmol/L quinidine (see later).

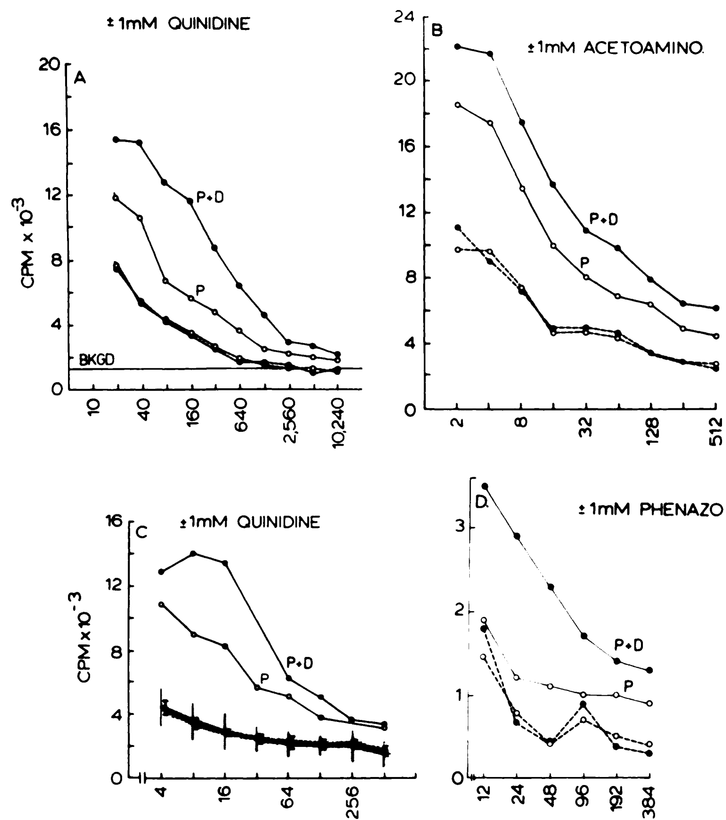

Fig 1. Antiplatelet antibody titers of serum from four patients with drug-dependent antiplatelet antibody: (A) Quinidine, (B) acetaminophen, (C) quinidine, (D) phenazopyridine. Washed platelets were adsorbed onto wells of plastic microtiter plates and then incubated with serial dilutions (in 1% BSA-PBS) of patient or control globulin fraction or serum in the presence or absence of 1 mmol/L drug. Wells were washed with 1% BSA-PBS, treated with ^{125}I-staphylococcal protein A, and then processed for radioactivity as described in Materials and Methods. Patient plus 1 mmol/L drug, P + D ●—●; patient without drug, P O—O; control subject plus 1 mmol/L drug, ●---●; control subjects without drug, O---O. BKGD refers to background counts obtained from wells in which globulin fractions were not added. Each point is the average of duplicate measurements. Panels A through D are representative of three to five experiments. The shaded area in Panel C represents the mean ± 2 SD for six control subjects. The dotted line represents the mean for three control subjects in the presence of drug.

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Figure 1B demonstrates similar findings with the serum of a patient with acetaminophen-dependent binding of antiplatelet antibody to platelets at a titer of >1:512 and drug concentration of 1 mmol/L. Note the enhanced binding of antiserum to platelets in the absence of drug. When this patient's serum was restudied 138 days later, non–drug-dependent antibody was no longer present, whereas the initial stored serum was still positive. Drug-dependent antibody was still positive at a titer of >1:64. When this patient's platelets were studied 138 days later for reactivity with the initial non–drug-dependent antibody, the reaction was positive, indicating that the antiserum was an autoantibody.

Figure 1C demonstrates similar findings with the serum of a second patient with quinidine-dependent binding of antiplatelet antibody to platelets at a titer of 1:128 and drug concentration of 1 mmol/L. The shaded area represents the ± 2 SD range for binding of control sera to normal platelets in the absence of drug. The dotted line represents binding of control sera in the presence of drug. Note the enhanced binding of antiserum to platelets in the absence of drug. When this patient's serum was restudied 23 days later, both drug-dependent and non–drug-dependent antibodies were no longer detectable, whereas the initial stored serum was still positive for both antibodies. When this patient's platelets were studied 162 days later for reactivity with the non–drug-dependent antibody of his initial stored serum, the titer was positive at 1:256, indicating that the antiserum was an autoantibody.

Figure 1D demonstrates similar findings with the serum of a patient with phenazopyridine-dependent binding of antiplatelet antibody to platelets at a titer of >1:384 and drug concentration of 1 mmol/L. Note the enhanced binding of serum to platelets in the absence of drug.

The binding of drug-dependent IgG bound per platelet was estimated by subtracting non–drug-dependent binding from drug-dependent binding, assuming a 2:1 stoichiometry of protein A for IgG[19] and a 75% binding capacity as assayed by Amersham. Binding curves for quinidine and phenazopyridine are plotted in Fig 2A and B. Note the saturation kinetic binding for both drugs.

Simultaneous v sequential addition of serum and drug to platelets. The effect of simultaneous addition of serum and quinidine to platelets followed by washing was compared with the sequential addition of serum, followed by washing, followed by quinidine, followed by washing. The combination of drug and serum was required to demonstrate drug-dependent antibody binding (data not shown). Similar results were obtained with phenazopyridine-dependent antiplatelet antibody (Fig 3).

Effect of 56 °C heat inactivation of drug-dependent antiplatelet antisera on binding of serum IgG to platelets. Experiments with heat-inactivated sera demonstrated the lack of requirement of heat-labile complement components for serum antibody binding to platelets in the presence of quinidine. Enhancement of binding to platelets in the presence of quinidine was the same whether the patient's serum was mixed 1:1 with fresh normal serum, whether the patient's serum was heated to 56 °C for 30 minutes and then mixed 1:1 with fresh normal serum, or whether heated patient's serum was mixed 1:1 with heated fresh normal serum. Similar results were obtained with phenazopyridine (data not shown).

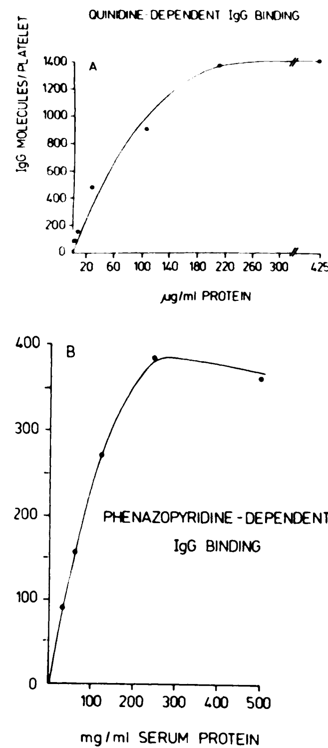

Fig 2. Quinidine- and phenazopyridine-dependent bindng of IgG to washed platelets. Drug-dependent binding of IgG was subtracted from non–drug-dependent binding of IgG as determined from the cpm of ^{125}I-staphylococcal protein A binding to platelets.

Purified $F(ab')_2$ fragment of the quinidine-dependent antiplatelet antibody. Figure 4B demonstrates the results of an experiment obtained with purified $F(ab')_2$ fragments of the

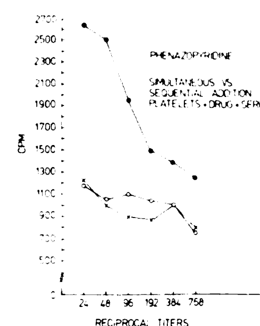

Fig 3. Effect of simultaneous *v* sequential addition of drug plus serum (drug-dependent antibody) to washed platelets. Patient serum plus 1 mmol/L phenazopyridine was added simultaneously for one hour before washing with 1% BSA-PBS, addition of ^{125}I-staphylococcal protein A, and processing for radioactivity (●—●); patient serum added first to washed platelets for 30 minutes followed by washing, followed by addition of drug for 30 minutes, followed by washing, followed by addition of ^{125}I-staphylococcal protein A, ○—○; drug added first for 30 minutes, followed by washing, followed by addition of serum for 30 minutes, followed by washing, followed by addition of ^{125}I-staphylococcal protein A, X—X. Each point is the average of triplicate measurements.

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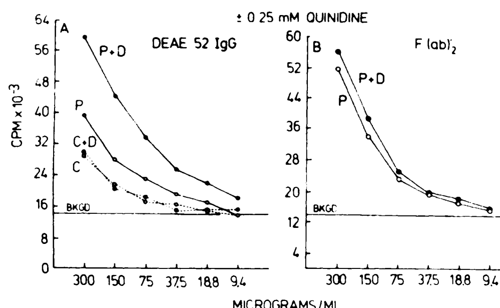

Fig 4. Antiplatelet antibody titers of purified IgG (DEAE-52 chromatography) and $F(ab')_2$ fragment of a patient with quinidine-induced thrombocytopenic purpura. (A) Purified IgG. (B) $F(ab')_2$ fragment. Purified patient or control IgG and $F(ab')_2$ fragments were serially diluted and incubated with washed platelets adsorbed to the wells of microtiter plates in the presence and absence of 0.25 mmol/L quinidine sulfate, as in Fig 1. After washing with 1% BSA-PBS, rabbit antihuman IgG (1:640 dilution) was applied to the wells before further washing and addition of ^{125}I-staphylococcal protein A. Wells were then processed for radioactivity as in Fig 1. Patient IgG or $F(ab)_2'$ plus drug, P + D ●—●; patient IgG or $F(ab')_2$ without drug, P O—O; control IgG with (●---●) or without drug, C O---O. BKGD refers to background cpm obtained from wells in which human IgG was not added. Each point is the average of duplicate measurements. This experiment is representative of five experiments.

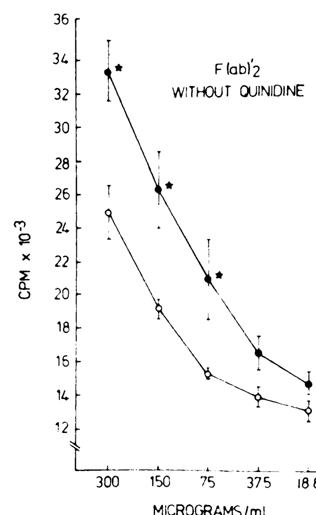

Fig 5. Antiplatelet antibody titer of $F(ab')_2$ fragment of IgG from a patient with quinidine-induced thrombocytopenic purpura, compared with the $F(ab')_2$ fragment of a normal subject tested in the absence of drug. Patient $F(ab')_2$ in the absence of drug, ●—●; control subject $F(ab')_2$ in the absence of drug, O—O. Each point represents the mean of four experiments performed in duplicate. SEM is given. Stars refer to statistically significant differences (>2 SD).

patient's IgG. This experiment was performed at the same time and in the same microtiter plate as the purified IgG experiment depicted in Fig 4A. Binding of the pepsin digest-preparation $F(ab')_2$ fragment of the patient's IgG was not enhanced by addition of 0.25 mmol/L quinidine. Similar results were obtained in four experiments using the patient's $F(ab')_2$ fragment as well as four other control $F(ab')_2$ fragments.

Enhanced binding of patient's serum, IgG or $F(ab')_2$ fragment in the absence of drug. Table 1 demonstrates the enhanced binding of patient's sera to platelets in the absence of quinidine, using five control sera. The average ratio of patient to control binding from five experiments was positive (>1.3) at a serum dilution of 1:160. Similar positive results were obtained with purified IgG of the patient's serum compared with control IgG at a titer of 1:320.

Figure 5 demonstrates the enhanced binding of the patient's $F(ab')_2$ fragment in four experiments compared with four different control $F(ab')_2$ fragments at different concentrations of $F(ab')_2$.

Table 1. Specificity of Patient Serum or IgG for Platelets in the Absence of Quinidine

Dilution	Patient Serum-Control Serum* (n = 5)	Patient IgG-Control IgG (n = 1)
1:20	1.37	1.63
1:40	1.66	1.43
1:80	1.79	1.35
1:160	1.47	1.44
1:320		1.33

*Ratio of radioactivity obtained representing binding of patient's serum to platelets divided by binding of control serum to platelets. Ratio of 1.3 is 2 SD greater than mean.

DISCUSSION

The mechanism of one quinidine-dependent patient's binding of antibody to platelets appears to require the Fc fragment of the patient's drug-dependent IgG since $F(ab')_2$ binding was not enhanced in the presence of drug. Similar findings were recently reported by Van Leeuwen et al[20] as well as ourselves.[21] However, two recent abstracts[22,23] provide evidence that drug-dependent binding is Fab-mediated. It is possible, therefore, that drug-dependent antibody-binding mechanisms are heterogeneous.

Because drug-dependent binding has been shown to fix complement, we examined the possibility that drug–IgG–complement complexes might bind by way of a platelet C3b receptor. This was shown not to be the case, since binding was not altered by heating the patient's serum or by adding fresh serum to the patient's heated serum. Furthermore, an assay that was capable of detecting 50 molecules of C3b receptor per platelet gave negative results with washed human platelets. (This assay was kindly performed by Dr Victor Nussensweig at New York University Medical School.) The simultaneous requirement of the patient's serum and the drug was necessary for binding, indicating that the complex of antibody and drug bound to the platelet, rather than the sequential binding of platelet plus drug plus serum. This later observation confirms the initial observations of Ackroyd.[1-3]

Binding of drug-dependent IgG to platelets was determined for quinidine and phenazopyridine by subtracting drug-dependent binding from non–drug-dependent binding. Saturation kinetics were obtained for both drugs.

Non–drug-dependent binding of antibody to platelets was demonstrated with the serum of all four patients with drug-dependent thrombocytopenic purpura secondary to

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quinidine, acetaminophen, and phenazopyridine, using a highly sensitive immunologic assay. One quinidine patient's serum was studied more carefully, using purified IgG and purified $F(ab')_2$ fragment. The reaction was positive in four to five experiments using four to five different control sera, IgG fractions, or $F(ab')_2$ fragments. These findings were alluded to in a previous report from our laboratory,[10] using an indirect assay, wherein we noted five of 26 patients with drug-dependent immunologic purpura who had positive platelet factor 3 immunoinjury antiplatelet antibody titers of 1:8, 1:8, 1:12, 1:12, and 1:16 without the addition of drug, which increased in titer to 1:32, 1:20, 1:32, 1:32, and 1:64, respectively, by addition of drug (thioguanine, dexamyl, Dilantin, ampicillin, and sulfamethoxazole). These findings are also compatible with the findings of Kelton et al,[11] who noted prolonged thrombocytopenia (longer than one month) in four patients with drug-induced immunologic thrombocytopenic purpura while off the drug (longer than the anticipated clearance time for the drug). Drug-dependent antiplatelet antibody was detectable when the patient's platelet count had returned to normal. It is of interest in this regard that Kekomaki et al,[24] using a platelet binding assay similar to ours, have recently described a male patient with quinidine-dependent platelet antibody who also appeared to have serum binding to platelets in the absence of drug.

The significance of non–drug-dependent antiplatelet antibody in the sera of ten patients (five males and five females) with drug-dependent antibody should be interpreted with caution. It is conceivable that its presence may be coincidental, unrelated to the development of drug-induced immunologic purpura, and possibly of chronic autoimmune or alloimmune nature. However, none of the four patients presently studied or the five patients previously studied by our group had chronic autoimmune thrombocytopenia or a past history of autoimmune disease. Indeed, one quinidine patient (1c) as well as the acetaminophen patient (1b) no longer had detectable non–drug-dependent antibody 23 days and 138 days, respectively, after testing of their initial positive sera. Furthermore, their respective initial positive sera were also shown to be reactive with their autologous platelets 23 and 138 days later, indicating that the antibody was autoimmune, transiently detectable, and not alloimmune. The serum of quinidine patient 1a was also screened against a panel of 40 lymphocyte preparations and shown to be nonreactive for alloantibodies.

Thus two types of antibody appear to be present in some patients with drug-dependent antiplatelet antibody: a drug-dependent antibody that binds first to a drug and then nonspecifically to a platelet Fc receptor by way of its Fc domain, and a non–drug-dependent antibody that binds specifically by way of its active $F(ab')_2$ domain to platelet antigenic determinants. It is proposed that in some drug-dependent immunologic thrombocytopenias the drug does bind to the platelet to produce antigenic determinants for antibody production. Some antibody is directed against the drug, whereas other antibody is directed against the platelet carrier region of the drug or some other region on the platelet that becomes exposed or antigenic after binding of drug to the platelet. A precedent for such a reaction has been described by Paul et al,[25] who noted that antibody formed in response to hapten-carrier conjugates (2,4-dinitrophenyl poly-L-lysine conjugates) have significant carrier specificity.

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