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Antibodies in Sulfonamide-Induced Immune Thrombocytopenia Recognize Calcium-Dependent Epitopes on the Glycoprotein IIb/IIIa Complex

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Drug-dependent IgG antibodies (DDAb) induced by sulfamethoxazole (SMX) and sulfisoxazole (SIX) were identified by flow cytometry in 15 patients who developed thrombocytopenia while taking one of these medications. Fourteen of the 15 DDAb were specific solely for the glycoprotein (GP)IIb/IIIa complex, and 13 of these reacted wholly or in part with epitopes present only on the intact GPIIb/IIIa heterodimer. None of 12 SMX-induced DDAb cross-reacted with SIX, but one of three SIX-induced antibodies reacted with SMX. Each of 10 SMX-induced DDAb tested reacted with the N1-acetyl metabolite of SMX, but only one reacted fully with the N4-acetyl derivative. Detection of the SMX- and SIX-dependent antibodies was facilitated by using bovine serum albumin (BSA) to achieve suspension of these weakly soluble drugs in an aqueous medium. Our findings indicate that DDAb induced by SMX and SIX, in contrast to those induced by quinidine and quinine, are mainly specific for GPIIb/IIIa and react preferentially with calcium-dependent epitopes present only on the intact GPIIb/IIIa heterodimer.
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DRUG-INDUCED thrombocytopenia (DITP) contributes significantly to morbidity and, occasionally, mortality in patients treated with a wide range of medications.[1-6] Although quinidine, quinine, heparin, and gold salts cause DITP with the greatest frequency, more than 100 different medications have been implicated in its pathogenesis.[4] Cases of DITP caused by sulfonamide antibiotics were recorded as early as 1943,[7] and numerous reports of this association have appeared since that time.[2,5] However, drug-dependent antibodies (DDAb) reactive with platelets have been identified in only a few instances.[2,8-11] Failure to detect DDAb with greater frequency in patients with sulfonamide-induced thrombocytopenia could reflect insensitivity of available assays, the poor solubility of many sulfonamide drugs in an aqueous medium, induction of thrombocytopenia by metabolites, rather than the primary drug,[11] or restriction of the binding of DDAb to platelets expressing certain alloantigens.[12] Recently, we applied flow cytometry to the detection of DDAb induced by quinine and quinidine and found that this technique is highly sensitive and specific for DDAb detection.[13] One sulfamethoxazole (SMX)-dependent antibody that could be identified by flow cytometry, but not by other methods, was described in that report. Here, we describe optimization of the flow-cytometric assay for DDAb induced by SMX and sulfisoxazole (SIX) and the use of this technique to identify sulfonamide-dependent DDAb in a series of patients in whom DITP was suspected. We also provide a preliminary characterization of this class of DDAb with emphasis on the ways in which they differ from DDAb induced by quinine and quinidine.

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MATERIALS AND METHODS

Patient sera. Sera from patients who developed thrombocytopenia while being treated with SMX or SIX were obtained from the Platelet Antibody Reference Laboratory of The Blood Center of Southeastern Wisconsin to which they were referred for serologic evaluation. A total of 50 sera were screened by flow cytometry for IgG antibodies reactive with platelets in the presence of drug as described below. Fifteen of the 50 sera were found to contain SMX- or SIX-dependent, platelet-reactive antibodies (see Results). Patients whose sera contained these DDAb ranged in age from 3 to 81 years. Seven were male and eight were female. SMX (in the form of SMX-trimethoprim) and SIX had been prescribed for indications such as otitis media and urinary tract infection. None of the patients were known to have human immunodeficiency virus type-1 (HIV-1) infection. To date, follow-up data have been obtained on 11 of 15 patients with antibody. None were known to be thrombocytopenic before taking SMX or SIX, and platelet levels returned to normal within 2 weeks after discontinuation of the drug in each instance.

Reagents. Fluorescein-conjugated, affinity-purified $F(ab')_2$ goat-antihuman IgG Fc and phycoerythrin (PE)-conjugated $F(ab')_2$ donkey antihuman IgM Fc were purchased from Jackson Immunoresearch Laboratory (Westgrove, PA). Monoclonal antibodies AP-2, specific for the glycoprotein (GP)IIb/IIIa complex, AP-3, specific for GPIIIa, and AP-1, specific for GPIb, were obtained, respectively, from Drs Thomas Kunicki, Peter Newman, and Robert Montgomery of the Blood Research Institute, Blood Center of Southeastern Wisconsin. SMX, SIX, and bovine serum albumin (BSA) were purchased from Sigma Chemical, St Louis, MO. The metabolites N1-acetyl and N4-acetyl sulfamethoxazole were a gift from Hoffman-LaRoche (Nutley, NJ).

Platelet samples. Platelet-rich plasma was isolated from blood of normal group O donors anticoagulated with ACD-A anticoagulant at a blood to anticoagulant ratio of 6:1. Dimethylsulfoxide (DMSO) was added slowly to concentrated platelets suspended in autologous plasma to a final concentration of 7.5% with stirring over a 15-minute period. The final concentration of platelets was $1.5 \times 10^6/\mu L$. The platelets were aliquoted and frozen at −80°C. When required for an assay, platelets were thawed at 37°C, washed three times in 0.027 mol/L phosphate-buffer isotonic saline, pH 7.1, containing 9 mmol/L EDTA and 0.1% BSA (PBS-albumin), and resuspended in PBS-albumin. In preliminary studies, it was found that frozen platelets were as satisfactory as fresh platelets for flow-cytometric assays and expressed normal amounts of GPIb/IX and GPIIb/IIIa as determined by binding of monoclonal antibody specific for these GP.

Flow-cytometric assay. The reaction mixture (0.1 mL) consisted of 2.5×10^7 platelets, 30 μL of drug at the desired concentration in 5% BSA, and 60 μL of patient serum. After incubation for 40 minutes at room temperature, the platelets were washed three times in phosphate-buffered NaCl, pH 7.4, containing 1% BSA (PBS-albumin) and drug at the same concentration as in the primary incu-

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bation. The washed platelets were resuspended in 200 μL of a 1:150 dilution of FITC-labeled anti-human IgG $F(ab')_2$ or PE-labeled anti-human IgM $F(ab')_2$, and were incubated in the dark at room temperature for 20 minutes. They were then washed once and resuspended in 1 mL PBS-albumin. In preliminary studies, we found that drug was not required in washes performed after addition of FITC-labeled anti-IgG. Platelet-bound FITC and PE were analyzed by flow cytometry (FACStar; Becton-Dickinson, Mountain View, CA), and mean fluorescence intensity in the linear mode was determined.[13] Controls consisting of patient serum without drug and normal serum with and without drug were routinely tested with each assay and did not differ significantly from one another. Mean fluorescence intensity obtained with a patient's serum in the presence of an implicated drug was divided by fluorescence intensity without drug, and this ratio was used as an index of drug-dependent deposition of IgG (or IgM) on the target platelets. Ten normal sera tested with and without drug yielded an average ratio of 0.97 ± .027 (SD) relative to a normal reference standard. Patient sera yielding a ratio of 2.0 or greater were considered to be positive for drug-dependent, platelet-reactive antibody.

Immunoprecipitation. Immunoprecipitation studies were performed using platelets labeled with biotin, enabling the precipitated proteins to be separated by electrophoresis, transferred to a polyvinylidine difluoride (PVDF) membrane, tagged with Streptavidin-horseradish peroxidase (Streptavidin-HRP; Amersham, Arlington Heights, IL), and detected by chemiluminescence generated from the action of HRP on substrate. A similar approach has been used by others to identify rat[14] and human[15] erythrocyte antigens recognized by polyclonal antibodies and human platelet glycoproteins recognized by monoclonal antibodies.[16,17]

Platelets were isolated from blood anticoagulated with EDTA, washed three times with PBS-albumin, pH 7.4, containing 0.1% EDTA, and resuspended in .01 mol/L phosphate-buffered saline (PBS), pH 7.4. NHS-LC-Biotin (Pierce Biochemical, Rockford, IL), (5 mmol/L final concentration) was added to a 1-mL suspension of 2×10^9 platelets. The mixture was incubated at 4°C for 30 minutes, washed three times in PBS containing 0.1% EDTA, and resuspended in PBS. In preliminary studies, it was found that biotinylation under these conditions did not affect the binding of DDAb to platelets determined by flow cytometry. A 0.1-mL suspension of 10^8 biotinylated platelets was incubated with 0.1 mL of patient serum or normal serum with or without drug (1.2 mmol/L), and the mixture was gently agitated at room temperature for 1 hour. The sensitized platelets were washed in PBS containing drug at the same concentration used for sensitization and were solubilized in 0.1 mL of buffer containing 20 mmol/L Tris-HCl, pH 8.5, 0.15 mol/L NaCl (TBS buffer), 0.2 mmol/L leupeptin, 1.4 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, and 1.2 mmol/L drug for 30 minutes at 4°C. The lysate was centrifuged at 13,000*g* for 30 minutes. Protein A-Sepharose CL-4 beads (Pharmacia, Piscataway, NJ) were preincubated in 2% BSA, 20 mmol/L TBS buffer, pH 8.5, at room temperature for 30 minutes, washed three times in the buffer used to lyse sensitized platelets, and resuspended in buffer at a 50% concentration. A 50-μL aliquot of beads was incubated with the platelet lysate at room temperature for 30 minutes, washed three times in 20 mmol/L TBS buffer, pH 8.5, containing drug at the same concentration as in the primary mixture, resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.125 mmol/L Tris-HCl, 20% glycerol, 1% SDS, .002% bromphenol blue, pH 6.8) and boiled for 6 minutes.

The eluate was electrophoresed on a 5% to 15% gradient SDS-polyacrylamide gel. The gel was then equilibrated in CAPS/methanol buffer and transferred to PVDF microporous membranes (Millipore, Bedford, MA) using a Genie electrophoretic blotter (Idea Scientific, Minneapolis, MN) at room temperature at 12 V for 1.5 hours. The membrane was blocked for 30 minutes with TBS buffer (10 mmol/L Tris, 0.15 mol/L NaCl, 2% BSA, 2% Tween-20, pH 7.4) and washed three times with Tris-buffered saline (10 mmol/L Tris, 0.15 NaCl, 0.1% Tween-20, pH 7.4) for 10 minutes each. Streptavidin-HRP conjugate diluted 1:4,000 in TBS buffer was then added and incubated at room temperature for 60 minutes. After washing four times (15 minutes each) with TBS buffer, the membrane was coated with a mixture of hydrogen peroxide/Luminol (ECL Detection System; Amersham Life Sciences, Arlington Heights, IL), wrapped inside a clear plastic sheet, and placed in an x-ray film cassette. The membrane was covered with a sheet of film (Hyperfilm-ESL; Amersham Life Sciences) and exposed at room temperature for various lengths of time.

Measurement of SMX and SIX levels. Levels of SMX and SIX in solution were determined by the Bratton-Marshall assay[18] by Dr Basil Doumas of the Department of Pathology, Medical College of Wisconsin.

RESULTS

Optimization of the flow-cytometric assay for SMX- and SIX-dependent antibodies. SMX and SIX are only slightly soluble in aqueous solution at neutral pH, and negative reactions were regularly obtained when drug dissolved in PBS, pH 7.4, for 1 hour was used to assay for DDAb induced by these medications. Positive reactions (see below) were obtained with drug solubilized in PBS by incubation overnight with stirring and occasional addition of 0.1 mol/L NaOH to maintain pH. It is known that SMX and SIX bind to albumin and circulate in plasma primarily in the albumin-bound form.[19] We found that both drugs could be solubilized at a concentration of 2 mg/mL within a few minutes in PBS, pH 7.4, containing 5% BSA. Drug resuspended in albumin was as effective as drug solubilized by overnight incubation in buffer in promoting the binding of SMX- and SIX-dependent antibodies to platelets. For convenience, drug suspended in albumin was used in experimental studies.

For maximum binding of DDAb to target platelets to be sustained, it was essential for drug to be present in the solutions used to wash platelets before adding FITC- or PE-labeled secondary antibody (Fig 1). With weaker antibodies, negative reactions were obtained unless this precaution was taken. In the studies to be described, wash solutions containing 1.2 mmol/L drug were routinely used. Individual DDAb reacted equally well against group O platelets from eight different donors. Platelets from three different group O donors were used interchangeably in experimental studies.

Detection of SMX- and SIX-induced DDAb. Fifteen of 50 sera samples screened for SMX- and SIX-dependent antibodies gave unequivocally positive reactions when tested with normal target platelets in the presence, but not in the absence, of drug (12 of 46 tested with SMX and three of four tested with SIX). DDAb were not detected in sera from 15 randomly selected normal subjects or in any of 28 nonthrombocytopenic patients who were taking SMX or had taken this drug within the previous 2 months. Some of the thrombocytopenic patients in whom DDAb were not detected were taking other drugs in addition to SMX or SIX, and it is possible that one of those medications caused the thrombocytopenia in some cases. In most cases, testing was requested as part of the general work-up of a patient pre-

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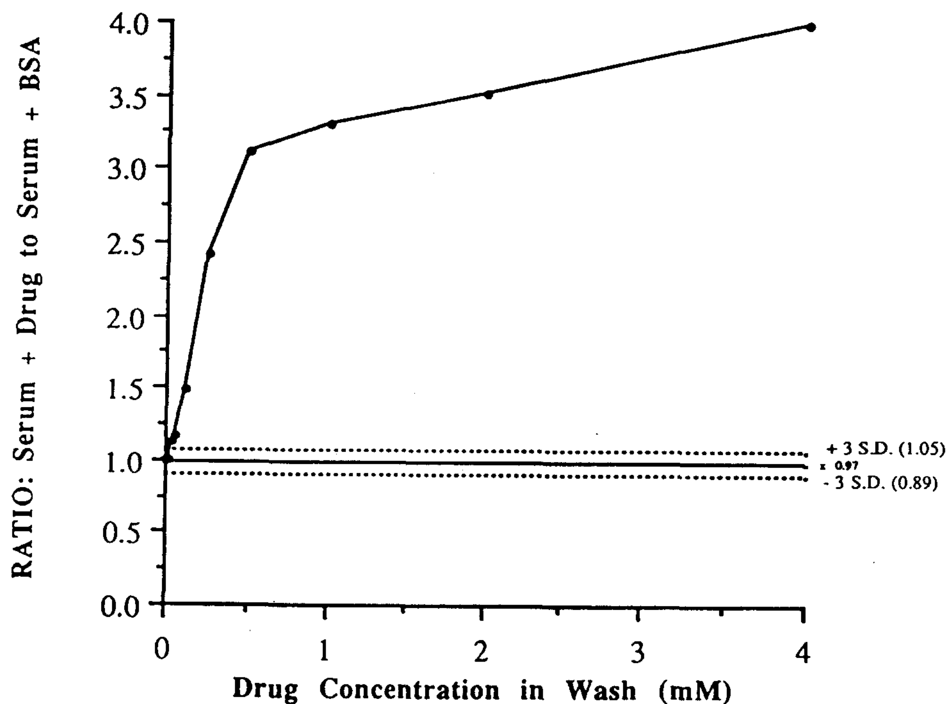

Fig 1. Effect of washing in buffer containing different concentrations of drug on binding of the SMX-dependent antibody from patient no. 1 to platelets. The primary reaction mixture consisted of 60 μL drug-dependent antibody diluted 1:10 in buffer, 2.5 × 10^7 washed platelets, and 1.2 mmol/L SMX. The sensitized platelets were washed three times in buffer containing drug at the indicated concentrations before adding fluorescein-labeled $F(ab')_2$ anti-IgG. Ordinate depicts mean platelet fluorescence intensity relative to fluorescence intensity obtained without drug in the primary incubation. Horizontal lines depict mean relative values obtained in the absence of drug ± 3 SD. At drug concentrations greater than 5.0 mmol/L, relative fluorescence intensity leveled off at approximately 4.3.

senting with thrombocytopenia. Therefore, reduced platelet levels were probably unrelated to drug sensitivity in many instances.

Reactions of a typical SMX-dependent DDAb (patient no. 1) against normal target platelets in flow cytometry are shown in Fig 2. No binding of IgG occurred in the absence of drug or with normal serum in the presence of drug.

Reactions of the 15 sera samples containing DDAb with normal target platelets are listed in Table 1. Ratios (fluorescence intensity with drug:fluorescence intensity without drug) ranged from 2.6:1 to 140:1. Two of the sera samples (patients no. 4 and 12) contained DDAb of both IgG and IgM classes. No IgM activity was detected in the other 13 sera samples. All sera were tested at least twice with similar results. In general, sera giving the highest binding ratios were those with the highest titers. Serum no. 1, which gave a binding ratio of 140:1, could be detected at a dilution of 1:60 in buffer.

Reactions of DDAb with platelets from a patient with type I Glanzmann's thrombasthenia. Fourteen of 15 sera samples failed to react or reacted only weakly (ratio < 2.0) with platelets from a patient with type I Glanzmann's thrombasthenia (GT) lacking detectable GPIIb/IIIa (Table 1). However, serum from one patient (no. 11) reacted as well with GT platelets as with normal platelets. The same GT platelets gave strong, drug-dependent reactions with serum from a patient with quinine-induced thrombocytopenia known to contain antibodies reactive with the GPIb/IX complex (not shown).

Inhibition of DDAb binding by the monoclonal antibody, AP-2. These findings suggested that all but one of the 15 DDAb were specific for a site or sites on GPIIb/IIIa. We therefore studied the binding of these DDAb to platelets preincubated with saturating quantities of murine monoclonal antibody AP-2, specific for the GPIIb/IIIa complex. Binding of seven of the DDAb (no. 1, 3, 5, 7, 8, 12, and 13) was markedly inhibited (>88%) by AP-2 (Table 1). This monoclonal antibody inhibited three sera (no. 2, 4, and 15) only partially (24% to 34%) and had little or no effect on four others (no. 6, 9, 10, and 14). No inhibition of DDAb binding occurred when platelets were preincubated with saturating quantities of normal mouse IgG or monoclonal antibody AP-1, specific for GPIb.

Reactions of DDAb with platelets treated at 37°C in EDTA. Treatment of platelets with EDTA at 37°C at pH 7.4 or above causes dissociation of the GPIIb/IIIa complex in the platelet membrane, accompanied by loss of AP-2 binding.[20,21] As shown in Fig 3, treatment of platelets with 5 mmol/L EDTA at 37°C for 30 minutes or more abolished binding of the DDAb in serum of patient no. 1 and of monoclonal antibody AP-2 (complex-specific), but not AP-3 (GPIIIa-specific). In all, EDTA treatment of platelets at 37°C greatly reduced (0% to 25% of control) the binding of 11 DDAb (no. 1, 3 to 5, 7, 8, 10, and 12 to 15) and partially reduced (37% to 50% of control) the binding of two others (no. 2 and 6) (Table 1). The DDAb from patient no. 9 bound as well to EDTA-treated platelets as to untreated platelets. The same was true of serum no. 11, the only one that reacted with GT platelets. EDTA-treated platelets reacted as well as untreated platelets with a Pl^{A1}-specific alloantibody, with monoclonal antibody AP-3 specific for GPIIIa, and with a quinine-induced DDAb specific for GPIIIa and GPIb (data not shown). Each of the DDAb reacted fully with platelets treated with EDTA at room temperature (shown for patient no. 1 in Fig 3) or at 37°C in the presence of 1 mmol/L calcium EDTA (not shown).

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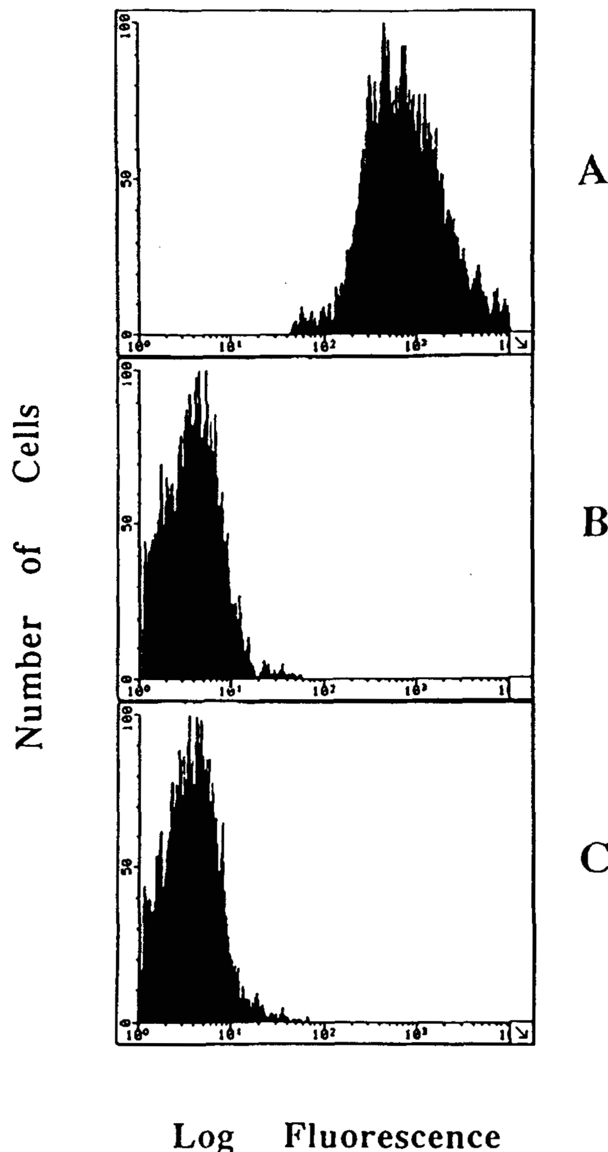

Fig 2. Typical histogram obtained with DDAb induced by sulfonamide antibiotics. Serum from patient no. 1 (60 μL) was incubated with 2.5 × 10^7 normal platelets in the presence (A) and absence (B) of 1.2 mmol/L SMX in 1% BSA. After three washes in buffer containing drug at the same concentration as in the primary mixture, platelet-bound IgG was detected with fluorescein-labeled anti-IgG Fc. (C) Results obtained with normal serum and SMX at the same concentration as in (A).

Immunoprecipitation with SMX-induced DDAb. Results of an immunoprecipitation study using biotin-labeled platelets and the SMX-induced DDAb from patient no. 1 are shown in Fig 4. Bands corresponding to GPIIb and GPIIIa were precipitated in the presence, but not in the absence, of SMX, and no bands were obtained with normal serum in the presence or absence of drug. Similar results were obtained with sera from patients no. 5 and 12 (not shown). The remaining DDAb have not yet been studied by this technique. Unfortunately, not enough serum was available to perform immunoprecipitation studies with the DDAb from patient no. 11, the only one that reacted with GT platelets.

Effect of DDAb recognizing the GPIIb/IIIa complex on platelet function. Monoclonal AP-2 inhibits the binding of fibrinogen to the GPIIb/IIIa complex on activated platelets and blocks platelet aggregation in a plasma medium.[20] We therefore studied whether DDAb that were blocked by AP-2 exerted a similar effect on platelet function. Sera from patients no. 1 and 12, in quantities capable of saturating their targets, failed to inhibit platelet aggregation induced by adenosine diphosphate (2 × 10^{-6} mol/L) in the presence or absence of drug. In contrast, almost complete inhibition was achieved with AP-2.

Reactions of DDAb induced by SMX and SIX with the opposite drug and with SMX metabolites. The structures of SMX and SIX differ only at the site of attachment of the isoxazole ring to the sulfamoyl bridge connecting the two aromatic groups and the presence of an additional methyl group on the isoxazole ring of SIX (Fig 5). Structures of the two major metabolites of SMX, N1-acetyl and N4-acetyl SMX, are also shown. Because one report suggested that antibodies associated with SMX-induced thrombocytopenia may be specific for the N4-acetyl metabolite,[11] we tested 10 of the 12 sera containing SMX-dependent antibodies with these metabolites using BSA for solubilization. As shown in Table 2, each serum reacted as strongly with N1-acetyl SMX as with SMX itself. However, only two positive reactions, one weak (serum no. 1) and one strong (serum no. 6), were obtained with N4-acetyl SMX. The 12 DDAb induced by SMX were tested with SIX, and the three DDAb induced by SIX were tested with SMX (Table 2). Only the DDAb

Table 1. Reactions of SMX- and SIX-Induced Antibodies With Various Target Platelet Preparations

Patient No.	Drug	Target Platelets: Normal	GT	AP-2-Blocked	37°C EDTA-Treated
1	SMX	100* (140)†	0*	0*	9*
2	SMX	100 (2.5)	13	73	50
3	SMX	100 (9.3)	0	2	1
4	SMX	100 (15)	14	66	24
5	SMX	100 (75)	0	0	4
6	SMX	100 (22)	0	110	37
7	SMX	100 (37)	0	0	0
8	SMX	100 (97)	2	12	7
9	SMX	100 (60)	0	100	93
10	SMX	100 (13)	2	95	6
11	SMX	100 (13)	124	NT	90
12	SMX	100 (13)	0	2	4
13	SIX	100 (14)	1	2	3
14	SIX	100 (5.3)	13	80	0
15	SIX	100 (14)	0	76	24

Abbreviations: NT, not tested; GT, Glanzmann's thrombasthenia.

*Mean platelet fluorescence in the presence of drug as a percentage of the value obtained with normal platelets in presence of drug (average of two or more determinations).

† Ratio of mean platelet fluorescence in the presence of drug to that obtained in the absence of drug (average of two or more determinations).

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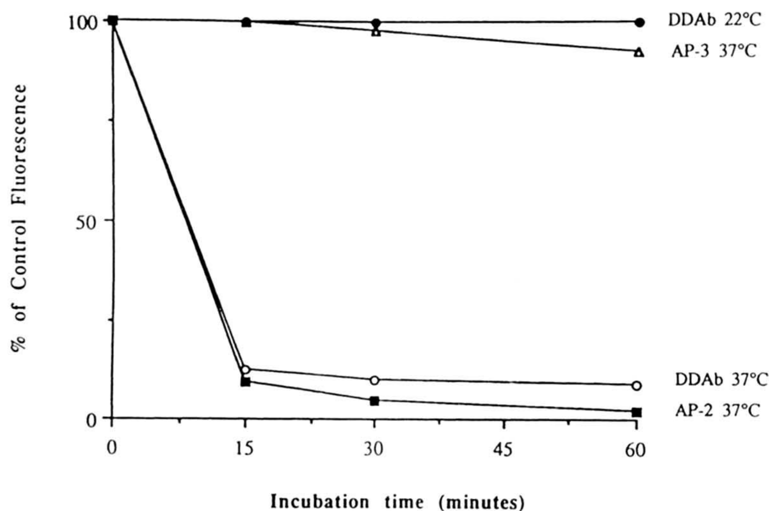

Fig 3. Effect of treating of platelets with 5 mmol/L EDTA at 37°C or 22°C on binding of SMX-induced antibody from patient no. 1 and monoclonals AP-2 and AP-3. Captions at the right indicate temperature of EDTA treatment and antibody used. Treatment with EDTA at 37°C abolished the binding of SMX-induced antibody and monoclonal AP-2, specific for the GPIIb/IIIa complex, but did not affect binding of monoclonal AP-3, specific for GPIIIa. Treatment with EDTA at 22°C was without effect on binding of SMX-induced antibody (●) or monoclonals AP-2 and AP-3 (not shown).

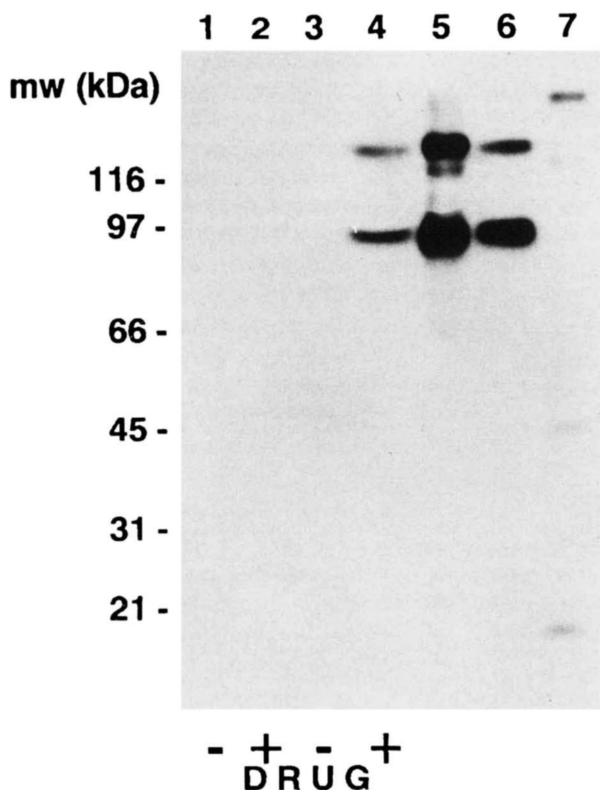

Fig 4. Immunoprecipitation of glycoproteins from biotin-labeled platelets. (Lanes 1 and 2) Normal serum with and without sulfamethoxazole (SMX); (lanes 3 and 4) serum from patient no. 1 without and with SMX; (lane 5) alloantibody specific for P1^{A1}; (lane 6) monoclonal antibody AP-2 specific for GPIIb/IIIa; (lane 7) monoclonal antibody AP-1 specific for GPIb. Electrophoresis was performed under nonreducing conditions for lanes 1 to 6 and reducing conditions for lane 7. Precipitated proteins were detected by chemiluminescence using a Streptavidin-HRP probe as described in Materials and Methods. The precipitated proteins in lanes 4 to 6 are GPIIb and GPIIIa. Precipitation of GPIb (above) and GPIX (~18 kD) by monoclonal AP-1 is shown in lane 7. The faint 45-kD band precipitated by AP-1 is unidentified.

of patient no. 13 induced by SIX cross-reacted, in this case with SMX. Unexpectedly, this reaction was about six times as strong as that obtained with the drug that caused the sensitization (SIX).

The 34 serum samples from thrombocytopenic patients taking SMX that gave negative reactions with that drug were also tested with N1-acetyl- and N4-acetyl SMX. No positive reactions were obtained.

DISCUSSION

Immunologically mediated injury to peripheral blood cells and their marrow precursors is a recognized side effect of many medications, but the mechanism(s) by which drugs produce tissue damage are, in general, poorly understood. For unknown reasons, platelets are targeted more often by drug-induced antibodies than red blood cells or neutrophils. In studies performed more than a decade ago, we[22] and others[23] showed that platelet-reactive DDAb induced by quinine and quinidine react preferentially with the GPIb/IX complex, the platelet receptor for von Willebrand factor. Subsequently, it was learned that the GPIIb/IIIa complex can also be a target,[24-26] and that DDAb specific for GPIb/IX or GPIIb/IIIa are heterogenous with respect to the epitopes on these glycoprotein complexes to which they bind.[24,27]

Our studies show that SMX and SIX also induce antibodies that react with platelets in the presence of drug at pharmacologic concentrations (0.2 to 0.3 mmol/L). However, DDAb induced by these sulfonamide compounds differ strikingly from those induced by quinine and quinidine in that 14 of 15 sera failed to react with platelets from a patient with type I GT and were, therefore, specific only for GPIIb, GPIIIa, or the GPIIb/IIIa complex (Table 1). Reactions with GPIIb/IIIa were directly confirmed with serum samples 1, 5, and 12 by immunoprecipitation.

The reactions of these 14 sera against different platelet preparations provide a basis for presumptive classification of their target specificities (Table 3). In EDTA at 37°C, the GPIIb/IIIa complex in the membrane of intact platelets dissociates, as shown by the failure of complex-specific monoclonal antibodies to bind and the appearance of free

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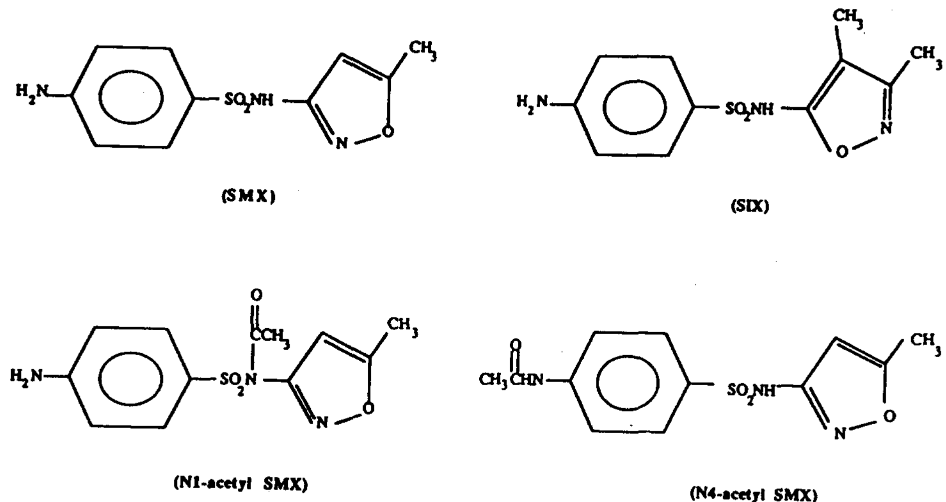

Fig 5. Structures of SMX, SIX, and the SMX metabolites, N1-acetyl SMX and N4-acetyl SMX.

GPIIb and GPIIIa following solubilization[20,21,28] (Fig 3). From their negative or weak (<25% of control) reactions with platelets treated with EDTA at 37°C, it appears that 11 of these 14 DDAb require the intact GPIIb/IIIa heterodimer for binding. Seven of these sera (group I) failed to bind (<12% of control) to platelets blocked with monoclonal AP-2, and therefore appear to recognize an epitope close to the AP-2 binding site. Three of the complex-specific DDAb (no. 10, 14, and 15; group II) bound readily (>75% of control) to platelets blocked with AP-2 and therefore appear to recognize an epitope on the heterodimer distant from the AP-2 binding site. Serum no. 9 reacted both with platelets blocked by AP-2 and with EDTA-treated platelets and presumably recognizes an epitope expressed on dissociated GPIIb or GPIIIa (group III). Partial (34%) inhibition by AP-2 of the binding of serum 4, which failed to react with EDTA-treated platelets, is consistent with the possibility that it contains a combination of group I and group II antibodies. Reactions of serum samples no. 2 and 6 were more difficult to analyze. Because these sera were not blocked by AP-2, and reacted only partially (50% and 37% of control) with EDTA-treated platelets, they may contain a combination of group II and group III DDAb. Serum no. 11 was the only one of the 15 DDAb that reacted with platelets from a patient with type I GT. Unfortunately, not enough of this serum was available to determine whether its target is GPIb/IX or some other platelet membrane component.

Monoclonal AP-2 inhibits platelet aggregation in response to adenosine diphosphate (ADP) and blocks the binding of fibrinogen to activated platelets.[20] Therefore, failure of the antibodies of patients no. 1 and 12, which were blocked by AP-2, to inhibit platelet aggregation in response to ADP was unexpected. A possible explanation is that type I antibodies recognize an epitope that is lost when AP-2 binds, but is different from the AP-2 binding site itself.

Table 2. Reactions of SMX- and SIX-Induced Antibodies With SMX, SIX, and Metabolites of SMX

Patient No.	Implicated Drug	Drug Tested: SMX	SIX	N1-Acetyl SMX	N4-Acetyl SMX
1	SMX	100*	0*	100*	15*
2	SMX	100	0	NT	NT
3	SMX	100	4	84	0
4	SMX	100	1	78	6
5	SMX	100	0	100	0
6	SMX	100	0	116	122
7	SMX	100	0	78	0
8	SMX	100	0	93	1
9	SMX	100	0	90	0
10	SMX	100	0	120	3
11	SMX	100	0	NT	NT
12	SMX	100	1	93	5
13	SIX	643	100	NT	NT
14	SIX	0	100	NT	NT
15	SIX	0	100	NT	NT

* Mean platelet fluorescence as a percentage of that obtained in testing with the sensitizing drug.

Table 3. Grouping of SMX and SIX-Induced Antibodies According to Target Specificities

Group No.	Epitope(s) Recognized	Reactions With Platelets: GT	AP-2-Blocked	37°C, EDTA-Treated	Patient Serum
I	GP IIb/IIIa near AP-2 binding site	No	No	No	1, 3, 5, 7, 8, 12, 13
II	GP IIb/IIIa away from AP-2 binding site	No	Yes	No	10, 14, 15
III	Dissociated GPIIb or GPIIIa	No	Yes	Yes	9
	Target(s) other than GPIIb/IIIa	Yes	NT	Yes	11
	Undetermined	No	Partial	No	4
	Undetermined	No	Yes	Partial	2, 6

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Although the GPIIb/IIIa complex remains intact in platelets exposed to EDTA at room temperature, this treatment causes conformational changes that reduce the binding of some complex-specific monoclonal antibodies[29] and creates epitopes for monoclonal antibody PMI-I[30] and naturally occurring antibodies found occasionally in patients and normal subjects.[31,32] It is apparent from our studies that the changes induced in GPIIb/IIIa by EDTA at room temperature do not affect binding of SMX- or SIX-induced DDAb. Recent studies by Calvete et al provide evidence that complexed GPIIb and GPIIIa molecules contact each other at numerous sites where there is hydropathic complementarity of peptide sequences.[33] Therefore, many different epitopes are potentially available on GPIIb/IIIa for binding of complex-specific DDAb. Further studies are needed to determine whether SMX- and SIX-induced DDAb react preferentially with only a few regions of the heterodimer or bind at many different sites.

We[34] and others[6,35] have suggested that drugs capable of causing DITP interact reversibly with the platelet membrane to induce conformational changes (neoantigens) or combinatorial epitopes recognized by DDAb. SMX and SIX are closely related structurally (Fig 5), yet only one of the 15 DDAb we studied reacted with both drugs. Therefore, for this model of DDAb-platelet interaction to hold, it must be further postulated that the neoantigens induced by drugs are sensitive to minor changes in drug structure, as suggested by previous studies of DDAb induced by quinidine and quinine.[36] From Table 2, it can be seen that each of 10 SMX-induced DDAb specific for GPIIb/IIIa reacted as well with the N1-acetyl metabolite of SMX as with SMX itself, but that only one (serum no. 6) reacted fully with the N4-acetyl metabolite. None of the 12 SMX-induced DDAb reacted with platelets in the presence of SIX, which contains an additional methyl group on the isoxazole ring. Thus, a structural modification in the sulfamoyl bridge connecting the two aromatic groups of SMX is tolerated, but modification of either aromatic group greatly reduces the ability of the molecule to promote DDAb binding. It is apparent that the SIX-induced antibody in serum no. 13, which bound to platelets more strongly in the presence of SMX than with the provocative drug, is an exception to this rule. Our findings are consistent with the possibility that one or both of the ring structures of SMX and SIX interact with GPIIb/IIIa to produce a conformational change or form a complex recognized by DDAb. It is of interest that quinidine and quinine, like SMX and SIX, also consist of two aromatic structures (quinuclidine and quinacrine rings) linked by a flexible bridge.

Our studies provide further evidence of the usefulness of flow cytometry for detection of DDAb reactive with platelets. Various assays have been used in previous studies of DDAb induced by SMX and SIX,[2,8-11] but the largest number of DDAb detected in any one study appears to be three.[2] As with DDAb induced by quinine or quinidine,[34] it is important that drug be present both in the primary mixture and in the subsequent washing steps to achieve maximum sensitivity (Fig 1). Adequate concentrations of drug can readily be achieved by using BSA to facilitate suspension in an aqueous medium.

SMX is commonly used in the treatment and prophylaxis of *Pneumocystis carinii* infection in patients with acquired immunodeficiency syndrome (AIDS). Autoimmune thrombocytopenia develops in 10% to 20% of patients infected with HIV-1 and may precede clinical symptoms of immunodeficiency.[37] Flow cytometry may be helpful to distinguish platelet-reactive antibodies induced by SMX from autoantibodies in such patients.

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