Rifampicin-dependent antibodies bind a similar or identical epitope to glycoprotein IX–specific quinine-dependent antibodies

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The drug-dependent antibody of a patient with rifampicin-induced thrombocytopenia was characterized using the antigen-capture enzyme-linked immunosorbent assay (MAIPA assay), flow cytometry, and immunoprecipitation. The antibody was found to bind glycoprotein (GP) Ib-IX but not GPllb-IIIa because (1) it immunoprecipitated drug-dependently the former but not the latter glycoprotein complex and (2) the MAIPA assay showed strong rifampicin-dependent antibody binding when anti-GPllb-IX monoclonal antibodies (mAbs) (AK2 and FMC25) but not anti-GPllb-IIIa mAbs (AP2, SZ21, and SZ22) were used to capture the antigen. The antibody binding site was further localized to the GPIX subunit of the GPllb-IX complex because flow cytometric analysis revealed drug-dependent antibody binding to L cells transfected with human GPllb and GPIX complementary DNA (L βlX cells) but not with human GPllb and GPIX complementary DNA (L αβ cells). Finally, in the MAIPA assay, the rifampicin-dependent antibody almost completely cross-blocked the binding of the anti-GPIX mAb (SZ1) to platelets. Similar cross-blocking of SZ1 binding to platelets by the quinine-dependent antibodies was also observed. This finding not only confirms that the epitope of the rifampicin-dependent antibody is on GPIX but it is also identical to or located in close proximity to that of the quinine-dependent antibody and SZ1. Further characterization of the epitopes of these antibodies may have important implications for a general understanding of the mechanism of drug-induced thrombocytopenia. (Blood. 2000; 95:1988-1992)

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and the streptavidin-HRP conjugate (Amersham, Bucks, United Kingdom) were purchased as indicated.

All monoclonal antibodies (mAb) were of the IgG class. MOPC21 (Becton & Dickinson, San Jose, CA), a murine IgG1 myeloma protein, was used as a control immunoglobulin, and SZ1, SZ21, and SZ22 were purchased from Immunotech (Marseille, France). The sheep anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody was purchased from Silenius (Hawthorn, Australia), and the rabbit anti-human FITC-conjugated secondary antibody from Dako.

AK2 and FMC25 mAbs, which are directed against epitopes on various parts of the human GPIb-IX complex, were obtained from Dr M. Berndt (Melbourne, Australia). AP2, a mAb that is directed against an epitope on GPIb-IIIa, was a kind gift from Dr T. J. Kunicki (La Jolla, CA).

**Patient**

Mrs N.P., a 66-year-old woman, was diagnosed with pulmonary tuberculosis but no other bleeding. Physical examination revealed petechiae on her legs and spontaneous bruising. Physical examination revealed petechiae on her legs but no other bleeding. Blood counts showed a severe thrombocytopenia: platelets 9 × 10^9/L, hemoglobin 165 g/L, and white blood cells 5.0 × 10^9/L. A diagnosis of drug-induced thrombocytopenia was made, and all drugs she was taking were stopped. She was begun on 50 mg of prednisone daily. A test for drug-dependent antibodies using an antigen-capture assay—MAIPA—demonstrated a rifampicin-dependent antiplatelet antibody with specificity against GPIb-IX complex, but no niacinoid-dependent antibody was detected. A bone marrow aspirate revealed normal numbers of megakaryocytes and erythroid and myeloid precursors, consistent with a thrombocytopenia due to increased peripheral platelet destruction. Her platelet count rose to 124 × 10^9/L, 189 × 10^9/L, and 214 × 10^9/L on days 4, 6, and 7, respectively, after withdrawal of antituberculosis drugs. Prednisone was then stopped.

**Cell lines**

Chinese hamster ovary (CHO) DUK (dihydrofolate reductase–negative [DHFR–]) cells and mouse L (tk–) cells stably transfected with complementary DNA (cDNA) encoding the GPIb-IX subunits in various combinations were produced in one of our laboratories (J.A.L.) as previously described.6 The cloning of the cDNAs for GPIbα, GPIββ, and GPIγ, and GPIγ has been reported previously.7,8 The 3 cDNAs (each containing the entire coding sequence and the 3′-untranslated region) were cloned separately into the eukaryotic vector pDZ (a kind gift from Dr K. Berker, Seattle, WA), in which transcription is driven by the adenovirus major late promoter and the SV40 enhancer.

**Methods**

**Transfection of CHO and L cells with GPIb-IX genes.** The CHO cells were transfected with the following combinations of GPIb-IX subunits cDNAs: (a) GPIbα, GPIββ, and GPIγ, (b) GPIbαs and GPIββ, (c) GPIbα and GPIγ, and (d) GPIbβ and GPIγ. The L cells were transfected with (a) GPIbαs and GPIββ and (b) GPIbβ and GPIγ. Expression of the GPIb-IX subunits in the cell lines was substantiated by Northern blot analysis to determine messenger RNA (mRNA) and by flow cytometry and enzyme-linked immunosorbent assay (ELISA) to ensure protein expression of the subunits on the cell surface. In all cell lines, the appropriate GPIb-IX subunits were expressed on the cell surface except in the CHO cells transfected with GPIbαs and GPIγ cDNA. In this cell line, GPIbα was expressed on the cell surface, but GPIγ was located in the cytoplasm when detected by flow cytometry.

**Enzyme-linked immunosorbent assay.** The ELISA was performed as previously described.9

**MAIPA assay.** The MAIPA assay was performed as previously described with minor modifications.10 Group O platelets (2 × 10^11 per tube) were washed once in phosphate-buffered saline (PBS/1% EDTA buffer, resuspended in 100 µL PBS/2% [w/v] BSA, and added to 50 µL of patient serum with or without 5 µL of rifampicin (final concentration, 70 µg/mL). The positive control for the assay was normal pooled platelets at a concentration of 2 × 10^11/100 µL plus 50 µL of patient serum known to contain an anti-PLA2 antibody.

**Flow cytometry.** Flow cytometry was performed on a FACStar Plus flow cytometer (Becton & Dickinson) fitted with a 100-nm air-cooled argon ion laser using the 488-nm green line for fluorescence excitation. The cell emission spectra were collected on FL1 (green) using a band pass filter 530 DF 30. Dead cells were excluded using propidium iodide on FL3 using a 700 LP filter. Cells or group O platelets for flow cytometry were prepared as described previously.10 Primary antibody incubation, mAb (10 µg/mL) or patient serum (1:20 dilution), was performed for 10 minutes at room temperature in the presence or absence of rifampicin (700 µg/mL). In experiments carried out in the presence of rifampicin, the working or wash buffer contained the drug beyond this stage at a concentration of 700 or 600 µg/mL, respectively.

**Biotin labeling of glycoprotein Ib-IX subunits.** The platelets (2 × 10^10) were incubated with 168 µmol of sulfoconjugated biotin, and the free biotin was quenched with excess glycine. The platelets were then incubated with one of the following: a mouse IgG1 control (MOPC21; 10 µg/mL), the GPIβ-specific mAb (GRP; 10 µg/mL), or AB or patient serum (40 µL of each with or without the drug at 700 µg/mL) for 30 minutes. In experiments performed in the presence of rifampicin, the working or wash buffer contained the drug beyond this stage at a concentration of 700 or 600 µg/mL, respectively. After 3 washes, the platelets were lysed by resuspension in 500 µL of 0.01 mol/L triethanolamine-buffered saline containing 0.5% Triton X-100, 0.05% Tween-20, and protease inhibitors (PMSF 2 mmol/L, leupeptin 10 µmol/L, aprotinin 10 µmol/L, EDTA 5 mL/L) and incubated for 1 hour. Following centrifugation at 12 000g for 30 minutes, the supernatant (450 µL) was collected; 1 × 10^7 sheep anti-mouse–coated Dynabeads, or 2.25 × 10^7 Dynabeads coated with goat anti-rabbit IgG linked to rabbit anti-human IgG, were added to the mAb or the human serum immunoprecipitates, respectively. After incubation for 2 hours, the Dynabeads were washed 5 times using the MBC-E-1 magnet before resuspension in 10 µL 2 × Laemmli buffer11 and storage at −80°C until analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**SDS-PAGE and detection of proteins.** The 20-µL samples for SDS-PAGE analysis were treated with 0.5 mol/L DTT and boiled for 5 minutes. Free DTT was quenched by 4 µL of 0.5 mol/L iodoacetamide. SDS-PAGE was performed according to the method of Laemmli using 12% Tris-glycine gels (Bio-Rad, Hercules, CA). After electrophoresis, the proteins were transferred to polyvinylidine difluoride (PVDF) membrane. Membranes were blocked overnight in 5% [w/v] skim milk (Diploma) and the following morning washed 5 times in PBS/0.05% Tween-20. Streptavidin HRP diluted 1:2000 in 2% BSA/PBS/0.05% Tween-20 was incubated with the membrane for 60 minutes. After 5 washes, the presence of a signal was detected using the Western blot chemiluminescence reagents according to the manufacturer’s instructions.

**Results**

**Rifampicin-dependent antibody binds to GPIb-IX and not to GPIb-IIIa.**

The patient serum was analyzed using the MAIPA assay. Antibody binding was only seen in the presence of rifampicin. The serum showed rifampicin-dependent antibody binding to platelets when the GPIbα-specific mAb, AK2, was used to capture the antigen (GPIb-IX complex). Similarly, strong rifampicin-dependent antibody binding occurred when the non cross-blocking GPIβ-specific mAb, FMC25, captured the antigen (Figure 1). When a GPIb-IIIa–specific mAb, AP2, SZ21, or SZ22, was used as the capture antibody, a negative result was obtained. These data indicate that the rifampicin-dependent serum contains an antibody that reacts with the GPIb-IX but not GPIb-IIIa complex.

When immunoprecipitation experiments were performed using
human AB or patient serum with and without rifampicin, the GPIb-IX complex components were isolated only in the presence of the patient serum and rifampicin (Figure 2). When the experiment was repeated in the presence of a nonimmune mouse IgG1 (MOPC21) and the GPIX-specific mAb, GRP, immunoprecipitation of the GPIb-IX complex components was observed with GRP in both the presence and absence of the drug, but no specific band was observed with MOPC21. The immunoprecipitation of the GPIb-IX subunits by the patient serum in the presence of rifampicin indicates that the drug-dependent antibody has specificity for the GPIb-IX complex.

**Further localization of rifampicin-dependent antibody binding to GPIX**

Flow cytometry was used to test the binding of the patient antibody to platelets and CHO cells stably transfected with GPIX. A negative control of AB serum detected no specific antibody binding in the presence of the drug on platelets (Figure 3) or CHO cells (not shown). Rifampicin-dependent antibody binding was observed on both the platelets and the CHO αβIX cells. These results confirm that the rifampicin-dependent antibody binds to the GPIb-IX complex.

Flow cytometry was also used to test the binding of the patient antibody to mouse L cells that had been stably transfected with either the human GPIbβ and GPIX cDNA (L βIX cells) or the human GPIbα and GPIbβ cDNA (L αβ cells). Binding was not observed in the absence of rifampicin. In the presence of rifampicin, the drug-dependent antibody bound only to L βIX cells and not to the L αβ cells (Figure 4). These data indicate that the rifampicin-dependent antibody binds specifically to the GPIX subunit of the GPIb-IX complex—GPIX is the only component not present on the L αβ cells.

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**Figure 1. Binding of rifampicin-dependent antibody to platelet surface glycoproteins.** MAIPA assay, an antigen-capture ELISA, was performed using S221, S222, and A2, anti-GPIb-IIIa mAbs, to capture the GPIb-IIIa complex or AK2 (an anti-GPIb mAb) or FMC25 (a non-cross-blocking anti-GPIX mAb) to capture the GPIb-IX complex. The studies were performed in the presence and absence of rifampicin with patient serum (Pt) or the control (AB) serum. Samples were assayed in the presence or absence of rifampin. Binding was not observed with the AB serum in the presence or absence of rifampicin with any of the mAbs. The patient serum did not bind in the absence of rifampin. In the presence of rifampicin, the patient serum gave a positive result in the presence of GPIb-specific mAb AK2 and GPIX-specific mAb FMC25. A negative result was observed with GPIb-IIIa mAbs S21, S222 (data not shown), and A2. These data suggest that the antibody(ies) in the patient serum reacted with GPIb-IX complex but not with the GPIb-IIIa complex.

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**Figure 2. Immunoprecipitation of the GPIb-IX complex using the rifampicin-dependent antibodies.** After labeling the surface proteins of the platelets with biotin, the GPIb-IX complex was immunoprecipitated utilizing AB serum, patient serum, a nonspecific murine IgG1 (MOPC21), and the GPIX-specific mAb, GRP, all in the presence or absence of the drug as indicated. The platelets were lysed, and the immunoprecipitated proteins were collected using Dynabeads coated with the appropriate capture antibodies. The proteins were analyzed by SDS-PAGE, transferred to PVDF membrane, and detected by streptavidin HRP and chemiluminescence reagents. The GPIb-IX complex (GPIbα, 143-kd band; GPIbβ, 24-kd band; and GPIX, 20-kd band) was only detected with the patient serum in the presence of the drug (lane 4) and with the anti-GPIX mAb (GRP) in both the absence (lane 7) and presence (lane 8) of the drug. Nonspecific bands (165 kd and 65 kd) were seen both with the test, and control antibodies in the presence or absence of the drug and were present in no particular pattern.

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**Figure 3. Binding of rifampicin-dependent antibodies to platelets and CHO αβIX cells.** The platelets or CHO αβIX cells were labeled with primary antibody (patient serum or AB serum) in the presence or absence of rifampicin, followed by an FITC-conjugated secondary antibody (rabbit anti-human IgG), and examined by flow cytometry. The AB serum did not bind to the platelets in the presence or absence of the drug (A). A similar result was observed when the CHO αβIX cells were labeled with AB serum in the presence or absence of rifampicin (not shown). The patient serum bound to the platelets (B) and the CHO αβIX cells (C) in the presence of the drug. The shaded peak in each graph represents the binding of the serum in the absence of the drug. Pt indicates patient.

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**Figure 4. Binding of the rifampicin-dependent antibodies to L βIX and L αβ cells.** The L βIX and L αβ cells were incubated with patient serum in the presence or absence of rifampicin, followed by an FITC-conjugated secondary antibody, and examined by flow cytometry. The patient serum did not bind in the absence of the drug (shaded peak). The patient serum bound to the L βIX cells (A) but not to the L αβ cells (B) in the presence of the drug. The shaded peak in each graph represents the binding of the serum in the absence of the drug. The figure illustrates the results from 1 experiment that is representative of the results observed on the 3 occasions the experiment was performed.
Rifampicin- and quinine-induced antibodies bind a similar or identical epitope on GPIX

The MAIPA assay is an antigen-capture ELISA that uses an mAb to capture GPIb-IX complex to which the human drug-dependent antibody has attached to the GP complex at a site distant from the mAb-binding site (Figure 5A). If the human antibody and the murine mAb-binding sites coincide or are in close proximity to each other, the human antibody will cross-block the binding of the mAb, the antigen is not captured, and a negative result is obtained (Figure 5B).

When the experiment was repeated using the anti-GPIX mAb, SZ1, a negative result was obtained because the rifampicin-dependent antibody bound to platelet GPIb-IX blocked the antigen capture by SZ21 (Figure 6A). As we have shown previously (Figure 1), antigen capture was not inhibited when other non-cross-blocking mAbs (GPIbc-specific, AK2, and GPIX-specific, FMC25) were used, and a positive result was obtained with each of these mAbs. These data confirm that the specificity of the antibody in the absence of the drug; Qn, quinine; Rif, rifampicin; Pt, patient.

Discussion

In this study, we have shown that the antibody of a patient with rifampicin-induced thrombocytopenia interacts drug-dependently with the platelet GPIb-IX complex, and not the GPIb-IIIa complex, using flow cytometry, the MAIPA assay, and immuno-precipitation.

GPIb is a major sialoglycoprotein on the platelet cell surface, with approximately 25 000 copies per platelet. GPIb is composed of 2 subunits, the α subunit of approximately 143 kd that is disulphide-bonded to a smaller β subunit of about 24 kd. GPIb is noncovalently linked to GPIX that has a molecular mass of 20 kd. The 3 glycoproteins have leucine-rich motifs, and they exist as a heterodimeric complex in the platelet membrane.5,6,15 The subunits of GPb-IX are encoded by separate genes.7,5,15

To determine which component of the GPIb-IX complex, the rifampicin-dependent antibody, was binding to, we used CHO and mouse L cells stably transfected with various components of this complex as described in “Methods.” Drug-dependent binding of the patient antibody was observed on the CHO αIX cells that contained all 3 components of the complex. When the mouse L cells were transfected with 2 of the 3 components of the complex, binding was only observed on the L βIX cells in the presence of the drug. Binding was not observed on the L αβ cells, indicating that neither of these components contained the epitope recognized by the drug-induced antibodies. These results indicate that the rifampicin-dependent antibodies bind an epitope on GPIX on the platelet surface. The CHO cell line transfected with GP Ibα and GPIX cDNA was not tested because it expressed only GPIb on the cell surface, but GPIX was present in the cytoplasm (see “Methods”).

The rifampicin-dependent antibody was able to inhibit the binding of the mAb SZ1, specific for GPIX, in the competitive MAIPA assay. The binding of this mAb was also inhibited by binding of the quinine-dependent antibody in the same assay, shown in this study and previously reported by our group.17 It is interesting that 2 other drug-induced antibodies could also block the binding of the mAb SZ1 to platelets. Gentilini et al18 recently reported that the ranitidine-induced antibody blocked the binding of SZ1 to platelets, and we have observed the same inhibitory effect previously on the binding by quinidine-induced antibodies.1,19,20 These observations suggest that these 4 drug-induced antibodies and the mAb SZ1 bind to either the same site or sites very close to each other. Because the binding sites of 4 drug-induced antibodies have been mapped to this region of GPIX, it is possible that the epitopes of many other drug-induced antibodies may also be localized to this...
site. The reason for the colocalization of the binding sites of these 4 antibodies is at present unclear because the drugs involved are chemically or structurally dissimilar, except for quinine and its optic isomer, quinidine.

In summary, this is the first study to map the epitope of the rifampicin-dependent antibody to the GPIX subunit of the GPIb-IX complex—more specifically, to a domain that is also the common binding site of 3 other drug-induced antibodies. This region of GPIX obviously plays an important role in epitope formation for drug-induced antibodies. Further elucidation of the characteristics of this region on GPIX is clearly required, and it will provide useful insights into the mechanisms of drug-induced thrombocytopenias.

Acknowledgment

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References

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