Antibodies causing thrombocytopenia in patients treated with RGD-
mimetic platelet inhibitors recognize ligand-specific conformers of α\textsubscript{IIb}/β\textsubscript{3} integrin

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ABSTRACT

RGD-mimetic platelet inhibitors act by occupying the RGD recognition site of \( \alpha_{\text{IIb/IIIa}}/\beta_3 \) integrin (GPIIb/IIIa), thereby preventing the activated integrin from reacting with fibrinogen. Thrombocytopenia is a well-known side effect of treatment with this class of drugs and is caused by antibodies, often naturally occurring, that recognize \( \alpha_{\text{IIb/IIIa}}/\beta_3 \) in a complex with the drug being administered. RGD peptide and RGD-mimetic drugs are known to induce epitopes (ligand-induced binding sites, LIBS) in \( \alpha_{\text{IIb/IIIa}}/\beta_3 \) that are recognized by certain monoclonal antibodies (mAbs). It has been speculated, but not shown experimentally, that antibodies from patients who develop thrombocytopenia when treated with an RGD-mimetic inhibitor similarly recognize LIBS determinants. We addressed this question by comparing the reactions of patient antibodies and LIBS-specific mAbs against \( \alpha_{\text{IIb/IIIa}}/\beta_3 \) in a complex with RGD and RGD-mimetic drugs and by examining the ability of selected non-LIBS mAbs to block binding of patient antibodies to the liganded integrin. Findings made provide evidence that the patient antibodies recognize subtle, drug-induced structural changes in the integrin head region that are clustered about the RGD recognition site. The target epitopes differ from classical LIBS determinants, however, both in their location and by virtue of being largely drug-specific.
INTRODUCTION

Ligand-mimetic platelet inhibitors bind specifically to the arginine-glycine-aspartic acid (RGD) recognition site of $\alpha_{\text{IIb}}/\beta_3$ integrin (GPIIb/IIIa), thereby preventing the activated integrin from reacting with fibrinogen and participating in platelet thrombus formation$^{1,2}$. Two such drugs, tirofiban and eptifibatide, have been shown to reduce adverse complications in patients treated with percutaneous transluminal coronary angioplasty$^{3,4}$ and are in widespread clinical use. Between 0.1 and 2.0 % of patients treated with tirofiban, eptifibatide and other drugs of this class evaluated in clinical trials experienced acute thrombocytopenia, often severe, within a few hours of starting treatment$^5$, a complication now known to be caused by antibodies that recognize $\alpha_{\text{IIb}}/\beta_3$ in a complex with the ligand mimetic drug being administered$^{6-10}$. A unique feature of such antibodies is that they can occur naturally in persons never previously exposed to one of these drugs, enabling thrombocytopenia to develop within a few hours of starting treatment$^7,11$.

Various mechanisms have been identified by which drug-induced antibodies cause thrombocytopenia$^{11,12}$. In patients sensitive to drugs like quinine, certain antibiotics, anticonvulsants and many other medications, soluble drug promotes binding of an otherwise non-reactive immunoglobulin to a platelet membrane glycoprotein by a mechanism that is not fully understood but does not appear to involve a preferred docking site for drug on the target glycoprotein$^{11,13-16}$. In contrast, RGD-mimetic drugs bind to a well-defined recognition site at the
junction of the αIIb beta-propeller and the β3-beta A domain (also designated beta I) of αIIb/β3\textsuperscript{17-20} and induce structural changes in the integrin. Numerous murine monoclonal antibodies have been described that recognize conformational changes (ligand-induced binding sites or LIBS) induced in αIIb/β3 by RGD peptide and by RGD-mimetic platelet inhibitors\textsuperscript{21-24}. By analogy, it has been proposed that antibodies causing thrombocytopenia in patients treated with ligand-mimetic inhibitors likewise recognize structural changes (mimetic-induced binding site, MIBS) induced in the integrin by drug\textsuperscript{5,7,25} but this has not been confirmed by experiment. In this report, we present evidence that antibodies causing thrombocytopenia in patients treated with eptifibatide or tirofiban do recognize structural changes (neoepitopes) induced in αIIb/β3 by these drugs. However, the human antibodies differ from classical LIBS-specific monoclonals in that they are largely drug-specific and appear to recognize subtle, drug-induced structural rearrangements, MIBS, in the integrin head region rather than the more widely dispersed LIBS epitopes.

MATERIALS AND METHODS

Patient antibodies. Antibodies from 43 patients who developed thrombocytopenia following treatment with eptifibatide or tirofiban were initially detected in testing done by the Platelet and Neutrophil Immunology Laboratory of the BloodCenter of Wisconsin. Platelet nadirs in the affected patients averaged 19,000/µl (median 10,000/µl, range 1,000 -102,000/µl). Bleeding symptoms,
consisting in most cases of petechial hemorrhages and ecchymoses, were observed in most patients who had severe thrombocytopenia (platelets < 20,000/μl) and 12 were given platelet transfusions. Twenty patients (47%) had no bleeding symptoms. Nineteen of 34 eptifibatide and 5 of 5 tirofiban samples (62%) were from patients with no known prior exposure to these drugs.

**Drug-dependent antibody detection.** Reactions of patient antibodies with platelets pre-treated with ligand-mimetic drugs or RGDW peptide were characterized by flow cytometry using an LSRII flow cytometer (Becton-Dickinson, San Jose, CA) as previously described. In brief, 5.0 x 10^6 platelets isolated from citrated blood were combined with test serum and eptifibatide 2.4 μM, tirofiban 2.0 μM, xemilofiban 2.7 μM, orbofiban 3.0 μM or RGDW peptide 1.0 mM in a 50 μl volume in Hepes buffer (137mM NaCl, 2mM KCl, 12mM NaHCO₃, 0.3mM NaH₂PO₄, 5mM Hepes, 0.65mM CaCl₂, pH 7.4) and incubated at room temperature for 60 minutes. Platelets were then washed twice in Hepes-buffered saline containing drug at the same concentration as in the primary reaction mixture and bound antibody was detected with FITC-labeled Fab’₂ goat anti-human IgG (H+L) antibody (Jackson ImmunoResearch Laboratories). A reaction was considered to be positive if the signal (median fluorescence intensity) obtained with drug-treated platelets was at least twice the signal obtained with patient serum in the absence of drug and with normal serum in the presence of drug (ratio test serum/control serum > 2.0). Reactions positive by this criterion
invariably exceeded the mean signal obtained with randomly selected normal sera (N=994) by greater than 3.0 S.D.

Reagents. Unless otherwise stated, reagents were purchased from Sigma Aldrich (St Louis, MO). Protein G sepharose was from GE healthcare (Piscataway, NJ); FBS from Hyclone (Logan, UT); F12K media, PBS, G418 and gentamycin from Mediatech (Verndon, VA); Fugene 6 from Roche (Indianapolis, IN); sulfo-NHS LC-biotin from Pierce Biotechnology (Rockford, IL) and FITC goat (fab’)_2 anti-mouse IgG(H+L), FITC goat (fab’)_2 anti-human IgG(H+L), FITC goat (fab’)_2 anti-human IgG(Fc) and phycoerythrin (PE)-labeled streptavidin from Jackson Immunoresearch (West Grove, PA). Eptifibatide and tirofiban were purchased from a local pharmacy. Xemilofiban and orbofiban (active forms) were gifts from GD Searle when that company was an independent entity. RGDW peptide was synthesized and purified by the peptide core laboratory of BloodCenter of Wisconsin.

Monoclonal antibodies (mAbs). Murine monoclonal antibodies specific for α_{Ib/β3} were produced in BalbC mice immunized with α_{IIb/β3} purified from human platelets\textsuperscript{27}. Details of the immunization protocol and selection for hybridomas secreting α_{Iib/β3}-specific mAbs have been described previously\textsuperscript{28}. Other mAbs used were 10E5 (anti-α_{IIb}) from BS Coller, Rockefeller University, 7E3 (anti-β_{3}) from Centocor Inc, Malvern PA), AP3 (anti-β_{3}) and AP2 (anti-α_{Iib}/β_{3}) from the Hybridoma Core laboratory of the Blood Research Institute (Milwaukee, WI).
MAbs were biotinylated with sulfo-NHS-LC-Biotin (Pierce Biotechnology, Rockford, IL) according to manufacturer’s protocol. In preliminary studies, it was found that the biotinylation process itself did not affect the ability of mAbs used in the study to react with $\alpha_{\text{IIb/}}/\beta_3$.

**LIBS-specific monoclonals.** MAbs specific for ligand-induced binding sites (LIBS) in $\alpha_{\text{IIb/}}/\beta_3$ were identified by screening integrin-specific mAbs produced as described above at a concentration of 10.0 $\mu$g/ml against untreated platelets and platelets pre-treated with 1.0 mM RGDW peptide$^{24}$ and selecting those that gave a median fluorescence intensity signal in the presence of peptide at least three times greater than the signal obtained in the absence of peptide. The 14 LIBS-specific mAbs thus selected produced ratios (signal obtained with RGD-treated platelets to that obtained with untreated platelets) ranging from 3.0 to 43.7 (average 11.9, median 6.9). Other LIBS-specific mAbs used were AP5 specific for the N-terminus of $\beta_3$$^{24}$, D3 specific for the $\beta_3$ hybrid/EGF1 domain$^{29}$ (from L Jennings, University of Tennessee), LIBS-6 specific for a $\beta_3$ EGF domain$^{21}$ and PMI-1 specific for the $\alpha_{\text{IIb/}}$ calf-2 domain$^{30}$ (both from M Ginsburg, University of California San Diego). Reactions of the latter four mAbs with RGD-treated and untreated platelets were similar to those of our 14 newly produced LIBS-specific mAbs (average ratio RGD/no RGD 14.3, median 14.2, range 5.2-23.8).
Mapping of mAb binding sites to specific domains of αIIb and β3. Stably transfected CHO cell lines expressing mixed αIIb/β3 integrins (rat/human) were described previously. Monoclonal antibodies (mAbs) were mapped to the α or β subunit of αIIb/β3 on the basis of their reactions with CHO cell lines expressing human αIIb paired with rat β3 or vice versa. Following assignment to an α or β subunit, the mAbs were mapped to the beta propeller domain of αIIb or the beta A domain of β3 on the basis of their reactions with CHO cells expressing αIIb/β3 that was human except for substitution of rat sequence in one of these domains. For example, mAb 290.5 reacted with a mixed integrin consisting of human αIIb and rat β3 and was therefore αIIb-specific. Its binding site was then localized to the beta propeller domain of αIIb by showing that it failed to react when this domain alone consisted of rat sequence but reacted with the reciprocal construct containing only human beta propeller sequence.

Further characterization of mAb binding sites. Sites recognized by mAbs were further defined by investigating their ability to compete with one another for binding. For these studies, human platelets were isolated from whole blood anticoagulated with acid-citrate-dextrose (ACD) and were suspended in phosphate-buffered saline containing 1.0 % albumin. In preliminary studies, the quantity of each mAb required to saturate about 90% of the available platelet αIIb/β3 receptors was determined using flow cytometry. Platelets were then incubated with twice this amount of mAb for 30 minutes at room temperature to block binding sites. Biotinylated mAbs (0.5 ug) were then added to the mixture
and incubated for an additional 30 minutes. Platelets were then washed twice, suspended in 50 μl phycoerythrin (PE)–labeled streptavidin (1:200) for 15 minutes and diluted to 0.2 ml. Platelet-bound PE was measured by flow cytometry. Binding of biotinylated mAb was expressed as a percentage of the signal (median fluorescence intensity, MFI) obtained in the absence of a blocking antibody.

**Inhibition of patient antibody binding by selected mAbs.** Platelets were suspended in 1% BSA in Tyrodes/Hepes buffer (137 mM NaCl, 2 mM KCl, 12 mM NaHCO₃, 0.3 mM NaH₂PO₄, 5 mM Hepes, 0.65 mM CaCl₂, pH 7.4) and were first treated with the drug for which each antibody was specific and then with selected monoclonal antibodies at twice the amounts needed to produce 90% saturation of their targets. Human antibodies were then added in amounts that produced a signal about 90% of the signal obtained with unblocked, drug-treated platelets. Following incubation for 30 minutes at room temperature, platelets were washed once and bound antibody was detected with FITC-labeled goat (Fab’2 anti–human IgG (H + L chain specific). When abciximab was used for blocking, human antibody binding was detected with FITC-labeled goat (Fab’2 anti-human IgG (Fc). Results were expressed as a percentage of the signal (median fluorescence intensity) obtained when platelets were treated with drug and an irrelevant mAb.
Research approvals. Human studies were approved by the Institutional Review Board of the BloodCenter of Wisconsin. Murine studies were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin.

RESULTS

Patient antibodies are mainly specific for the drug that caused thrombocytopenia.

Antibodies from 43 patients who developed thrombocytopenia within 24 hours of treatment with eptifibatide (38 cases) or tirofiban (5 cases) were tested for reactions with intact platelets pre-treated with eptifibatide, tirofiban, the non-approved RGD-mimetic drugs xemilofiban and orbofiban, and RGDW peptide. As expected, all patient samples reacted with platelets pre-treated with the drug that caused thrombocytopenia (Table 1). As illustrated in Figures 1A and 1B for a typical patient with eptifibatide-induced thrombocytopenia, this was the only reaction observed with twenty-seven of 38 eptifibatide-dependent antibodies (72%) and three of five tirofiban-dependent antibodies (60%). However, antibodies from 11 of 38 cases of eptifibatide-induced thrombocytopenia and two of five cases of tirofiban-induced thrombocytopenia also recognized platelets pre-treated with one or more of the other agents (Table 1). Five of 38 sera from patients sensitive to eptifibatide cross-reacted with tirofiban, eight with
xemilofiban, three with orbofiban and seven with RGDW; two of five sera from patients with tirofiban-induced thrombocytopenia cross-reacted with xemilofiban and one of these also recognized eptifibatide-treated platelets. The cross-reaction of serum from a patient with eptifibatide-induced thrombocytopenia against platelets pre-treated with RGDW is shown in Figure 1C. A unique eptifibatide-dependent antibody reacted with platelets treated with all five agents (Figure 1D). With only two exceptions, reactions of the 13 cross-reacting sera were significantly stronger against platelets treated with the drug that caused thrombocytopenia than against platelets treated with any of the other four ligands.

In contrast to patient antibodies, LIBS-specific mAbs tended not to discriminate between ligands.

Figure 2 summarizes reactions of the 38 epifibatide-dependent antibodies, the 5 tirofiban-dependent antibodies and 18 LIBS-specific mAbs against platelets pre-treated with eptifibatide, tirofiban and RGDW peptide. To facilitate comparisons, the reaction of each drug-dependent antibody against platelets pre-treated with the drug that caused thrombocytopenia was assigned a value of 100 and reactions of the same antibody against platelets treated with the other ligands was expressed as a percentage of that value. Similarly, the signal obtained with each LIBS mAb against RGDW-treated platelets was assigned a value of 100 and results obtained with eptifibatide and tirofiban-treated platelets were
compared to that value. As a group, the LIBS-specific mAbs differed significantly from the patient antibodies in that their reactions were relatively independent of the ligand that occupied the RGD recognition site under our experimental conditions. In contrast, reactions of the patient antibodies were largely specific for the drug that caused thrombocytopenia.

**Binding sites for ten monoclonal antibodies (mAbs) were mapped to five subdomains in the \( \alpha_{\text{IIb}}/\beta_3 \) head region.**

Eptifibatide, tirofiban, and RGDW have distinctly different structures (Figure 3), making it extremely unlikely that cross-reactions observed with 13 patient antibodies (Figure 1, Table 1) are due to direct recognition of some common element of drug structure. An alternative possibility is that the patient antibodies recognize epitopes created by structural changes induced in \( \alpha_{\text{IIb}}/\beta_3 \) by the ligand-mimetic agents. If this is the case, failure of most eptifibatide-dependent antibodies to cross-react with tirofiban and vice versa (Figure 1, Table 1) suggests that these two agents, even though they both bind to \( \alpha_{\text{IIb}}/\beta_3 \) by mimicking the structure of RGD, induce different sets of epitopes in the integrin that can be distinguished by the patient antibodies. We examined this possibility by determining whether monoclonal antibodies specific for different epitopes in the “head” domains of the integrin might produce different patterns of inhibition when their ability to block binding of the patient antibodies to their targets was determined.
Ten mAbs specific for epitopes expressed on N-terminal domains of α\textsubscript{IIb} or β\textsubscript{3} (Table 2) were used for these studies. Specific amino acid residues on α\textsubscript{IIb} recognized by mAb 10E5 were previously defined by X-ray crystallography\textsuperscript{17} and approximate binding sites for four others (7E3, AP3, 312.6 and 312.8) were identified on the basis of their reactions with selectively mutated α\textsubscript{IIb}/β\textsubscript{3}\textsuperscript{28,31,32}.

Binding sites for the remaining 5 mAbs were localized to the α\textsubscript{IIb} beta propeller (290.5, 312.2 and 184.2) or the β\textsubscript{3}-beta A (AP2) or hybrid (330.1) domains on the basis of their reactions with CHO cells expressing chimeric forms of α\textsubscript{IIb}/β\textsubscript{3} in which a rat domain (α\textsubscript{IIb} beta propeller or β\textsubscript{3} beta A) was substituted for the human domain and vice versa as described in Methods. Each of the 10 mAbs was tested for its ability to block binding of the others labeled with biotin to platelet α\textsubscript{IIb}/β\textsubscript{3}. As expected, each mAb completely blocked its biotinylated counterpart (Table 3 diagonal). In addition, five pairs of mAbs (the α\textsubscript{IIb}-specific pairs 10E5/290.5, 184.2/312.8 and 312.2/312.6 and the β\textsubscript{3}-specific pairs 7E3/AP2 and AP3/330.1) completely blocked each other but had only partial or no effect on binding of the remaining eight (Table 3). Possible binding sites for the five pairs of mAbs on N-terminal domains of α\textsubscript{IIb} and β\textsubscript{3} based on these patterns of inhibition and prior knowledge of specific amino acid residues recognized by mAbs 10E5, 7E3, AP3, 312.6 and 312.8\textsuperscript{17,28,31-33} are illustrated in Figure 4, where ovals representing antibody footprints are constructed so that, when projected onto the integrin, they occupy a surface area of about 1000 sq. Å, consistent with structural studies showing that the total solvent-accessible
surface area buried in typical monoclonal antibody-antigen complexes ranges from 1300 to 2300 sq. Å (650-1150 sq. Å for each component of the complex)\textsuperscript{34-36}.

The five pairs of mAbs differed from one another in their ability to block binding of eptifibatide and tirofiban-dependent human antibodies to $\alpha_{\text{IIb/}\beta_3}$.

We then examined the ability of the 10 mAbs to inhibit drug-dependent binding to $\alpha_{\text{IIb/}\beta_3}$ of six human drug-dependent antibodies, three specific for eptifibatide (E1, E2, E3) and three specific for tirofiban (T1, T2, T3) that were available in quantities sufficient for these studies. Results are shown in Table 4. MAb 7E3, which recognizes a peptide loop in $\beta_3$ very close to the RGD binding site\textsuperscript{31}, completely blocked each human antibody. The same pattern of inhibition was obtained with the Fab fragment, abciximab, derived from 7E3\textsuperscript{31} (data not shown). MAb AP2, which competes with 7E3 for binding (Table 3), also inhibited each antibody by 34-96%. Patterns of inhibition distinctly different from that of mAbs 7E3 and AP2 were obtained with the other eight mAbs, however. For example, the mAb pair 10E5/290.5 inhibited antibodies E1 and E2 almost totally and inhibited E3 by 50-60% but had little effect on the tirofiban-dependent antibodies T1, T2 and T3. In contrast, the 312.2/312.6 pair inhibited E2 and E3 by 68-87% and inhibited T1 by 41-45% but had little effect on E1, T2 or T3. Several contrasting patterns of inhibition are illustrated in Figure 5.
Acute, severe thrombocytopenia was recognized in patients treated with tirofiban, eptifibatide and other RGD-mimetic platelet inhibitors soon after this class of drugs was introduced. When serologic studies showed that this complication is caused by antibodies specific for $\alpha_{\text{IIb}}/\beta_3$ in a complex with drug, it was suggested that the human antibodies, like LIBS-specific mAbs, might recognize epitopes created by conformational changes induced in the integrin by ligand. Our studies were designed to examine this possibility.

Of 43 patient antibodies studied, 13 reacted with platelets pre-treated with a ligand-mimetic drug in addition to the one that caused thrombocytopenia and/or with RGD-treated platelets (Table 1), consistent with the possibility that this subset of antibodies is specific for LIBS-like epitopes induced in common by various ligands. However, 30 patient antibodies recognized the integrin only in a complex with the drug that caused thrombocytopenia. This behavior differed from that of 18 different LIBS-specific mAbs, which reacted equally well with $\alpha_{\text{IIb}}/\beta_3$ in a complex with eptifibatide, tirofiban and RGD peptide under our experimental conditions (Figure 2). In its resting state, $\alpha_{\text{IIb}}/\beta_3$ assumes a bent configuration in which the head region is in close proximity to the lower leg pieces and epitopes recognized by LIBS mAbs are relatively inaccessible. LIBS antibody binding is favored by ligand-induced rearrangements leading to exposure of these sites. In general, LIBS-specific mAbs tend to recognize
epitopes in the α₁Iib calf domains, the β₃ hybrid domain and the more distal PSI, EGF-like and cystatin domains of β₃ (Table 2) that are totally or partially sequestered in the resting state¹⁷,²⁴. The 18 LIBS mAbs studied by us fit this pattern. The contrasting reaction patterns of LIBS mAbs and patient antibodies against α₁Iib/β₃ occupied by different ligands (Figure 2) suggested that the latter were not specific for classical LIBS determinants.

Drug-dependent binding of each of the patient antibodies was blocked by mAb 7E3 and by abciximab, a Fab fragment derived from 7E3, which recognizes the specificity-determining loop (SDL) of β₃-beta A and adjacent amino acid residues close to the metal ion-dependent adhesion site (MIDAS) and the RGD recognition site³¹,³³. A trivial explanation for this finding would be that abciximab and 7E3 dislodge drug from its binding site. However, abciximab has been shown not to compete with low molecular weight RGD-mimetic inhibitors for binding to the integrin⁴² or to displace bound inhibitor⁴³. Moreover, we found that RGD peptide induces the epitopes recognized by LIBS mAbs PMI-1, LIBS6, 319.1, 322.5 and AP5 even when abciximab is bound to the integrin (Supplemental Figure 1). To characterize the binding sites for patient antibodies more fully, we defined the ability of mAbs specific for subdomains of the α₁Iib/β₃ head structure distinct from the abciximab binding site to interfere with drug-dependent binding of six representative patient antibodies, three eptifibatide- and three tirofiban-specific. As shown in Table 4 and Figure 5, distinctly different patterns of inhibition were observed. For example, antibody E1 was completely
blocked by the 10E5/290.5 mAb pair specific for the cap subdomain of the $\alpha_{\text{IIb}}$ beta propeller\textsuperscript{17,28} but was relatively unaffected by two other mAb pairs (184.2/312.8 and 312.2/312.6) specific for other sites on the beta propeller. In contrast, antibody E2 was blocked by both the 10E5/290.5 and 312.2/312.6 mAb pairs. A plausible interpretation for these findings is that antibodies E1 and E2 recognize sites on the beta propeller located close to the cap subdomain but that their footprints are slightly different, enabling E2 to be blocked by both of the mAb pairs and E1 to be blocked only by 10E5/290.5. In contrast to antibodies E1 and E2, the tirofiban-dependent antibodies T1 and T3 were blocked completely only by 7E3 and a third tirofiban-dependent antibody, T2, was blocked by both 7E3 and AP2. These findings suggest that T1, T2, and T3 occupy at least two distinct footprints on the $\beta_3$-beta A domain. A schematic representation of the likely spatial relationships of binding sites for the six human antibodies is shown in Figure 6.

Evidence suggests that immune thrombocytopenia associated with drugs like quinine and many others is caused by antibodies that directly recognize some element of drug structure and bind to their target in such a way that drug is trapped at the antibody-antigen interface\textsuperscript{11,13,16}. The finding that mAb 7E3 and its Fab fragment abciximab, which bind to an epitope immediately adjacent to the RGD recognition site\textsuperscript{31}, prevented each of six patient antibodies from binding to $\alpha_{\text{IIb}}/\beta_3$ occupied by ligand (Table 4) is consistent with the possibility that the patient antibodies studied, like quinine-dependent antibodies, recognize bound
drug and adjacent amino acid residues. Other findings argue against this possibility, however. As shown in Table 1, 13 of 43 patient antibodies recognized αⅡb/β₃ occupied by a ligand other than the drug that caused thrombocytopenia. Cross-reactions of five antibodies from patients with eptifibatide-induced thrombocytopenia against αⅡb/β₃ occupied by tirofiban are especially noteworthy because these two drugs have very different structures (Figure 3), making it very unlikely that direct contact between antibody and drug is involved in antibody recognition. Moreover, most of the patients had no prior exposure to the drug that caused thrombocytopenia. Substances like tirofiban and eptifibatide are not found in nature and it is extremely unlikely that patients would have potent, naturally occurring antibodies that recognize these structures. Finally, because RGD-mimetic drugs all occupy the same binding pocket on the integrin₁⁹, footprints of the patient antibodies although not necessarily identical, would be tightly localized by a requirement that they contact drug itself. However, blocking studies with various mAbs (Table 4) indicate that the antibody footprints are distinctly different and that not all are overlapping despite being clustered about the 7E3 binding site. Finally, RGD and RGD-mimetic drugs are known to induce significant structural changes in αⅡb/β₃ that can be immunogenic²¹,²⁴,²⁹ whereas drugs like quinine appear to have no preferred binding site¹⁵,²⁰,⁴⁴ and are not known to induce structural changes. These considerations favor the possibility that antibodies causing thrombocytopenia in patients treated with ligand-mimetic drugs are distinctly different from those found in patients with quinine-associated thrombocytopenia, being specific for structural changes (neoepitopes, MIBS)
created adjacent to the RGD recognition site when ligand binds. Findings shown in Figure 2 and the blocking studies summarized in Table 4 indicate that the epitopes recognized are distinct from classical LIBS determinants, both in their locations and in being largely drug-specific.

Other possible interpretations require discussion. Intact mAbs were used for the blocking studies; the Fc component of an intact mAb, although flexible, might interfere with binding of another antibody (mAb or patient) some distance away from its binding site. This seems unlikely to have influenced our findings because the five pairs of mAbs used for blocking interfered only with each other and not with any of the other four pairs (Table 3). A second concern is that the blocking mAbs could, like LIBS-specific mAbs, have induced conformational changes in the integrin that modified epitopes recognized by one or more patient antibodies. LIBS mAbs do induce conformational changes in $\alpha_{\text{IIb}}/\beta_3$ by shifting its equilibrium toward an “active” conformation$^{23,24,29}$. However, none of the blocking mAbs or 7E3/abciximab had LIBS activity (Table 2) and, as noted, each affected the binding of only one other mAb (Table 3). Thirdly, ligand-induced structural change in integrins is a dynamic process, possibly involving several intermediate conformations$^{17,18}$, any one of which might conceivably be recognized preferentially by some patient antibodies. This is unlikely to have influenced our findings because saturating quantities of ligand-mimetic drugs were maintained in the reaction mixtures throughout the assays to maximize activation. Moreover, we performed a study in which platelets were treated with tirofiban and
eptifibatide at concentrations ranging from sub-threshold (0.005 ug/ml) to super-maximal (4.0 ug/ml) and found that patient antibody specificities were uniform throughout the entire concentration range (data not shown). Finally, it is possible that more than one ligand-dependent antibody was present in some patient samples, which could explain why patient antibody E3 was at least partially inhibited by each of the blocking mAbs (Table 4). Even if this were the case, the distinctly different inhibitory patterns obtained with the panel of blocking mAbs (Table 4, Figure 5) indicate that the individual patient antibodies are recognizing distinctly different epitopes in the integrin head region.

Further studies are needed to define the structural basis for drug-induced epitopes in the \( \alpha_{\text{IIb}}/\beta_3 \) head region recognized by eptifibatide- and tirofiban-dependent patient antibodies. RGD peptide and RGD-mimetic drugs bind to a defined pocket in the head region of \( \alpha_{\text{IIb}}/\beta_3 \) where a basic nitrogen of the ligand forms a hydrogen bond with Asp 224 of \( \alpha_{\text{IIb}} \) and an acidic group contacts the Mg++ in the \( \beta_3 \) MIDAS\textsuperscript{17,20}. Eptifibatide, tirofiban and RGDW peptide differ slightly in the distance between their positive and negative charges\textsuperscript{17,20} and mimetic compounds differ in the flexibility of covalent bonds that connect the Arg-mimetic group and Asp-mimetic groups. For example, tirofiban has a more flexible alkyl-oxy chain connecting to its piperidine moiety than the homoarginine connecting to the guanidinium group in eptifibatide. Varying degrees of flexibility among ligand-mimetics could influence allosteric effects on integrin conformation around the ligand binding pocket, leading to subtle ligand-mimetic specific
structural changes in the surrounding regions of the integrin and creating ligand mimetic-specific epitopes (MIBS) recognized by patient antibodies. Xiao et al resolved the crystal structures of constructs comprising αIIb residues 1-452 and β3 residues 1-440 in a complex with eptifibatide and tirofiban and compared these structures with that of unliganded αvβ3. Movements up to 5.3 Å in magnitude resulting from ligation were observed in various beta strands and helical structures of the β3-beta A domain but ligand-specific structural differences were not described. A possible explanation for this is that patient antibodies might recognize (and perhaps stabilize) drug-specific structural isomers that are present in aqueous solution but are lost upon crystallization. We recently succeeded in producing a murine monoclonal antibody that recognizes αIIb/β3 only in a complex with eptifibatide and thus closely mimics the behavior of antibodies that cause thrombocytopenia in patients treated with this drug. Further studies with this mAb may enable identification of a ligand-specific epitope at the structural level.

Thrombocytopenia in patients treated with RGD ligand-mimetic platelet inhibitors can be life-threatening and drugs that are equally effective but do not cause thrombocytopenia would be desirable. Zhu and co-workers recently described an inhibitor designated RUC-1, which like eptifibatide and tirofiban, blocks binding of fibrinogen to the activated integrin. However, RUC-1 contacts only Asp-224 and adjacent αIIb amino acid residues and fails to induce the “open” (activated) integrin configuration or the epitope recognized by the LIBS antibody.
APS^{20}. It seems possible that drugs of this type may be incapable of inducing structural changes recognized by ligand-dependent human antibodies and will therefore be much less likely to trigger thrombocytopenia.

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Authorship Contribution

RHA designed the study, provided oversight of experimental work and wrote the manuscript. DWB designed the study, provided oversight of experiments, carried out selected studies and wrote the manuscript. MR performed laboratory studies and contributed to experimental design. JZ aided in interpretation of results and in writing of the manuscript.
Conflicts of interest

The authors declare no conflicts of interest.

REFERENCES


<table>
<thead>
<tr>
<th>Cause of TP</th>
<th>N</th>
<th>Epti</th>
<th>Tiro</th>
<th>Xemi</th>
<th>Orbo</th>
<th>RGDW</th>
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<tr>
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<td>38</td>
<td>38</td>
<td>5</td>
<td>8</td>
<td>3</td>
<td>7</td>
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<td>Tirofiban</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>2</td>
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<td>0</td>
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*Values shown indicate number of samples that gave positive reactions against platelets treated with the indicated ligands. Epti = eptifibatide, Tiro = tirofiban, Xemi = xemilofibin, Orbo = orbofiban. TP = thrombocytopenia*
Table 2. Characteristics of monoclonal antibodies mapped to subdomains of the $\alpha_{IIb}/\beta_3$ head structure

<table>
<thead>
<tr>
<th>Designation</th>
<th>Glycoprotein</th>
<th>Specificity</th>
<th>Domain</th>
<th>Amino acids</th>
<th>Reference</th>
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<td>$\alpha_{IIb}$</td>
<td>Beta</td>
<td>propeller</td>
<td>77-82, 206, 208, 213-215</td>
<td>17</td>
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<tr>
<td>290.5</td>
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<td>Beta</td>
<td>propeller</td>
<td></td>
<td></td>
</tr>
<tr>
<td>184.2</td>
<td>$\alpha_{IIb}$</td>
<td>Beta</td>
<td>propeller</td>
<td></td>
<td></td>
</tr>
<tr>
<td>312.8</td>
<td>$\alpha_{IIb}$</td>
<td>Beta</td>
<td>propeller</td>
<td></td>
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<td>Beta</td>
<td>propeller</td>
<td></td>
<td></td>
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<tr>
<td>312.6</td>
<td>$\alpha_{IIb}$</td>
<td>Beta</td>
<td>propeller</td>
<td></td>
<td>28</td>
</tr>
<tr>
<td>PMI-1</td>
<td>LIBS-$\alpha_{IIb}$</td>
<td>Calf-2</td>
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<td>844-859</td>
<td>30</td>
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<tr>
<td>7E3</td>
<td>$\beta_3$</td>
<td>Beta</td>
<td>A</td>
<td>129, 177-184</td>
<td>32</td>
</tr>
<tr>
<td>AP2</td>
<td>$\beta_3$</td>
<td>Beta</td>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP3</td>
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<td>PSI/hybrid</td>
<td></td>
<td>50-62</td>
<td>33</td>
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<td>330.1</td>
<td>$\beta_3$</td>
<td>PSI/hybrid</td>
<td></td>
<td></td>
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<tr>
<td>AP5</td>
<td>LIBS-$\beta_3$</td>
<td>PSI</td>
<td></td>
<td>1-6</td>
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<td>LIBS-$\beta_3$</td>
<td>Hybrid/EGF</td>
<td></td>
<td></td>
<td>29</td>
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<tr>
<td>LIBS-6</td>
<td>LIBS-$\beta_3$</td>
<td>EGF</td>
<td></td>
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Table 3. Reciprocal blocking studies performed with 10 α\textsubscript{IIb}/β\textsubscript{3}-specific monoclonal antibodies*

<table>
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<tr>
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<th>10E5</th>
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<th>184.2</th>
<th>312.8</th>
<th>312.2</th>
<th>312.6</th>
<th>7E3</th>
<th>AP2</th>
<th>AP3</th>
<th>330.1</th>
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<td>23.9</td>
<td>5.7</td>
<td>8.6</td>
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<td>100.0</td>
<td>12.5</td>
<td>14.1</td>
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<td>25.7</td>
<td>4.0</td>
<td>15.4</td>
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<td>24.9</td>
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<td>99.0</td>
<td>23.4</td>
<td>14.6</td>
<td>28.9</td>
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<td>33.4</td>
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<td>37.1</td>
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<td>100.0</td>
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<td>41.6</td>
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<td>43.0</td>
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<td>20.3</td>
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<td>3.7</td>
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<tr>
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<td>13.6</td>
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<td>29.4</td>
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<td>4.5</td>
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<td>24.0</td>
<td>10.0</td>
<td>14.1</td>
<td>25.1</td>
<td>10.3</td>
<td>99.6</td>
<td>100.0</td>
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</table>

* Platelets were incubated with saturating quantities of the unlabeled (blocking) monoclonal antibodies (mAb) listed in the first horizontal row or with buffer alone. After a single wash with buffer solution, biotinylated mAbs listed in the first column were added and bound antibody was detected with PE-labeled streptavidin using flow cytometry. Values shown (average of triplicate determinations) indicate the extent to which “blocking” mAbs reduced the signal obtained with unblocked platelets (expressed as percent inhibition). Reactions inhibited by 45% or more are shaded light gray; reactions inhibited by 90% or more are shaded dark gray.
Table 4. Inhibition of eptifibatide- and tirofiban-dependent patient antibodies by GPIIb- and GPIIIa-specific monoclonals*

<table>
<thead>
<tr>
<th>Pt aby</th>
<th>Monoclonal antibodies</th>
<th>10E5</th>
<th>290.5</th>
<th>184.2</th>
<th>312.8</th>
<th>312.2</th>
<th>312.6</th>
<th>7E3</th>
<th>AP2</th>
<th>AP3</th>
<th>330.1</th>
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<tbody>
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<td>97.0</td>
<td>96.6</td>
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<td>99.4</td>
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<tr>
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<tr>
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<td>45.2</td>
<td>41.1</td>
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<td>93.2</td>
<td>44.9</td>
<td>40.9</td>
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<td>17.6</td>
<td>25.6</td>
<td>97.5</td>
<td>95.8</td>
<td>17.8</td>
<td>4.5</td>
</tr>
<tr>
<td>T3</td>
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<td>16.1</td>
<td>11.1</td>
<td>5.7</td>
<td>22.9</td>
<td>17.4</td>
<td>29.3</td>
<td>93.7</td>
<td>34.4</td>
<td>44.8</td>
<td>58.2</td>
</tr>
</tbody>
</table>

* Platelets were treated with tirofiban or eptifibatide and were then incubated with saturating quantities of the indicated monoclonal antibodies or with buffer alone. Antibodies (aby) from patients (Pt) who experienced thrombocytopenia after treatment with eptifibatide (E1, E2, E3) or tirofiban T1, T2, T3) were then added and platelet-bound human IgG was measured by flow cytometry. Values shown (average of triplicate determinations) indicate the extent to which each mAb reduced the signal obtained with platelets treated with eptifibatide or tirofiban alone (expressed as percent inhibition). Reactions inhibited by 45% or more are shaded light gray; those inhibited by 90% or more are shaded dark gray.
FIGURE LEGENDS

Figure 1: Representative reactions of patient antibodies. Patient samples were tested against platelets pre-treated with eptifibatide (E), tirofiban (T), xemilofiban (X), orbofiban (O) and RDGW peptide (R). A. Serum from a typical patient with eptifibatide-induced thrombocytopenia reacted with platelets pre-treated with eptifibatide (solid tracing) but not with untreated platelets (dashed tracing) using flow cytometry. B. The same serum failed to recognize platelets pre-treated with T, X, O and R. C. A second patient sample reacted with platelets pre-treated with E or R. D. A third antibody recognized platelets pre-treated with each of the five ligands. Reactions of patient serum with untreated platelets and normal serum with treated platelets were negative (not shown). Horizontal dashed line indicates upper limit of normal (mean + 3.0 SD). In figures 1B, 1C and 1D, values on the ordinate indicate ratio of median fluorescence intensity value obtained with ligand-treated platelets to value obtained with untreated platelets.

Figure 2. Reactions of patient antibodies and LIBS-specific mAbs against platelets pre-treated with saturating concentrations of eptifibatide (E), tirofiban (T) and RGDW peptide (R) (flow cytometry). For each antibody group, signals (median fluorescence intensity) obtained with E (left), T (center) and R (right) were expressed as “100 percent” and signals obtained with other
ligands were expressed as a percent of this value. Bars denote averages for 38 eptifibatide, 5 tirofiban and 18 LIBS antibodies. *** = p < 0.001 compared with signal obtained with E (left), T (center) and R (right). NS = not significant.

Figure 3. Structures of eptifibatide, tirofiban and RGDW peptide.

Figure 4. Ribbon diagram of the $\alpha_{\text{IIb}}/\beta_3$ head region indicating possible binding footprints of 5 monoclonal antibody pairs. Structural coordinates were from Protein Data Bank (3FCS) (19) viewed with WebLab viewer Pro 3.7 (from Molecular Simulations Inc, Princeton NJ). Amino acid residues 606-959 of $\alpha_{\text{IIb}}$ and 561-690 of $\beta_3$ were omitted for the sake of clarity. Alpha helical structures are red and beta sheets are turquoise. Solid ovals represent likely footprints of mAbs 10E5, 7E3, AP3 and RGD peptide based on prior crystallographic (17) or mutagenic (32, 33) studies. Dashed ovals represent likely binding footprints of seven other mAbs inferred from their reactions with chimeric $\alpha_{\text{IIb}}/\beta_3$ constructs and from blocking studies summarized in Table 3.

Figure 5. Monoclonal antibody pairs produce different patterns of inhibition when tested for their ability to block binding of patient antibodies. Platelets pre-treated with eptifibatide or tirofiban were incubated with saturating quantities of one of ten “blocking” mAbs. The signal (median fluorescence intensity)
obtained with a patient antibody was then determined by flow cytometry and was expressed as a percentage of the signal obtained in the absence of a blocking mAb (% relative binding). Upper frame: The eptifibatide-dependent antibody E2 was blocked almost completely by mAb pairs 10E5/290.5 and 312.2/312.8 specific for the \( \alpha_{\text{IIb}} \) beta propeller and by 7E3 but was relatively unaffected by the other 5 mAbs. Lower frame: In contrast, the tirofiban-dependent antibody T2 was completely blocked by 7E3/AP2 but not by the other four pairs of monoclonals. Values shown are the average of triplicate measurements. Brackets indicate +/- 1.0 S.D.

Figure 6. Schematic representation of likely “footprints” for patient antibodies E1, E2 and E3 and T1, T2 and T3 based on inhibition of antibody binding by mAbs specific for subdomains of the \( \alpha_{\text{IIb}} \) and \( \beta_3 \) head regions. The footprint for mAb 7E3 (and abciximab) is shown by the dashed oval. “RGD” indicates approximate location of the binding site for RGD peptide, eptifibatide and tirofiban.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Antibodies causing thrombocytopenia in patients treated with RGD-mimetic platelet inhibitors recognize ligand-specific conformers of $\alpha_{IIb/\beta3}$ integrin

Daniel W. Bougie, Mark Rasmussen, Jieqing Zhu and Richard H. Aster