Antibodies causing thrombocytopenia in patients treated with RGD-mimetic platelet inhibitors recognize ligand-specific conformers of $\alpha_{IIb}/\beta_3$ integrin

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Arginine-glycine-aspartic acid (RGD)-mimetic platelet inhibitors act by occupying the RGD recognition site of $\alpha_{IIb}/\beta_3$ 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 Abs, often naturally occurring, that recognize $\alpha_{IIb}/\beta_3$ 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 $\alpha_{IIb}/\beta_3$ that are recognized by certain Abs. It has been speculated, but not shown experimentally, that Abs 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 Abs and LIBS-specific mAbs against $\alpha_{IIb}/\beta_3$ in a complex with RGD and RGD-mimetic drugs, and by examining the ability of selected non-LIBS mAbs to block binding of patient Abs to the liganded integrin. Findings made provide evidence that the patient Abs recognize subtle, drug-induced structural changes in the integrin head region that are clustered about the RGD recognition site. The target epitopes differ from classic LIBS determinants, however, both in their location and by virtue of being largely drug-specific. (Blood. 2012;119(26):6317-6325)

Introduction

Ligand-mimetic platelet inhibitors bind specifically to the arginine-glycine-aspartic acid (RGD) recognition site of $\alpha_{IIb}/\beta_3$ 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 angioplasty³ 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 Abs that recognize $\alpha_{IIb}/\beta_3$ in a complex with the ligand mimetic drug being administered.⁶ ⁷ ⁸ A unique feature of such Abs 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 Abs cause thrombocytopenia.¹¹ ¹² In patients sensitive to drugs like quinine, certain antibiotics, anticonvulsants, and many other medications, a soluble drug promotes binding of an otherwise nonreactive Ig to a platelet membrane glycoprotein by a mechanism that is not fully understood but does not appear to involve a preferred docking site for a drug on the target glycoprotein.¹¹ ¹³ ¹⁴ ¹⁵ In contrast, RGD-mimetic drugs bind to a well-defined recognition site at the junction of the $\alpha_{IIb}$ β-propeller and the $\beta_3$-β A domain (also designated β I) of $\alpha_{IIb}/\beta_3$,¹⁷ ¹⁸ ¹⁹ and induce structural changes in the integrin. Numerous murine mAbs have been described that recognize conformational changes (ligand-induced binding sites [LIBS]) induced in $\alpha_{IIb}/\beta_3$ by RGD peptide and by RGD-mimetic platelet inhibitors.²¹ ²² By analogy, it has been proposed that Abs causing thrombocytopenia in patients treated with ligand-mimetic inhibitors likewise recognize structural changes (mimetic-induced binding site [MIBS]) induced in the integrin by drug²³ ²⁴ but this has not been confirmed by experiment. In this report, we present evidence that Abs causing thrombocytopenia in patients treated with eptifibatide or tirofiban do recognize structural changes (neoepitopes) induced in $\alpha_{IIb}/\beta_3$ by these drugs. However, the human Abs differ from classic 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.

Methods

Patient Abs

Abs from 43 patients who developed thrombocytopenia after treatment with eptifibatide or tirofiban were initially detected in testing done by the Platelet and Neutrophil Immunology Laboratory (BloodCenter of Wisconsin). Platelet nadirs in the affected patients averaged 19 000/µL (median 10 000/µL, range 1000-102 000/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 were given platelet transfusions. Twenty (47%) of 43 patients had no bleeding symptoms. Nineteen of 34 eptifibatide and 5 of 5 tirofiban samples (or 24 [62%] of 39 samples) were from patients with no known prior exposure to these drugs.

Drug-dependent Ab detection

Reactions of patient Abs with platelets pretreated with ligand-mimetic drugs or RGDW peptide were characterized by flow cytometry using an
LSRII flow cytometer (BD Biosciences) as previously described. In brief, 5.0 \times 10^6 platelets isolated from citrated blood were combined with test serum and eptifibatide 2.4\,\mu M, tirofiban 2.0\,\mu M, xemilofiban 2.7\,\mu M, orbofiban 3.0\,\mu M, or RGDW peptide 1.0\,\mu M in a 50-\mu L volume in HEPES buffer (137\,\text{mM} \text{NaCl}, 2\,\text{mM} \text{KCl}, 12\,\text{mM} \text{NaHCO}_3, 0.3\,\text{mM} \text{NaH}_2\text{PO}_4, 5\,\text{mM} \text{HEPES}, 0.65\,\text{mM} \text{CaCl}_2, \text{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 Ab was detected with FITC-labeled Fab\(_2\) goat anti-human IgG (H + L) Ab (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 median signal obtained with randomly selected normal sera (N = 994) by > 3.0 SD.

Reagents

Unless otherwise stated, reagents were purchased from Sigma-Aldrich. Protein G sepharose was obtained from GE Healthcare; FBS was obtained from HyClone; F12K media, PBS, G418, and gentamycin were obtained from Mediatech; Eugenol 6 was obtained from Roche; sulfo-NHS-LC-biotin was obtained from Pierce Biotechnology, and FITC goat (Fab\(_2\)) anti-mouse IgG (H + L), FITC goat (Fab\(_3\)) anti-human IgG (Fc), and PE-labeled streptavidin were obtained from Jackson ImmunoResearch Laboratories. Eptifibatide and tirofiban were purchased from a local pharmacy. Xemilofiban and orbofiban (active forms) were gifts from G. D. Searle when that company was an independent entity. RGDW peptide was synthesized and purified by the Peptide Core Laboratory (BloodCenter of Wisconsin).

mAbs

Murine mAbs specific for \(\alpha_{\text{IIb}}\beta_{3}\) were produced in BALB/c mice immunized with \(\alpha_{\text{IIb}}\beta_{3}\)-purified from human platelets. Details of the immunization protocol and selection for hybridomas secreting \(\alpha_{\text{IIb}}\beta_{3}\)-specific mAbs have been described previously. Other mAbs used were 10E5 (anti-\(\alpha_{\text{IIb}}\)) from B. S. Coller (Rockefeller University), 7E3 (anti-\(\beta_{3}\)) from Centocor Inc, AP3 (anti-\(\beta_{3}\)) and AP2 (anti-\(\alpha_{\text{IIb}}\beta_{3}\)) from the Hybridoma Core Laboratory (Blood Research Institute). mAbs were biotinylated with sulfo-NHS-LC-Biotin (Pierce Biotechnology) according to the 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 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 M against untreated platelets and platelets pretreated with 1.0\,\text{mM} RGDW peptide and selecting those that gave a median fluorescence intensity of at least 3 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 \(\beta_{3}\), D3 specific for the \(\beta_{3}\) hybrid/EGF1 domain (from L. Jennings, University of Tennessee), LIBS-6 specific for a \(\beta_{3}\) EGF domain, and PMI-1 specific for the \(\alpha_{\text{IIb}}\) calf-2 domain (both from M. Ginsburg, University of California, San Diego). Reactions of the latter 4 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 \(\alpha_{\text{IIb}}\) and \(\beta_{3}\)

Stably transfected CHO cell lines expressing mixed \(\alpha_{\text{IIb}}\beta_{3}\) integrins (rat/human) were described previously. mAbs were mapped to the \(\alpha\) or \(\beta\) subunit of \(\alpha_{\text{IIb}}\beta_{3}\) on the basis of their reactions with CHO cell lines expressing human \(\alpha_{\text{IIb}}\) paired with rat \(\beta_{3}\) or vice versa. After assignment to an \(\alpha\) or \(\beta\) subunit, the mAbs were mapped to the \(\beta\) propeller domain of \(\alpha_{\text{IIb}}\) or the \(\beta\) A domain of \(\beta_{3}\) on the basis of their reactions with CHO cells expressing \(\alpha_{\text{IIb}}\beta_{3}\) that was human except for substitution of a rat sequence in one of these domains. For example, mAb 290.5 reacted with a mixed integrin consisting of human \(\alpha_{\text{IIb}}\) and rat \(\beta_{3}\) and was therefore \(\alpha_{\text{IIb}}\) specific. Its binding site was then localized to the \(\beta\) propeller domain of \(\alpha_{\text{IIb}}\) by showing that it failed to react when this domain alone consisted of a rat sequence but reacted with the reciprocal construct containing only a 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 PBS containing 1.0% albumin. In preliminary studies, the quantity of each mAb required to saturate about 90% of the available platelet \(\alpha_{\text{IIb}}\beta_{3}\) 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 \\mu M) were then added to the mixture and incubated for an additional 30 minutes. Platelets were then washed twice, suspended in 50 \\mu L of PE-labeled streptavidin (1:200) for 15 minutes and diluted to 0.2 \\mu L. Platelet-bound PE was measured by flow cytometry. Binding of biotinylated mAb was expressed as a percentage of the signal (median fluorescence intensity) obtained in the absence of a blocking Ab.

Inhibition of patient Ab binding by selected mAbs

Platelets were suspended in 1% BSA in Tyrode/HEPES buffer (137\,\text{mM} \text{NaCl}, 2\,\text{mM} \text{KCl}, 12\,\text{mM} \text{NaHCO}_3, 0.3\,\text{mM} \text{NaH}_2\text{PO}_4, 5\,\text{mM} \text{HEPES}, 0.65\,\text{mM} \text{CaCl}_2, \text{pH} 7.4) and were first treated with the drug for which each Ab was specific and then with selected mAbs at twice the amounts needed to produce 90% saturation of their targets. Human Abs were then added in amounts that produced a signal ~ 90% of the signal obtained with unblocked, drug-treated platelets. After incubation for 30 minutes at room temperature, platelets were washed once and bound Ab was detected with FITC-labeled goat (Fab\(_3\)) anti-human IgG (H + L chain–specific). When abciximab was used for blocking, human Ab binding was detected with FITC-labeled goat (Fab\(_3\)) 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 Abs are mainly specific for the drug that caused thrombocytopenia

Abs from 43 patients who developed thrombocytopenia within 24 hours of treatment with eptifibatide (38 cases) or tirofiban...
their reactions were relatively independent of the ligand that platelets were compared with that value. As a group, the LIBS-100, and results obtained with eptifibatide- and tirofiban-treated LIBS mAb against RGDW-treated platelets was assigned a value of a percentage of that value. Similarly, the signal obtained with each cytopenia was assigned a value of 100 and reactions of the same Ab against platelets pretreated with the drug that caused thrombocytopenia was assigned a value of 100 and reactions of the same Ab against platelets pretreated with the drug that caused thrombocytopenia (Table 1). As illustrated in Figure 1A and B for a typical patient with eptifibatide-induced thrombocytopenia, this was the only reaction observed with 27 (72%) of 38 eptifibatide-dependent Abs and 3 (60%) of 5 tirofiban-dependent Abs. However, Abs from 11 of 38 cases of eptifibatide-induced thrombocytopenia and 2 of 5 cases of tirofiban-induced thrombocytopenia also recognized platelets pretreated with one or more of the other agents (Table 1). Five of 38 sera from patients sensitive to eptifibatide cross-reacted with tirofiban, 8 with xemilofiban, 3 with orbofiban, and 7 with RGDW; 2 of 5 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 pretreated with RGDW is shown in Figure 1C. A unique eptifibatide-dependent Ab reacted with platelets treated with all 5 agents (Figure 1D). With only 2 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 4 ligands.

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

Figure 2 summarizes reactions of the 38 eptifibatide-dependent Abs, the 5 tirofiban-dependent Abs, and 18 LIBS-specific mAbs against platelets pretreated with eptifibatide, tirofiban, and RGDW peptide. To facilitate comparisons, the reaction of each drug-dependent Ab against platelets treated with the drug that caused thrombocytopenia was assigned a value of 100 and reactions of the same Ab 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 with that value. As a group, the LIBS-specific mAbs differed significantly from the patient Abs 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 Abs were largely specific for the drug that caused thrombocytopenia.

Binding sites for 10 mAbs were mapped to 5 subdomains in the αIIb/β3 head region

Eptifibatide, tirofiban, and RGDW have distinctly different structures (Figure 3), making it extremely unlikely that cross-reactions observed with 13 patient Abs (Figure 1, Table 1) are due to direct recognition of some common element of drug structure. An alternative possibility is that the patient Abs recognize epitopes created by structural changes induced in αIIb/β3 by the ligand-mimetic agents. If this is the case, failure of most eptifibatide-dependent Abs to cross-react with tirofiban and vice versa (Figure 1, Table 1) suggests that these 2 agents, even though they both bind to αIIb/β3 by mimicking the structure of RGD, induce different sets of epitopes in the integrin that can be distinguished by the patient Abs. We examined this possibility by determining whether mAbs specific for different epitopes in the “head” domains of the integrin

![Figure 1](image1.png)  
**Figure 1. Representative reactions of patient Abs.** Patient samples were tested against platelets pretreated with eptifibatide (E), tirofiban (T), xemilofiban (X), orbofiban (O), and RGDW peptide (R). (A) Serum from a typical patient with eptifibatide-induced thrombocytopenia reacted with platelets pretreated with eptifibatide (solid tracing) but not with untreated platelets (dashed tracing) using flow cytometry. (B) The same serum failed to recognize platelets pretreated with T, X, O, and R. (C) A second patient sample reacted with platelets pretreated with E or R. (D) A third Ab recognized platelets pretreated with each of the 5 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). (B-D) Values on the ordinate indicate ratio of median fluorescence intensity value obtained with ligand-treated platelets to value obtained with untreated platelets.

![Figure 2](image2.png)  
**Figure 2. Reactions of patient Abs and LIBS-specific mAbs against platelets pretreated with saturating concentrations of eptifibatide (E), tirofiban (T), and RGDW peptide (R; flow cytometry).** For each Ab group, signals (median fluorescence intensity) obtained with (left) E, (center) T, and (right) R were expressed as “100%” and signals obtained with other ligands were expressed as a percentage of this value. Bars denote averages for 38 eptifibatide, 5 tirofiban, and 18 LIBS Abs. ***P < .001 compared with signal obtained with (left) E, (center) T, and (right) R. NS indicates not significant.
might produce different patterns of inhibition when their ability to
block binding of the patient Abs to their targets was determined.

Ten mAbs specific for epitopes expressed on N-terminal
domains of $\alpha_{IIb}$ or $\beta_3$ (Table 2) were used for these studies. Specific
amino acid residues on $\alpha_{IIb}$, recognized by mAb 10E5 were
previously defined by x-ray crystallography$^{17}$ and approximate
binding sites for 4 others (7E3, AP3, 312.6, and 312.8) were
identified on the basis of their reactions with selectively mutated
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domains of $\alpha_{IIb}$ or $\beta_3$ (Table 2), and approximate
binding sites for 4 others (7E3, AP3, 312.6, and 312.8) were
identified on the basis of their reactions with selectively mutated
$\alpha_{IIb}/\beta_3$,$^{28,31,32}$ Binding sites for the remaining 5 mAbs were
localized to the $\alpha_{IIb}$ $\beta$ propeller (290.5, 312.2, and 184.2) or the
$\beta_3$-$\beta$ A (AP2) or hybrid (330.1) domains on the basis of their
reactions with CHO cells expressing chimeric forms of
$\alpha_{IIb}/\beta_3$ in which a rat domain ($\alpha_{IIb}$ $\beta$ propeller or $\beta_3$-$\beta$ A) was substituted for
the human domain and vice versa as described in “Mapping of
mAb-binding sites to specific domains of $\alpha_{IIb}$ and $\beta_3$. Each of the
10 mAbs was tested for its ability to block binding of the others
labeled with biotin to platelet $\alpha_{IIb}/\beta_3$. As expected, each mAb
completely blocked its biotinylated counterpart (Table 3
diagonal). In addition, 5 pairs of mAbs (the $\alpha_{IIb}$-specific pairs
10E5/290.5, 184.2/312.8, and 312.2/312.6, and the $\beta_3$-specific
pairs 7E3/AP2 and AP3/330.1) completely blocked each other but
had only partial or no effect on binding of the remaining 8
(Table 3). Possible binding sites for the 5 pairs of mAbs on
N-terminal domains of $\alpha_{IIb}$ and $\beta_3$ 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$^{17,28,31-33}$ are
illustrated in Figure 4, where ovals representing Ab footprints are
constructed so that, when projected onto the integrin, they occupy a
surface area of $\sim$ 1000 sq Å, consistent with structural studies
showing that the total solvent-accessible surface area buried in
typical mAb-Ag complexes ranges from 1300 to 2300 sq Å
(650-1150 sq Å for each component of the complex).$^{34-36}$

**The 5 pairs of mAbs differed from one another in their ability to
block binding of eptifibatide and tirofiban-dependent human
Abs to $\alpha_{IIb}/\beta_3$**

We then examined the ability of the 10 mAbs to inhibit drug-
dependent binding to $\alpha_{IIb}/\beta_3$ of 6 human drug-dependent Abs,
3 specific for eptifibatide (E1, E2, E3), and 3 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,$^{31}$
completely blocked each human Ab. The same pattern of inhibition
was obtained with the Fab fragment, abciximab, derived from
7E3$^{31}$ (data not shown). mAb AP2, which competes with 7E3 for
binding (Table 3), also inhibited each Ab by 34%-96%. However,
patterns of inhibition distinctly different from that of mAbs
7E3 and AP2 were obtained with the other 8 mAbs. For example,
the mAb pair 10E5/290.5 inhibited Abs E1 and E2 almost totally,
and inhibited E3 by 50%-60% but had little effect on the
tirofiban-dependent Abs 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.

**Discussion**

Acute, severe thrombocytopenia was recognized in patients treated
with tirofiban, eptifibatide, and other RGD-mimetic platelet inhibi-
tors soon after this class of drugs was introduced.$^5$ When serologic

\begin{table}
\centering
\caption{Characteristics of mAbs mapped to subdomains of the $\alpha_{IIb}/\beta_3$ head structure}
\begin{tabular}{|c|c|c|c|}
\hline
Designation & Glycoprotein & Domain & Amino acids & Reference \\
\hline
10E5 & $\alpha_{IIb}$ & Beta propeller & 77-82, 206, 208, 213-215 & 17 \\
290.5 & $\alpha_{IIb}$ & Beta propeller & & \\
184.2 & $\alpha_{IIb}$ & Beta propeller & & \\
312.8 & $\alpha_{IIb}$ & Beta propeller & & 28 \\
312.2 & $\alpha_{IIb}$ & Beta propeller & & \\
312.6 & $\alpha_{IIb}$ & Beta propeller & & 28 \\
PMI-1 & LIBS-$\alpha_{IIb}$ & Calf-2 & 844-859 & 30 \\
7E3 & $\beta_3$ & Beta A & 129, 177-184 & 31 \\
AP2 & $\beta_3$ & Beta A & & \\
AP3 & $\beta_3$ & PSI/hybrid & 50-62 & 32 \\
330.1 & $\beta_3$ & PSI/hybrid & & \\
AP5 & LIBS-$\beta_3$ & PSI & 1-6 & 24 \\
D3 & LIBS-$\beta_3$ & Hybrid/EGF & & 29 \\
LIBS-6 & LIBS-$\beta_3$ & EGF & & 21 \\
\hline
\end{tabular}
\end{table}

$^*$LIBS indicates ligand-induced binding site; PSI, plexin-semaphorin-integrin; and EGF, epidermal growth factor.
studies showed that this complication is caused by Abs specific for α_{IIb}/β_{3} in a complex with drug,7,9,10,26 it was suggested that the human Abs, like LIBS-specific mAbs, might recognize epitopes created by conformational changes induced in the integrin by ligand.5,7,25 Our studies were designed to examine this possibility. Of 43 patient Abs studied, 13 reacted with platelets pretreated 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 Abs is specific for LIBS-like epitopes induced in common by various ligands. However, 30 patient Abs 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 α_{IIb}/β_{3} in a complex with eptifibatide, tirofiban, and RGD peptide under our experimental conditions (Figure 2). In its resting state, α_{IIb}/β_{3} assumes a bent configuration in which the head region is in close proximity to the lower leg pieces17,37 and epitopes recognized by LIBS mAbs are relatively inaccessible.17,19,38 LIBS Ab binding is favored by ligand-induced rearrangements leading to exposure of these sites.19,39-41 In general, LIBS-specific mAbs tend to recognize epitopes in the α_{IIb} calf domains, the β_{3} hybrid domain, and the more distal PSI, epidermal growth factor–like, and cystatin domains of β_{3} (Table 2) that are totally or partially sequestered in the resting state.17,24 The 18 LIBS mAbs we studied fit this pattern. The contrasting reaction patterns of LIBS mAbs and patient Abs against α_{IIb}/β_{3} occupied by different ligands (Figure 2) suggested that the latter were not specific for classic LIBS determinants.

Table 3. Reciprocal blocking studies performed with 10 α_{IIb}/β_{3}–specific mAbs

<table>
<thead>
<tr>
<th>Blocking (unlabeled) monoclonal</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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<td>99.4</td>
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<td>7.8</td>
<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>
</tr>
</tbody>
</table>

Platelets were incubated with saturating quantities of the unlabeled (blocking) mAbs 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 Ab 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 percentage of inhibition).

Bold indicates reactions inhibited by 90% or more; and underlined values, reactions inhibited by 45% or more.

Figure 4. Ribbon diagram of the α_{IIb}/β_{3} head region indicating possible binding footprints of 5 mAb pairs. Structural coordinates were from Protein Data Bank (3FCS)19 viewed with WebLab viewer Pro 3.7 (Molecular Simulations Inc). Amino acid residues 606-959 of α_{IIb} and 561-690 of β_{3} were omitted for the sake of clarity. Alpha helical structures are red, and β sheets are turquoise. Solid ovals represent likely footprints of mAbs 10E5, 7E3, AP3, and RGD peptide based on prior crystallographic17 or mutagenic31,32 studies. Dashed ovals represent likely binding footprints of 7 other mAbs inferred from their reactions with chimeric α_{IIb}/β_{3} constructs and from blocking studies summarized in Table 3.
Drug-dependent binding of each of the patient Abs was blocked by mAb 7E3 and by abciximab, a Fab fragment derived from 7E3, which recognizes the specificity-determining loop of β3-β A and adjacent amino acid residues close to the metal ion-dependent adhesion site and the RGD recognition site.32,33 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 inhibitor.43 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, available on the Blood Web site; see the Supplemental Materials link at the top of the online article). To characterize the binding sites for patient Abs more fully, we defined the ability of mAbs specific for subdomains of the αIIb/β3 head structure distinct from the abiciximab-binding site to interfere with drug-dependent binding of 6 representative patient Abs, 3 epifibatide- and 3 tirofiban-specific. As shown in Table 4 and Figure 5, distinctly different patterns of inhibition were observed. For example, Ab E1 was completely blocked by the 10E5/290.5 mAb pair specific for the cap subdomain of the αIIbβ propeller17,28 but was relatively unaffected by 2 other mAb pairs (184.2/312.8 and 312.2/312.6) specific for other sites on the β propeller. In contrast, Ab E2 was blocked by both the 10E5/290.5 and 312.2/312.6 mAb pairs. A plausible interpretation for these findings is that Abs E1 and E2 recognize sites on the β 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 Abs E1 and E2, the tirofiban-dependent Abs T1 and T3 were blocked completely only by 7E3 and a third tirofiban-dependent Ab, T2, was blocked by both 7E3 and AP2. These findings suggest that T1, T2, and T3 occupy at least 2 distinct footprints on the β3-β A domain. A schematic representation of the likely spatial relationships of binding sites for the 6 human Abs is shown in Figure 6.

Evidence suggests that immune thrombocytopenia associated with drugs like quinine and many others is caused by Abs that directly recognize some element of drug structure and bind to their target in such a way that the drug is trapped at the Ab-Ag interface.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,33 prevented each of 6 patient Abs from binding to αIIb/β3 occupied by ligand (Table 4) is consistent with the possibility that the patient Abs studied, like quinine-dependent Abs, recognize bound drug and adjacent amino acid residues. However, other findings argue against this possibility. As shown in Table 1, 13 of 43 patient Abs recognized αIIb/β3 occupied by a ligand other than the drug that caused thrombocytopenia. Cross-reactions of 5 Abs from patients with epifibatide-induced thrombocytopenia against αIIb/β3 occupied by tirofiban are especially noteworthy because these 2 drugs have very different structures (Figure 3), making it very unlikely that direct contact between Ab and drug is involved in Ab 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 Abs that recognize these structures. Finally, because RGD-mimetic drugs all occupy the same binding pocket on the integrin,19 footprints of the patient Abs 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 Ab 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 immunogenic,21,24,29 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 Abs 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 classic 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 Ab (mAb or patient) some distance away from its binding site. This seems unlikely to have influenced our findings because the 5 pairs of mAbs used for blocking interfered only with each other and not with any of the other 4 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 Abs. LIBS mAbs do induce conformational changes in αIIb/β3 by shifting its equilibrium toward an “active” conforma-

tion.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). Third, 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 Abs. 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 subthreshold (0.005 μg/mL) to supermaximal (4.0 μg/mL) and found that patient Ab specificities were uniform throughout the entire concentration range (data not shown). Finally, it is possible that more than one ligand-dependent Ab was present in some patient samples, which could explain why patient Ab 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 Abs are recognizing distinctively different epitopes in the integrin head region.

Further studies are needed to define the structural basis for drug-induced epitopes in the αIIb/β3 head region recognized by eptifibatide- and tirofiban-dependent patient Abs. RGD peptide and RGD-mimetic drugs bind to a defined pocket in the head region of αIIb/β3 where a basic nitrogen of the ligand forms a hydrogen bond with Asp224 of αIIb and an acidic group contacts the Mg$^{2+}$ in the β3 metal ion-dependent adhesion site.17,20 Eptifibatide, tirofiban, and RGDW peptide differ slightly in the distance between their positive and negative charges,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 Abs. 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 αIIb/β3.17,45 Movements up to 5.3 Å in magnitude resulting from ligation were observed in various ß strands and helical structures of the β3-ß4 domain but ligand-specific structural differences were not described.17 A possible explanation for this is that patient Abs might recognize (and perhaps stabilize) drug-specific structural isomers that are present in aqueous solution but are lost on crystallization. We recently succeeded in producing a murine mAb that recognizes αIIb/β3 only in a complex with eptifibatide and thus closely mimics the behavior of Abs 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-threatening5,7,46,47 and drugs that are equally effective but do not cause thrombocytopenia would be desirable. Zhu and coworkers recently described an inhibitor designated RUC-1, which like eptifibatide and tirofiban, blocks
binding of fibrinogen to the activated integrin.\(^20\) However, RUC-1 contacts only Asp224 and adjacent \(\alpha_{\text{IIb}}\) amino acid residues and fails to induce the “open” (activated) integrin configuration or the epitope recognized by the LIBS mAb APS.\(^20\) It seems possible that drugs of this type may be incapable of inducing structural changes recognized by ligand-dependent human Abs and will therefore be much less likely to trigger thrombocytopenia.

Acknowledgments

The authors thank the Platelet and Neutrophil Immunology Laboratory (BloodCenter of Wisconsin, Brian Curtis, PhD, Director) for patient materials, Trudy Holyst (Peptide Core Laboratory) for peptide synthesis, and Renée Bordeaux and Erin Ytte (Hybridoma Core Laboratory) for production of mAbs.

References


This work was supported by grants HL-13629 and HL-44612 from the National Heart, Lung, and Blood Institute.

Authorship

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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This work was supported by grants HL-13629 and HL-44612 from the National Heart, Lung, and Blood Institute.


Antibodies causing thrombocytopenia in patients treated with RGD-mimetic platelet inhibitors recognize ligand-specific conformers of \( \alpha_{\text{IIb}}/\beta_3 \) integrin

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