A Novel Anti-platelet Monoclonal Antibody (3C7)
Specific for the Complex of Integrin αIIbβ3 Inhibits Platelet Aggregation and Adhesion*

Ping Chen†, Chong-Xiu Sun‡, and Jian-Ning Liu§
From the Institute of Molecular Medicine and State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, 22 Hankou Road, Nanjing 210093, China

Activation or ligand binding induces conformational changes in αIIbβ3, resulting in exposure of neoeptopes named ligand-induced binding sites. We reported here a novel monoclonal antibody developed by using Chinese hamster ovary (CHO) cells expressing an activated αIIbβ3 mutant (CHO αIIbβ3Δ177) as the immunogen. This IgG2b named 3C7 was specific for the complex of αIIbβ3 as demonstrated by flow cytometry, immunoprecipitation, and EDTA chelating. The binding of 3C7 to platelets increased significantly when platelets were activated by ADP/thrombin or occupied by RGDS peptides, fibrinogen, or PAC-1, suggesting that 3C7 was an anti-ligand-induced binding site antibody. The antibody failed to bind to the CHO cells expressing another αIIbβ3 mutant (β3Y178A) suggesting that the Cys177-Cys184 loop of β3 was likely the epitope for 3C7. 3C7 inhibited platelet aggregation, which was initiated by ADP or thrombin in a dose-dependent manner (IC50 of 5.6 and 0.05 μg/ml, respectively). The antibody also inhibited platelet adhesion to immobilized fibrinogen but not to fibronectin or collagen. These findings suggested that 3C7 was a potent antagonist of integrin αIIbβ3 and a potential anti-thrombotic agent.

Platelet aggregation and adhesion are the central events in thrombosis and homeostasis (1). Integrin αIIbβ3 (platelet glycoprotein GPIIb/IIIa) is the fibrinogen receptor of platelets. It is in a resting state in the circulation but is activated during platelet aggregation and adhesion. Although the mechanism is not yet fully elucidated, the activation is believed to be induced by conformational changes of αIIbβ3, resulting in a higher affinity for fibrinogen and other adhesive molecules (2). When stimulated by platelet agonists such as ADP or thrombin, integrin αIIbβ3 undergoes rapid conformational changes, which expose fibrinogen binding sites and enables the rapid formation of platelet clots (3). Ligand binding to activated integrin αIIbβ3 further induces expression of neoeptopes or ligand-induced binding sites (LIBS)1 (4, 5).

Platelet activation, aggregation, and adhesion are importantly involved in acute coronary syndromes and following certain intravascular therapeutic interventions (6–9). The central role of integrin αIIbβ3 in thrombosis has led to the development of pharmaceutical agents that block interactions between integrin αIIbβ3 and fibrinogen. The αIIbβ3 antagonists are capable of inhibiting platelet adhesion and aggregation and formation of platelet thrombi at the site of plaque rupture or plaque fissure (10). Because platelet-rich rather than fibrin-rich thrombosis was found to be responsible for many acute complications of angioplasty, the blockade of platelet glycoprotein IIb/IIIa receptor was appreciated as valuable in interventional cardiology. Currently there are three integrin αIIbβ3 antagonists used clinically, abciximab (11), tirofiban (12), and eptifibatide (13). Abciximab is a chimeric Fab created based on a murine monoclonal antibody 7E3. Its mechanism of action is thought to be a spatial hindrance of the receptor as opposed to the RGD binding site. Eptifibatide is a synthetic heptapeptide, and tirofiban is a non-peptide antagonist, both mimicking the structure of RGD. All exhibit high affinity binding to integrin αIIbβ3, inhibit ex vivo platelet aggregation, and have proven useful clinically (14).

In the present study, we examined the in vitro anti-platelet effect and binding property of 3C7, a novel monoclonal antibody against the complex of integrin αIIbβ3 raised with CHO cells expressing an activated integrin αIIbβ3 mutant as the immunogen. We found that 3C7 inhibited fibrinogen-mediated platelet aggregation induced by ADP or thrombin. It blocked platelet adhesion to immobilized fibrinogen but not to fibronectin or collagen. The binding of fibrinogen to platelets was partially blocked by 3C7. More interestingly, 3C7 bound to both non-activated and activated platelets. The binding and affinity increased significantly when platelets were activated by ADP/thrombin or occupied by ligands for the integrin. To our knowledge, 3C7 is the first anti-LIBS monoclonal antibody specific for the complex of αIIbβ3.

EXPERIMENTAL PROCEDURES

Materials—Lipopectamine reagent, pcDNA 3.1(−) Zeo vector, antibiotics G418 sulfate, and Zeocin were purchased from Invitrogen. The RGDS peptide was obtained from Sigma-Aldrich. mAb SZ21 (anti-β3) and SZ22 (anti-αIIb) were from Jiangsu Institute of Hematology (Suzhou, China). The mAb PAC-1 (anti-activated αIIbβ3) was purchased from 3D Biosciences. The mutant BEST kit was from TaKaRa (Dalian, China). Immobilized protein G, sulfo-NHS-LC-biotin, horseradish peroxidase-conjugated goat anti-mouse IgG, and chemiluminescent substrate kit were from Pierce. Fluorescein-conjugated donkey anti-mouse IgG was obtained from Rockland (Gilbertsville, PA). The TMB peroxidase-sub-
The total amount added. Nonspecific binding was determined in the bent assay using avidin coated on microtiter wells and subtracted from determine the amount of total 3C7 binding, the free 3C7 in the super-
cloned by limiting dilution. Expression of individual subunits
Positive colonies were isolated by cylinder cloning and further sub-
sequencing. Mutant GAGATATACATGA-3/H11032 was introduced mutations were confirmed by DNA
membrane. The membrane was blocked and probed for2ha troom
temperature with SZ22 and SZ22. After several washes, the membrane
was incubated with the secondary goat anti-mouse IgG conjugated to horseradish peroxidase and finally developed using the chemilumines-
cence ECL kit.

Flow Cytometry Analysis—Binding of antibodies to platelets and CHO cells expressing integrins were analyzed by flow cytometry as previous described (16). In brief, washed platelets (1×10^7/ml) or cells (5×10^6/ml) were incubated with the primary antibody for 30 min at 4 °C followed by incubation with FITC-conjugated donkey anti-mouse IgG and analyzed on a FACScan (Becton Dickinson). For some experiments, washed platelets were exposed to different treatments prior to incubation with 3C7. To assess the effect of the separate subunit (αIIb or β3), CHO cells expressing αIIbβ3 were firstly treated by incubation with 5 mM EDTA at 37 °C or room temperature for 30 min. To study the effect of the activation state of αIIbβ3, washed platelets were pretreated with 0.1 mM ADP or 0.5 units/ml thrombin at room temperature for 20 min. To study the effect of ligand occupation, washed platelets were incubated directly with 1 mM RGDS at room temperature for 20 min or with fibrinogen or PAC-1 for 45 min after platelets were activated with 0.1 mM ADP or 0.5 units/ml thrombin before addition of 3C7. Finally, to investigate the effect of 3C7 on fibrinogen binding, platelets were activ-
ated with 0.1 mM ADP, then incubated with 300 μg/ml FITC-fibrino-
gen in the presence of various concentrations of 3C7 for 15 min at 37 °C and analyzed by flow cytometry (17).

Platelet Aggregation and Adhesion—Platelet aggregation was performed using citrated platelet-rich plasma. After incubation at 37 °C for 5 min with various concentrations of 3C7, aggregation was initiated by 10 μM ADP or 0.35 units/ml thrombin. The maximal platelet aggregation within 5 min was recorded using an aggregometer. Platelet adhesion to immobilized ligand was done as described previously (18) with one modification; platelets were labeled with sulfo-NHS-LC-Biotin instead of 51Cr. Briefly, 2×10^7 labeled platelets were incubated with various concentrations of 3C7 and then added to each well coated with fibrinogen, fibronectin, or collagen and allowed to adhere at 37 °C for 1 h. The non-adherent platelets were aspirated off before the addition of horseradish peroxidase-conjugated streptavidin to be developed with the TMB substrate. The extent of adhesion at each concentration of 3C7 was detected by comparison with the standard curve and expressed as the percentage of the control in which platelets were not preincubated with 3C7.

RESULTS

Generation and Characterization of Monoclonal Antibody—
Monoclonal antibodies were produced by fusing FO cells with splenocytes from mice immunized with CHO cells expressing αIIbβ3A717, an αIIbβ3 mutant with an active conformation (19). Ten days after fusion, 80% of hybridoma supernatants were positive in enzyme-linked immunosorbent assay against CHO/ αIIbβ3A717 cells. Specific clones were further screened for their reactivity with αIIbβ3. The antibody produced by a positive clone (named 3C7) was identified as IgG2a-immunoglobulin and purified by an affinity column of protein G. Flow cytometry analysis revealed that 3C7 had no binding to CHO cells expressing either β3 or αIIb alone, or αIIIb. In contrast, 3C7

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**Fig. 1.** Specific reactivity of 3C7 with cells expressing αIIbβ3. Cells were washed and incubated with 3C7 followed by FITC-conjugated donkey anti-mouse IgG. Then the cells were washed and analyzed by flow cytometry. A, CHO/IIb; B, CHO/β3; C, CHO/β3; D, CHO/αIIbβ3; E, CHO/αIIbβ3; F, human platelets.
recognized α_{IIIb}β_{3} expressed on CHO cells or platelets (Fig. 1). 3C7 was unable to probe α_{IIIb}β_{3} reduced or non-reduced in Western blot (data not shown).

3C7 Is Specific for α_{IIIb}β_{3} Complex—Because β_{3} is required for the post-translation process of α_{IIIb} (20), the lack of 3C7 binding to α_{IIIb} transfected CHO cells (Fig. 1B) is insufficient to conclude that 3C7 does not recognize α_{IIIb}. Therefore, we used immunoprecipitation to confirm this property of 3C7. As shown in Fig. 2, although anti-β_{3} mAb SZ21 precipitated β_{3} from the lysate of CHO/α_{IIIb}, 3C7 failed to precipitate α_{IIIb} or β_{3} using the lysate of CHO/α_{IIIb} or CHO/α_{IIIb}β_{3}, suggesting that it was not against α_{IIIb} or α_{β}. By contrast, two bands corresponding to α_{IIIb} and β_{3} were found in the 3C7 immunoprecipitation against the lysate of platelets or CHO/α_{IIIb}β_{3} (Fig. 2). Additionally, 3C7 was confirmed to be specific for intact α_{IIIb}β_{3} using EDTA chelating. The CHO cells expressing the complex of α_{IIIb}β_{3} were firstly treated with 5 mM EDTA at 37 °C for 30 min, which was proved to dissociate the complex of α_{IIIb}β_{3} (21), and then binding of 3C7 was assayed by flow cytometry. As a control, the cells were preincubated with 5 mM EDTA at room temperature for 30 min, which is known not to affect the structure of the complex. As shown in Fig. 3, the binding of 3C7 to the cells pretreated with 5 mM EDTA at 37 °C was diminished to the level of background. In contrast, binding of 3C7 to the control cells was not affected as compared with the cells without any treatment.

3C7 Recognizes a LIBS Epitope on Integrin α_{IIIb}β_{3} in Platelets—Because 3C7 was raised by immunizing mice with CHO cells expressing the activated state of α_{IIIb}β_{3} (α_{IIIb}β_{3}Δ17), its binding properties to activated platelets were therefore consequently investigated. First, using platelets activated by ADP or thrombin, 3C7 binding was increased by more than 5-fold (Fig. 4A). Second, 3C7 binding was also increased after the addition of RGDS, fibrinogen or PAC-1 (Fig. 4B). The binding affinity of 3C7 to platelets was also measured in the presence of different platelet ligands. The K_{d} for resting platelets and for platelets activated by ADP or occupied with RGDS were 4.60 ± 2.13, 1.11 ± 0.99, and 1.30 ± 1.22 nM (n = 3), respectively. These data indicated that 3C7 recognized a LIBS epitope. It was reported that certain anti-LIBS antibodies activated α_{IIIb}β_{3} and promoted fibrinogen binding to platelets (22, 23). However, 3C7 was not an activating antibody (Fig. 4, C and D).

Platelet Aggregation Initiated by ADP or Thrombin Was Blocked by 3C7—3C7 inhibited platelet aggregation induced by ADP or thrombin in a dose-dependent manner with an IC_{50} value of 5.6 μg/ml for ADP and 0.05 μg/ml for thrombin, respectively (Fig. 5).

Platelet Adhesion to Immobilized Fibrinogen Was Inhibited by 3C7—α_{IIIb}β_{3} is critical for platelet adhesion to immobilized
IC50 was measured as 0.3 μg/ml fibrinogen in the presence of 3C7. The labeled fibrinogen, binding of fibrinogen to ADP-stimulated platelets was measured with FACS in the presence of 3C7. The maximal platelet aggregation within 5 min was measured, and the aggregation at each concentration of 3C7 was expressed as the percentage of the maximal platelet aggregation in the absence of 3C7. Data were mean ± S.D. of at least three different determinations.

Therefore, Tyr178 was mutated (3Y178A, indicating that the Cys177–Cys184 loop and the expression of 3C7 was monitored using SZ21 (specific for αIIIb, indicating that the Cys177–Cys184 loop was unaffected by the mutation of Tyr178). As shown in Fig. 8, the expression of β3 was monitored using SZ21 (specific for β3) and was unaffected by the mutation of β3Y178A or β3Y178I. However, 3C7 had a full binding to αIIIbβ3Y178I and nearly no binding to αIIIbβ3Y178A, indicating that the Cys177–Cys184 loop was important for c7E3

**DISCUSSION**

Three types of anti-LIBS antibodies against αIIIb or β3 are described in the literature (a) with no effect on platelet aggregation (PMI-1, αIIIb-specific) (25, 26), (b) with inhibition of platelet aggregation (anti-LIBS-1, β3-specific) (4), and (c) with activation of αIIIbβ3 (D3GP3, anti-LIBS-2, -3, -6, β3-specific) (4, 5, 22). To our knowledge, 3C7 is the first anti-LIBS mAb against the complex of αIIIbβ3. It has a higher affinity for active platelets and inhibits both platelet aggregation and adhesion. Moreover, its binding to platelets is enhanced by additional ligand binding.

These differences may be attributed to our new approach, which used CHO cells expressing activated αIIIbβ3 as the immunogen. This was based on the assumption that αIIIbβ3 with different conformations could be expressed on CHO cells where it functioned similar to its counterparts in platelets. Our data confirmed that deletion of the β3 cytoplasmic domain induced conformational changes in the extracellular part of αIIIbβ3 and an exposure of neoepitope LIBS (19). Interestingly, 3C7 recognized the complex of αIIIbβ3 exclusively and did not react with αIIIb or β3. This was demonstrated by EDTA chelating, which caused dissociation of the complex. Our strategy allowed recognition of epitopes not exposed or inaccessible on the resting integrin. In contrast, all other anti-LIBS or activation-dependent mAbs were produced using either whole platelets (27),

**Fig. 5.** Effect of 3C7 on human platelet aggregation. Human PRP was incubated with various concentrations of 3C7 at 37 °C for 5 min. Platelet aggregation was induced by 10 μM ADP (top panel) or 0.35 units/ml thrombin (bottom panel). The maximal platelet aggregation within 5 min was measured, and the aggregation at each concentration of 3C7 was expressed as the percentage of the maximal platelet aggregation in the absence of 3C7. Data were mean ± S.D. of at least three different determinations.

**Fig. 6.** Effect of 3C7 on human platelet adhesion to immobilized ligands. Biotinylated platelets were incubated with various concentrations of 3C7 and then allowed to adhere to wells coated with indicated concentrations of fibrinogen (diamonds) and fibronectin (squares). After non-adherent platelets were removed by washing, adhered platelets were quantitated using horseradish peroxidase-conjugated streptavidin. The extent of adhesion was expressed as the percentage of control platelets adhered without preincubation with 3C7. Data were mean ± S.D. of at least three different experiments.

**Fig. 7.** Inhibition of 3C7 on fibrinogen binding to ADP activated platelets. ADP-treated platelets were incubated with FITC-fibrinogen in the presence of various concentrations of 3C7. Fibrinogen binding at each concentration of 3C7 was expressed as the percentage of the fluorescence intensity in the absence of 3C7. Data shown were mean ± S.D. of at least three different experiments.
explain the discrepant IC50 of 3C7 for its inhibitory effect on receptor-ligand binding. Replacement of the Cys 177–Cys 184 disulfide bond of 3C7 by epitope mapping with ligand mimetic mAbs (24). The disulfide-bonded loop for its role in antibody and ligand binding.

Although it is unclear how ligands interact with αITβ3, it is suggested that native ligands (e.g. fibrinogen) or ligand-mimetic antibodies may directly contact discontinuous binding sites at both subunits, which may constitute a ligand binding pocket (24). Three human αITβ3-specific mAbs (PAC-1 (27), OP-G2 (40), and LJ-CP3 (41)) have the RYD sequence that mimics RGD in their CDR3 regions, indicating that the RYD sequence may occupy the same space as RGD does (42). Although 3C7 inhibits fibrinogen binding to platelets, its binding to platelets is promoted by pretreatment of fibrinogen or RGDS. Therefore, the inhibitory effect of 3C7 seems not because of a direct occupancy of the RGDS binding site. However, when 3C7 binds, it blocks fibrinogen binding to platelets probably because of its spatial effect.

αITβ3 antagonists currently available either bind to the resting integrin or induce a transition of αITβ3 from a resting to ligand competent state (43). This has been implicated in thrombocytopenia occasionally reported after administration of such antagonists including abciximab in man (44,45). Thus, activation-dependent mAbs would be of great interest to develop for therapeutic use. Previous studies suggested that agents recognizing αITβ3 with high affinity and selectivity for the activated rather than resting integrin possess a high therapeutic potential for thromboembolic events (46). Activation-dependent antibodies mimicking fibrinogen may also alleviate certain adverse effects such as increased risk of bleeding encountered with abciximab in a clinic (44). Additionally, antibodies preferentially recognizing ligand-occupied conformations may be of value in diagnosis. In comparison with abciximab, 3C7 is comparable for its binding affinity and inhibitory activity to platelets but is highly specific for the complex of αITβ3. The clinical potential of the unique specificity of 3C7 needs to be evaluated in vivo with animal studies, because αITβ3, the sparse target of abciximab, is widely distributed in diverse tissues and involved in many physiological and pathological events.

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Fig. 8. Effect of Y178A mutation on αITβ3 binding to 3C7. A, CHO cells expressing αITβ3 WT, Y178A, and Y178I were stained with SZ21 and 3C7 followed by incubation with FITC-conjugated donkey anti-mouse IgG. Then the cells were washed and analyzed by flow cytometry. B, the relative fluorescence intensity of 3C7 binding was normalized to β2 expression using SZ21.

platelet membranes (26,28), purified αITβ3 (5), purified αITβ3 mixed with RGDS (4), or synthetic αITβ3 peptides (29) as the immunogen. In addition, a single-chain variable fragment specific for activated αITβ3 was obtained using phage display against activated platelets (30).

Efforts to raise activation-dependent antibodies using whole platelets activated prior to immunization encountered a number of technique difficulties. Our experiments demonstrated an effective approach to solve the problem. First, transfected cells preserve native conformations of the integrin. Second, it allows production of antibodies against different conformations of the integrin specifically made with site-directed mutations.

The disulfide-bonded loop (Cys187–Cys193 of β3, Cys189–Cys191 of β2, and Cys187–Cys184 of β3) laying on the upper surface of the β I domain and projecting from the surface containing sites adjacent to a metal ion-dependent adhesion site (31) is known to be critical for affinity and specificity of receptor-ligand binding. Replacement of the β3 loop with the β2 loop activates binding of αITβ3 to ICAM-1 (32). The mutation of β3 integrin (T188I) at this loop promotes cell spreading in human SCC4 keratinocyte (33). This loop also determines the differential regulation of Rho GTPases by β3 and β2 integrins in intracellular signaling events (34). Moreover, ligand binding sites are found close to or overlapping with residues at this loop by epitope mapping with ligand mimetic mAbs (24). The disruption of the Cys177–Cys184 disulfide bond of β3 affects binding of mAb (AP2, LM609, or c7E3) (35) as well as exposure of the LIBS epitope (35). In this report, we have demonstrated that the Cys177–Cys184 loop of β3 is also important for 3C7 recognizing a unique epitope for the complex of αITβ3. It further confirms the structural importance of this disulfide-bonded loop for its role in antibody and ligand binding.

3C7 preferentially binds to platelets stimulated by ADP or thrombin in vitro. Under the condition of our assay, platelet aggregation stimulated by ADP or thrombin was inhibited with a very different IC50. Both ADP (36) and thrombin (37) activate platelets via different G-protein-coupled receptors and lead to distinct structural rearrangements of αITβ3 (38) This may explain the discrepant IC50 of 3C7 for its inhibitory effect on ADP or thrombin-induced platelet aggregation. 3C7 inhibits platelet adhesion to immobilized fibrinogen but not to fibronectin or collagen. This may be because of the fact that platelet adhesion to fibronectin and collagen is also mediated by other integrins such as αVβ3, αVβ1, α3β1, and α5β1 (39).

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