Inhibition of Platelet-mediated, Tissue Factor–induced Thrombin Generation by the Mouse/Human Chimeric 7E3 Antibody

Potential Implications for the Effect of c7E3 Fab Treatment on Acute Thrombosis and "Clinical Restenosis"

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Abstract

The murine/human chimeric monoclonal antibody fragment (c7E3 Fab) blocks GPIIb/IIIa and $\alpha_v\beta_3$ receptors, inhibits platelet aggregation, and decreases the frequency of ischemic events after coronary artery angioplasty in patients at high risk of suffering such events. Although inhibition of platelet aggregation is likely to be the major mechanism of c7E3 Fab's effects, since activated platelets facilitate thrombin generation, it is possible that c7E3 Fab also decreases thrombin generation. To test this hypothesis, the effects of c7E3 Fab and other antiplatelet agents were tested in a thrombin generation assay triggered by tissue factor. c7E3 Fab produced dose-dependent inhibition of thrombin generation, reaching a plateau of 45–50% inhibition at concentrations $\geq$ 15 μg/ml. It also inhibited thrombin–antithrombin complex formation, prothrombin fragment F_{1+2} generation, platelet-derived growth factor and platelet factor 4 release, incorporation of thrombin into clots, and microparticle formation. Antibody 6D1, which blocks platelet GPIb binding of von Willebrand factor, had no effect on thrombin generation, whereas antibody 10E5, which blocks GPIIb/IIIa but not $\alpha_v\beta_3$ receptors decreased thrombin generation by $\sim$ 25%. Combining antibody LM609, which blocks $\alpha_v\beta_3$ receptors, with 10E5 increased the inhibition of thrombin generation to $\sim$ 32–41%. The platelets from three patients with Glanzmann thrombasthenia, who lacked GPIIb/IIIa receptors but had normal or increased $\alpha_v\beta_3$ receptors, supported $\sim$ 21% less thrombin generation than normal platelets. We conclude that thrombin generation initiated by tissue factor in the presence of platelets is significantly inhibited by c7E3 Fab, most likely in part through both GPIIb/IIIa and $\alpha_v\beta_3$ blockade, and that this effect may contribute to its antithrombotic properties. (*J. Clin. Invest.* 1996. 98:863–874.)

Key words: platelets • thrombin • glycoprotein IIb/IIIa • c7E3 Fab • restenosis

Introduction

Both platelets and coagulation proteins contribute to thrombotic cardiovascular disease via a complex set of interactions, and both antiplatelet agents and anticoagulants are effective against thrombosis. Antiplatelet agents are thought to work primarily by decreasing platelet aggregation, whereas anticoagulants are thought to work primarily by decreasing thrombin formation or inhibiting thrombin after it is formed. However, since activated platelets can facilitate thrombin generation by providing a catalytic surface on which coagulation reactions occur (1) and by releasing an activated form of Factor V (2), it is possible that antiplatelet agents may also function as anticoagulants in vivo. Both quantitative and qualitative mechanisms may contribute to an anticoagulant effect of potent antiplatelet agents: (*a*) a decrease in platelet aggregation and platelet thrombus formation may result in a local decrease in both the mass of platelet membranes on which thrombin can be generated and the number of platelets available to release activated Factor V (quantitative effects), and (*b*) inhibition of the platelet "activation" process may prevent platelet membranes from developing an enhanced catalytic efficiency and may inhibit release and surface expression of activated Factor V (qualitative effects) (1, 2).

The murine/human chimeric 7E3 monoclonal antibody fragment (c7E3 Fab)[1] blocks platelet GPIIb/IIIa and $\alpha_v\beta_3$ vitronectin receptors, producing inhibition of platelet aggregation and platelet thrombus formation (3). Data from the recent Phase III EPIC study indicate that c7E3 Fab decreases the frequency of ischemic events after coronary artery angioplasty or atherectomy in patients at high risk of suffering such events (4). Although the antiplatelet effect of c7E3 Fab in preventing direct mechanical obstruction of the blood vessel by inhibiting platelet aggregation is likely to be the major mechanism for its beneficial effect, it is possible that the potent inhibition of platelet thrombus formation by c7E3 Fab may result in decreased thrombin formation, which in turn could further decrease platelet activation and platelet thrombus formation. Thus, despite being an antiplatelet agent, c7E3 Fab may function as an anticoagulant. Moreover, since thrombin may cause smooth muscle cell migration and intimal hyperplasia (5), processes that may contribute to restenosis, it is possible that decreased thrombin generation by c7E3 Fab may play a role in

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Presented in part at the meeting of the American Society of Hematology, 2–6 December 1994, Nashville, TN. Portions of this work have appeared in abstract form (1994. *Blood.* 84:470a).

Received for publication 24 October 1995 and accepted in revised form 31 May 1996.

J. Clin. Invest.

0021-9738/96/08/0863/12 $2.00
Volume 98, Number 3, August 1996, 863–874

1. *Abbreviations used in this paper:* c7E3 Fab, Fab fragment of mouse human chimeric monoclonal antibody 7E3; DB-cAMP, dibutyryl cyclic AMP; F_{1+2}, prothrombin fragment 1 + 2; FSC, forward angle light scatter; iso-T6, (iso-S)FLLRN-NH_2; MTX, isobutyl-methyl-xanthine; NHS-LC-Biotin, sulfosuccinimidyl 6-(biotinamido) hexanoate; PF4, platelet factor 4; PPP, platelet-poor plasma; PRP, platelet-rich plasma; SSC, side angle light scatter; TAT, thrombin-antithrombin III.

the modest reduction in clinical restenosis observed after 6 mo in c7E3 Fab–treated patients (6).

In vivo studies are required to assess the total impact of c7E3 Fab treatment on thrombin generation after vascular injury. In vitro studies, however, can analyze the effect of c7E3 Fab on the phenomena related to platelet activation that result in enhanced thrombin generation. In the present study, we analyzed the effect of c7E3 Fab and other antiplatelet agents on platelet-dependent, tissue factor–induced thrombin production using modifications of a well characterized thrombin generation assay (7–10).

Methods

Chemicals. BSA, purified human thrombin, hirudin, *N*6,O^2-dibutyryladenosine 3′:5′-cyclic monophosphoric acid (dibutyryl cyclic AMP, DB-cAMP), 3-isobutyl-methyl-xanthine (MTX), prostaglandin I_2 (prostacyclin), Triton X-100, paraformaldehyde, and DMSO were obtained from Sigma Chemical Co. (St. Louis, MO). ADP and arachidonic acid were from Chrono-Log Corp. (Havertown, PA). *H*-D-Phe-Pip-Arg-pNA (S-2238) and human plasmin were from Chromogenix (Mölndal, Sweden). Aspirin was obtained from Mallinckrodt Inc. (Paris, KY). Ionophore A23187, aprotinin, and human fibrinogen were from Calbiochem Corp. (La Jolla, CA). *N*-2 hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (Hepes) was from Eastman Kodak Co. (Rochester, NY). Iodogen, papain, and sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-Biotin) were from Pierce Chemical Co. (Rockford, IL). Reptilase was from Stago (Asnières, France). Other chemicals were from Sigma Chemical Co. or Fisher Scientific Co. (Pittsburgh, PA). Calpeptin was from Alexis (Läufelfingen, Switzerland).

Antibodies. Purified murine monoclonal antibodies 10E5 [anti–GPIIb/IIIa ($\alpha_{IIb}\beta_3$; CD 41/CD61)] (11), 7E3 [(anti–GPIIb/IIIa +$\alpha_v\beta_3$; CD 41/CD61 + CD51/CD61)] (12), 6D1 (anti–GPIbα; CD 42b) (13), 6F1 [anti–GPIa/IIa ($\alpha_2\beta_1$; CD 49b)] (14), and 7H2 [anti–GPIIIa (β_3; CD 61)], which partially inhibits fibrinogen binding to platelets and platelet aggregation (15), have been previously described. 7H2 Fab, which has little effect on fibrinogen binding or platelet aggregation, was obtained by enzymatic digestion with immobilized papain as previously described (15). c7E3 Fab (anti–GPIIb/IIIa +$\alpha_v\beta_3$) (16), the drug used in the EPIC trial (4, 6), was obtained from Centocor (Malvern, PA). Antibody LM609 (anti–$\alpha_v\beta_3$, which inhibits $\alpha_v\beta_3$ function; CD51/CD62), and antibody LM142 (anti-α_v, which does not inhibit $\alpha_v\beta_3$ function; CD51) (17) were generously provided by Dr. David Cheresh (The Scripps Research Institute, La Jolla, CA). Antibodies 10E5 and LM609 were converted into Fab fragments by digestion with ficin according to the manufacturer's directions (Pierce Chemical Co.). Antibody AP3 (anti–GPIIIa; CD 61) (18) was generously provided by Dr. Peter Newman (Blood Center of Southeastern Wisconsin, Milwaukee, WI). Antibodies were diluted from stock solutions in 0.15 M NaCl, 0.01M Tris/HCl, 0.05% sodium azide, pH 7.4, or 0.15 M NaCl, 0.01 M sodium phosphate, 0.05% azide, pH 7.4, with 0.15 M NaCl, 0.01 M Tris/HCl, 0.05% sodium azide, pH 7.4, for use in the assays. In preliminary experiments we determined that azide did not affect the assay results at the concentrations used in the studies.

Biotinylation of antibodies was performed using NHS-LC-Biotin as previously described by Gretch et al. (19).

Defibrinated and native plasma. 200 ml of blood from each of 15 normal voluntary donors (blood group AB) was anticoagulated with 0.013 M sodium citrate. Platelet-poor plasma (PPP) was made by a double centrifugation (2,000 *g* for 10 min at 22°C, and then 5,000 *g* for 10 min at 4°C). The plasmas were pooled, frozen in dry ice/ethanol and stored at −20°C. A single batch of plasma was used for all PPP studies. PPP was defibrinated immediately before use by adding 1/50th vol reptilase, letting the clot form at 37°C for 5 min, keeping the clotted plasma at 0°C for 10 min, and then removing the clot by centrifugation. No clottable fibrinogen was found after this treatment.

Platelet-rich plasma (PRP) for thrombin generation assays. Blood from normal donors was anticoagulated with citrate as above and centrifuged for 10 min at 250 *g* at 22°C. The platelet count was adjusted to 300,000 per μl with PPP prepared from the PRP.

Gel-filtered platelets. Blood from normal donors was anticoagulated with acid-citrate dextrose formula A (ACD-A) (8.5:1.5, vol/vol). PRP was obtained by centrifugation (3 min at 800 *g* at 22°C), removed, and, after adding 1/10 vol of additional ACD-A, centrifuged for 10 min at 2,100 *g* at 22°C. The platelet pellet was reconstituted with 1 ml of TSBG [0.05 M Tris/HCl, 0.15 M NaCl, 0.5% BSA (wt/vol), 5 mM glucose, pH 7.4]. Platelets were then gel filtered through Sepharose 2B (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with TSBG, using the same buffer to elute the platelets.

Through the courtesy of Dr. Harvey Weiss (St. Lukes-Roosevelt Hospital, New York), platelets from three patients with Glanzmann thrombasthenia, who lack functional GPIIb/IIIa receptors as judged by an absence of ADP-induced platelet aggregation, were also gel filtered and studied. All three patients had markedly reduced binding (≤ 25% of normal) of one or more monoclonal antibodies to GPIIb/IIIa (10E5, 7E3, 7H2) and normal or increased platelet $\alpha_v\beta_3$ receptor expression as judged by the binding of radiolabeled antibodies LM609 and/or LM142; the latter studies were performed as previously described (20). One of the patients was studied on two different occasions.

Peptides. The thrombin receptor–activating peptide, racemic (iso-S)FLLRN-NH_2 (iso-T6), was synthesized as previously described (21). RGDF, which inhibits both GPIIb/IIIa and $\alpha_v\beta_3$, was synthesized as previously described (22).

Determination of thrombin generation supported by gel-filtered platelets. Thrombin generation was measured by an adaptation of our previously described techniques (7–10). In 12 × 75-mm polypropylene tubes (Becton Dickinson, Inc., Rutherford, NJ), 240 μl of defibrinated plasma was mixed with 50 μl of gel-filtered platelets and 10 μl of buffer (0.01 M Tris/HCl, 0.15 M NaCl, 0.05% azide, pH 7.4). In some experiments the buffer contained monoclonal antibodies, aspirin, peptides, DB-cAMP, MTX, or prostacyclin. Antibodies were added at 5 to 20 μg/ml final concentrations and incubated for 20 min at 37°C. Thrombin generation was initiated by adding 60 μl of recombinant tissue factor (Innovin; Baxter Healthcare Corp., Deerfield, IL) diluted 1:1,500 in $CaCl_2$ (16.7 mM). The final concentration of tissue factor chosen was 1⁄10th that required to clot PPP in 70 s (10). In some experiments, the volumes of all reagents were increased, but the proportions remained the same. Thrombin generation was assayed at 37°C by measuring thrombin amidolytic activity in samples obtained every 30 s for 15 min. Samples (10 μl) were removed from the incubation mixtures and added to a prewarmed (37°C) mixture of buffer [465 μl; 0.1 M NaCl, 0.05 M Tris/HCl, 0.05% BSA (wt/vol), 20 mM EDTA, pH 7.9] and chromogenic thrombin substrate S-2238 (25 μl; 4 mM). After 3.25 min at 37°C, the reaction was stopped by adding 300 μl of 1 M citric acid, and the OD_{405} was determined (Ultrospec III; Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The assay was calibrated by testing known amounts of purified human thrombin in each assay.

Thrombin generation was calculated from the free thrombin amidolytic activities by a computer program that takes into account the effects of thrombin's neutralization by antithrombin III and other serpins, as well as the reaction between thrombin and α_2-macroglobulin, which results in a complex that retains some chromogenic activity (8, 9, 23). Thrombin generation was expressed as the thrombin potential; that is, the total area under the curve of thrombin concentration vs time. The peak level of thrombin generation was also determined. Results are expressed as absolute values or as a percentage of a control sample that was assayed in parallel. We determined the intraassay coefficient of variation of thrombin potential for the modified assay used in the current study by performing 12 replicates and found it to be 2.9%. Platelets gel filtered 3 h before use in the assay gave results very similar to platelets used within 30 min of gel filtration (1.5±2.4% decrease; $n = 5$), and so platelets were used within 3 h of gel filtration in all experiments.

Assay of GPIIb/IIIa blockade by c7E3 Fab. Iodination of c7E3 Fab with ^{125}I was performed with Iodogen as previously described (20). To assay for GPIIb/IIIa receptor blockade by c7E3 Fab, 200 μl of defibrinated plasma plus gel-filtered platelets, with or without the addition of c7E3 Fab (5 to 50 μg/ml for 20 min at 37°C), was incubated with ^{125}I-labeled c7E3 Fab (20 μg/ml) for 10 min at 22°C. Platelet-bound ^{125}I-c7E3 Fab was separated from free ^{125}I-c7E3 Fab by centrifugation (5 min at 12,000 *g* at 22°C) through 30% sucrose in a microtube. The platelet bound ^{125}I-c7E3 Fab and supernatant ^{125}I-c7E3 Fab were determined separately after cutting off the tip of the tube containing the platelet pellet, and used to calculate the number of molecules bound per platelet (24).

Thrombin–antithrombin III (TAT) complexes and prothrombin fragment 1+2 (F_{1+2}). TAT complexes and F_{1+2} were determined by ELISA using commercially available kits (Enzygnost; Behring, Marburg, Germany). Samples (200 μl) were obtained from thrombin generation assays and thrombin generation was stopped by adding 40 μl of EDTA–hirudin buffer (0.15 M NaCl, 0.05 Tris/HCl, pH 7.4) containing 120 mM EDTA and 60 U/ml hirudin. Samples were immediately centrifuged (10 min at 2,100 *g* at 22°C) and the resultant supernatant fraction rapidly frozen and stored at −20°C.

Platelet aggregation and platelet counts. Platelet aggregation studies were carried out in an aggregometer (Chromo-Log Corp.) at 37°C with stirring. Citrated PRP or gel-filtered platelets suspended in 138 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 12 mM $NaHCO_3$, 2 mM $MgCl_2$, 10 mM Hepes, 5.5 mM glucose, 0.2% BSA (wt/vol), plus 0.3 g/l of human fibrinogen, pH 7.4, were introduced into the aggregometer cuvette at final platelet counts of 300,000–350,000 platelets per microliter. ADP (10 μM), arachidonic acid (1 mM), and the thrombin receptor–activating peptide iso-T6 (5 μM) were used as agonists.

To assess the production of platelet aggregates with volumes greater than 43 fl during the thrombin generation assay, platelet counts were performed at indicated times by removing 5-μl samples from the incubation mixture, diluting them with 20 ml of diluent (Isoton; Coulter Immunology, Hialeah, FL) and analyzing them in a resistive particle counter (Counter Coulter Z1; Coulter Immunology) using lower and upper thresholds of 4 and 43 fl.

Determination of PDGF. PDGF was measured by radioimmunoassay using ^{125}I-PDGF as a standard (Amersham Corp., Arlington Heights, IL). Samples (200 μl) were obtained from the thrombin generation assay and mixed with EDTA–hirudin buffer as for the TAT assay. Two PDGF positive controls were prepared by adding 60 μl of buffer containing 60 μM iso-T6 or 6% Triton X-100 (vol/vol) instead of tissue factor to the thrombin generation assay and incubating for 15 min at 37°C.

Determination of platelet factor 4 (PF4). PF4 was quantified by ELISA. Samples were obtained and thrombin generation was stopped as indicated above for the TAT and PDGF assays. Samples were centrifuged twice (15 min at 2,100 *g* at 22°C and 5 min at 12,000 *g* at 22°C) before freezing.

Microparticle determination by flow cytometry. Samples (50 μl) were obtained from the thrombin generation assay, mixed with 10 μl of EDTA–hirudin buffer, and fixed by adding 1/6th vol 6% paraformaldehyde (wt/vol) at 4°C. Microparticle formation was also investigated in gel-filtered platelets suspended in 137 mM NaCl, 2.7 mM KCl, 16 mM $NaHCO_3$, 5 mM $MgCl_2$, 35 mM Hepes, 5.5 mM glucose, 0.2% BSA (wt/vol), 2.5 mM $CaCl_2$, pH 7.4, and activated with 0.25 U/ml of thrombin or 5 μM ionophore A23187 for 15 min at 37°C. The gel-filtered platelets were then fixed as above. In some experiments, platelets were preincubated (20 min at 37°C) with 20 μg/ml of the c7E3 Fab or 10E5 antibodies.

Platelet microparticles were identified on the basis of their reactivity with antibody 6D1 (anti–GPIbα) and their characteristic light-scattering properties. Fixed platelets (5 μl) were incubated with biotin-labeled 6D1 (20 μg/ml) for 30 min at 22°C, and then 10 μl of FITC streptavidin (Becton Dickinson, Inc.) was added and incubated for 30 min at 22°C. Samples were then diluted in PBS (0.01 M sodium phosphate, 0.15 mM NaCl, pH 7.4). To assess the absolute number of microparticles present in a sample, 5 μl of a suspension containing 1.35×10^5 fluorescent beads of 6.6 μm diameter (Becton Dickinson, Inc.) were added to each diluted sample immediately before flow cytometric analysis to serve as an internal standard. The concentration of beads was determined by microscopy using a hemacytometer (American Optical Scientific Instruments, Buffalo, NY).

Samples were analyzed in a flow cytometer (EPICS Profile; Coulter Immunology) using software supplied by the manufacturer (EPICS Profile Software, version 2.4). The instrument was equipped with a 100 mW argon laser operated at 15 mW power at a wavelength of 488 nm. The flow cytometer was calibrated daily using fluorescent beads (Coulter Immunology). Platelets and microparticles were identified as particles giving a positive fluorescent signal at 530 nm with antibody 6D1. Subsequent analysis was limited to particles that were positive for 6D1 fluorescence. Quadrant analysis in dot-plot histograms was performed by plotting forward angle light scatter (FSC) as a function of side angle light scatter (SSC) at logarithmic settings to separate the added 6.6-μm beads (upper FSC, upper SSC), from the microparticles (lower FSC, lower SSC), and platelets, including small aggregates (upper FSC, lower SSC). The location of the platelets was confirmed using PRP in the absence of antibody labeling. The FSC threshold for distinguishing microparticles from platelets was set at ~0.8 μm diameter as calculated using calibrated beads (Becton Dickinson, Inc.). A minimum of 10,000 fluorescent particles were analyzed in each sample.

The absolute number of microparticles in a sample was calculated by the equation: (number of beads added to sample/number of beads actually counted) × number of microparticles actually counted. The percentage of microparticles was calculated using the formula: 100 × microparticles actually counted/ (microparticles+platelets actually counted).

Thrombin bound to clots. Clots were prepared at 37°C by adding 60 μl of tissue factor diluted in $CaCl_2$ to 240 μl of citrated PPP, 50 μl of either buffer or gel-filtered platelets (final concentration 300×10^9/liter), and 10 μl of buffer (0.01 M Tris/HCl, 0.15 M NaCl, pH 7.4) with or without antibodies. Clots were allowed to form for 30 min, and then were gently squeezed against the test tube wall. Clots were then washed overnight with 10 changes of PBS with 0.05% sodium azide (wt/vol). Clots (estimated vol of 10 μl) were then placed in 90 μl of human plasmin (1 casein U/ml) in the same buffer and dissolved for 16 h at 37°C. Aprotinin (50 KIU) was then added and 10-μl samples were removed and assayed for amidolytic activity as above, but with a 30-min incubation time. Since the effects of plasmin and aprotinin on thrombin amidolytic activity were found to be only modest (14.5±2.5% decrease, $n = 8$), they were not considered in the analysis.

Determination of thrombin generation in platelet-rich plasma. PRP (240 μl) was incubated with 60 μl of either buffer [0.1 M NaCl, 0.05 M Tris/HCl, 0.05% BSA (wt/vol), pH 7.35] alone or buffer containing the monoclonal antibody to be tested, for 10 min at 37°C with gentle stirring. Thrombin generation was initiated by adding either 60 μl of recombinant tissue factor diluted 1:4,000 in 0.1 M $CaCl_2$ or 60 μl of 0.1 M $CaCl_2$ alone. Samples were removed and assayed for thrombin activity as described for the gel-filtered platelet assay, except that sampling and stopping was done with pushbutton-equipped pipettes that automatically recorded the incubation time (~2 min). When the clot formed, it was wound on a plastic spatula and removed.

Statistical analysis. Results are expressed as the mean±SD. Student's *t* test was used for independent or paired data, and ANOVA was used for statistical comparisons. $P < 0.05$ was considered significant.

Results

Gel-filtered platelets

Effect of platelet count and ionophore A23187 activation on thrombin generation. Thrombin potential was profoundly dependent upon the number of platelets present in the incubation mixture (Fig. 1). Increasing the number of platelets resulted in

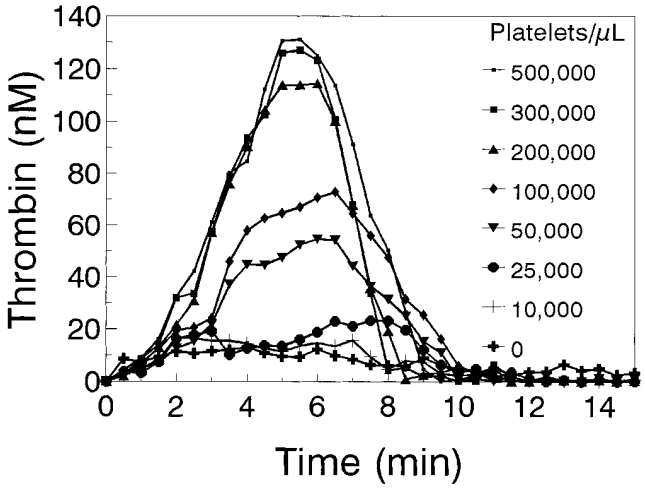

Figure 1. Effect of platelet count on thrombin generation in the reconstituted system. As described in Methods, defibrinated plasma and gel-filtered platelets were mixed together and then tissue factor was added at T = 0. Samples were removed at 30-s intervals and tested for thrombin amidolytic activity. The thrombin generated at each time point was then calculated and plotted. There was minimal thrombin generation in the absence of platelets, and thrombin generation plateaued at 300,000–500,000 platelets/μl. Results are from one of three to six experiments conducted at the different platelet counts.

increased thrombin potential ($r = 0.90$, $P < 0.002$), with near maximal thrombin generation at a platelet count of 300,000 per μl. Thus, this concentration was used in the subsequent studies unless otherwise indicated.

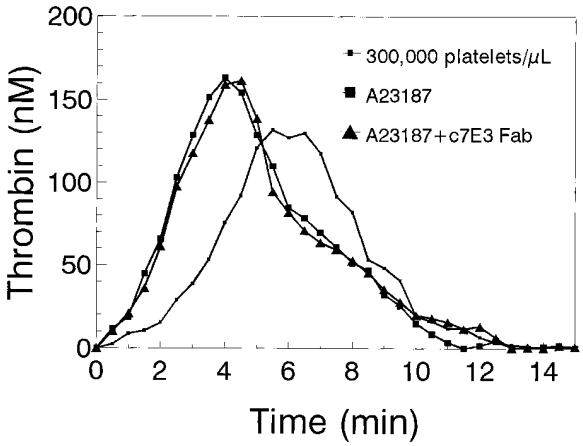

Figure 2. Effect of ionophore A23187 stimulation, alone and in combination with c7E3 Fab, on thrombin generation in the reconstituted system. Thrombin generation was measured as indicated in Methods using: (*a*) gel-filtered platelets (300,000 per μl), (*b*) gel-filtered platelets stimulated with 5 μM ionophore A23187 for 15 min at 37°C (A23187), or (*c*) gel-filtered platelets stimulated with A23187 followed by incubation with 20 μg/ml c7E3 Fab for 20 min at 37°C (A23187 + c7E3 Fab). Ionophore pretreatment resulted in a shortening of the lag time, an increase in peak thrombin concentration, and an increase in the thrombin potential (area under the curve). Addition of c7E3 Fab to platelets with ionophore did not significantly decrease thrombin generation. Results are from one of three similar experiments.

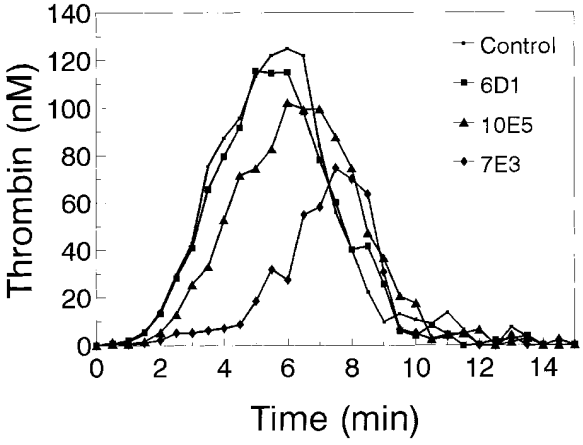

Figure 3. Effect of monoclonal antibodies 6D1, 10E5, and 7E3 on thrombin generation. Thrombin generation was measured as described in Methods with a final platelet count of 300,000 per μl. Samples were either untreated (*Control*) or preincubated with monoclonal antibodies 6D1 (anti–GPIbα), 10E5 (anti–GPIIb/IIIa), or 7E3 (anti–GPIIb/IIIa + $\alpha_v\beta_3$) at 20 μg/ml. Antibody 6D1 had minimal effect on thrombin potential, 10E5 reduced thrombin potential by ~23%, and 7E3 decreased thrombin potential by ~47%. Results are of one representative experiment from 10 to 18 separate experiments employing the different antibodies.

When gel-filtered platelets were treated with ionophore A23187 (5 μM) for 15 min at 37°C to produce microparticles before beginning the assay, the thrombin potential was increased by 20±4% and the peak thrombin concentration was 18±3% greater ($n = 4$; $P < 0.05$ for both parameters) (Fig. 2).

Effect of monoclonal antibodies 6D1, 6F1, 7E3, and c7E3 Fab. The effects of monoclonal antibodies directed against platelet membrane glycoproteins on thrombin generation are

Table I. Effect of Antibodies, Heparin, and the Peptide RGDF on Thrombin Generation Supported by Gel-filtered Platelets

Antibody or peptide	*n*	Thrombin potential	Peak thrombin concentration
		*% inhibition**	*% inhibition**
6D1 (20 μg/ml)	11	3±6‡	3±4
6F1 (20 μg/ml)	4	3±9	7±7
10E5 (20 μg/ml)	18	23±5§	27±7§
7E3 (20 μg/ml)	10	47±9§‖	47±6§‖
c7E3 Fab (5 μg/ml)	15	18±7¶	25±7§
c7E3 Fab (10 μg/ml)	16	38±9§‖	43±12§‖
c7E3 Fab (15 μg/ml)	15	47±5§‖	51±7§‖
c7E3 Fab (20 μg/ml)	31	47±5§‖	50±7§‖
c7E3 Fab (50 μg/ml)	3	46±5**‡‡	50±15**‡‡
RGDF (50 μM)	4	24±5**	32±6**
RGDF (100 μM)	4	44±9**	51±6**
RGDF (500 μM)	4	44±9**	52±5**
7H2 (20 μg/ml)	5	22±17**	30±13**
7H2 Fab (20 μg/ml)	5	9±14	15±14
AP3 (20 μg/ml)	4	24±5**	31±4**

*Compared to control of gel-filtered platelets; ‡mean±SD; **$P < 0.05$; ¶$P < 0.01$; §$P < 0.001$ compared with control; ‡‡$P < 0.05$; ‖$P < 0.001$ compared with 20 μg/ml 10E5.

Table II. Effects of Combinations of Anti–GPIIb/IIIa and Anti–$\alpha_V\beta_3$ Antibodies on Thrombin Generation Supported by Gel-filtered Platelets during the Thrombin Generation Assay

Antibody	*n*	Thrombin potential	Peak thrombin concentration
		*% inhibition**	*% inhibition**
A[‡]			
LM609 (20 μg/ml)	7	5±5[§]	12±6
10E5 (20 μg/ml)	7	23±4	26±8
10E5 + LM609	7	32±5[‖]	38±10[‖]
LM142 (20 μg/ml)	7	1±4	1±3
10E5 (20 μg/ml)	7	22±4	27±10
10E5 + LM142	7	23±4	28±11
LM609 (20 μg/ml)	3	6±2	9±2
c7E3 Fab (20 μg/ml)	3	48±2	46±3
c7E3 Fab + LM609	3	48±0	40±2
LM142 (20 μg/ml)	3	−1±5	−2±1
c7E3 Fab (20 μg/ml)	3	48±2	46±3
c7E3 Fab + LM142	3	47±1	41±3
B			
LM609 (20 μg/ml)	4	6±5	10±5
LM 609 Fab (20 μg/ml)	4	5±4	11±5
10E5 (20 μg/ml)	4	27±7	28±10
10E5 Fab (20 μg/ml)	4	23±6	24±19
10E5 + LM609	4	38±9[‖]	41±11[‖]
10E5 Fab + LM609 Fab	4	41±10[π]	43±7[π]

* Compared to control of gel-filtered platelets; [‡]experiments in groups *A* and *B* were conducted with different antibody preparations and were separated in time by more than 1 yr; [§]mean±SD; [‖]$P < 0.01$ compared with 10E5 alone; and [π]$P < 0.01$ compared with 10E5 Fab alone.

shown in Tables I and II, with selected effects depicted in Fig. 3. Antibodies 6D1 (anti–GPIbb) and 6F1 (anti–GPIa/IIa) had virtually no effect on thrombin generation compared to control (Table I). Antibody 10E5 (anti–GP IIb/IIIa), as well as its Fab fragment, in contrast, inhibited both thrombin potential and peak thrombin concentration by ~ 25% (Tables I and II).

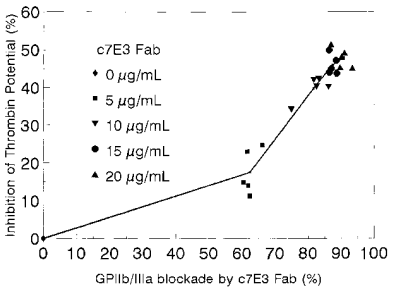

Figure 4. Correlation between inhibition of thrombin potential by c7E3 Fab and GPIIb/IIIa receptor blockade. Gel-filtered platelets (300,000 per μl) were incubated with increasing concentrations of c7E3 Fab for 20 min at 37°C and then tested in both the thrombin generation assay and in the GPIIb/IIIa receptor blockade assay. At ~ 60% GPIIb/IIIa receptor blockade, there was ~ 15% inhibition of thrombin potential. At higher levels of c7E3 Fab, there was nearly a linear relationship between inhibition of thrombin potential and GPIIb/IIIa receptor blockade, reaching a plateau of ~ 50% inhibition at ~ 90% receptor blockade. Results are from five similar experiments.

Table III. Effect of Monoclonal Antibodies on Platelet Counts during the Thrombin Generation Assay

Time	Control	c7E3 Fab	10E5
min	$\times 10^{-3}$ per μl ($n = 13$)	20 μg/ml $\times 10^{-3}$/μl ($n = 12$)	20 μg/ml $\times 10^{-3}$/μl ($n = 4$)
0	281±5*	283±14	261±12
3	188±20	256±12[‡]	250±4[§]
5	174±23	247±11[‡]	235±5[§]
10	110±20	243±12[‡]	209±3[§]
15	78±19	239±13[‡]	197±7[§]
Thrombin potential inhibition (%)		48±4[‡]	22±4[§]

*Mean±SD; [‡]$P < 0.001$ and [§]$P < 0.01$ compared with control. Samples were obtained at the indiciated times during the thrombin generation assay, diluted, and analyzed in a resistive particle counter. The instrument was set so that only single platelets or small platelet aggregates (≤ 43 fl) were counted.

Murine monoclonal 7E3 (20 μg/ml), which is directed against another epitope on the GPIIb/IIIa receptor and cross-reacts with the $\alpha_v\beta_3$ receptor, was significantly more potent than 10E5 in inhibiting thrombin potential (47%, $P < 0.001$ compared to inhibition by 10E5) and peak thrombin generation ($P < 0.001$). The chimeric Fab fragment of antibody 7E3, c7E3 Fab, produced dose-dependent inhibition of thrombin generation between 5 and 20 μg/ml, with no additional inhibition produced by 50 μg/ml. The results with c7E3 Fab were nearly identical to those produced by intact murine 7E3. In contrast to the differences between c7E3 Fab and 10E5 in the thrombin generation assay, both antibodies produced complete inhibition of platelet aggregation induced by ADP or isoT6 in PRP (data not shown).

In other experiments, inhibition of thrombin generation by c7E3 Fab was correlated with GPIIb/IIIa receptor blockade by c7E3 Fab (Fig. 4). Inhibition of thrombin generation appeared to follow a threshold pattern, with ~ 60% GPIIb/IIIa receptor blockade correlating with just ~ 15% inhibition of thrombin potential. At higher doses of c7E3 Fab, there was a nearly linear relationship between inhibition of thrombin potential and GPIIb/IIIa receptor blockade, plateauing at ~ 50% inhibition of thrombin potential with ~ 90% GPIIb/IIIa receptor blockade. The correlation between thrombin potential and platelet

Table IV. Thrombin Generation Using Platelets from Patients with Glanzmann Thrombasthenia

	Thrombin potential	Peak thrombin concentration
	*% inhibition**	*% inhibition**
Patient 1	26	36
	20	17
Patient 2	17	15
Patient 3	21	23

*Compared to a normal platelet control assayed at the same time.

Table V. Effect of Aspirin, Dibutyryl cyclic AMP, 2-Isobutyl-methyl-xanthine, and Prostacyclin on Thrombin Generation

	n	Thrombin potential	Thrombin Peak
		% inhibition	*% inhibition*
ASA			
In vitro* (100 μM)	4	9±4	12±3
In vitro (2 mM)	5	13±5‡	14±5
In vivo* (325 mg)	3	12±3‡	17±6‡
DB-cAMP (1 mM)	3	11±2	12±3
DB-cAMP (10 mM)	3	21±3‡	22±3‡
DB-cAMP (20 mM)	3	32±6‡	32±8‡
MTX (0.1 mM)	3	10±3	10±5‡
MTX (0.5 mM)	3	21±1‡	18±3‡
MTX (1 mM)	3	25±3‡	26±4‡
Prostacyclin (0.01 μM)	3	12±0‡	14±3‡
Prostacyclin (0.1 μM)	3	34±4‡	35±2‡

Mean±SD. ASA, aspirin. *In vitro, aspirin added to gel-filtered platelets before assay; in vivo, blood was obtained from a normal volunteer before and 2 h after taking 325 mg of aspirin by mouth; and ‡$P < 0.05$ compared with control.

count depicted in Fig. 1 permitted the conversion of the inhibition of thrombin potential by a given concentration of c7E3 Fab into an equivalent platelet count. When expressed in this manner, the highest dose of c7E3 Fab had the same effect as reducing the platelet count from 300,000 per μl to ~ 80,000 per μl, a reduction of ~ 73%.

To assess the mechanism by which c7E3 Fab inhibits thrombin generation, we performed single-platelet counts from samples obtained throughout the assay to monitor platelet aggregation. The results showed a steady decrease in single platelets or platelet aggregates < 43 fl vol over time in the control experiments, but significantly less decrease over time in samples treated with c7E3 Fab (Table III). Monoclonal antibody 10E5 (20 μg/ml) was less effective than c7E3 Fab in preventing the decrease in counts.

Studies with platelets from patients with Glanzmann thrombasthenia. To confirm a role for GPIIb/IIIa in supporting thrombin generation in this system, we also studied platelets from three unrelated patients with Glanzmann thrombasthenia. Compared to the results with control platelets, the onset of thrombin generation was delayed, thrombin potential was decreased between 17% and 26%, and peak thrombin generation was reduced by 15–36% (Table IV). The decrease in thrombin potential with platelets from patients with Glanzmann thrombasthenia (mean 21%) was very similar to that of normal platelets treated with antibody 10E5 (23±5%), but both were less than that observed with c7E3 Fab treatment of normal platelets (47±5%).

Effect of monoclonal antibodies LM609 and LM142, alone or in combination with 10E5 or c7E3 Fab. The greater ability of 7E3 (than 10E5) to inhibit thrombin generation in this system, and the similarity in results between 10E5-treated platelets and the platelets from the patients with Glanzmann thrombasthenia, suggested that the binding of 7E3 not only blocks GPIIb/IIIa, but also interferes with the development of platelet coagulant activity by another mechanism. Potentially, this may be related to 7E3's crossreactivity with the $\alpha_v\beta_3$ vitronectin receptor since 10E5 does not bind to $\alpha_v\beta_3$ and the Glanzmann patients we tested had normal or increased levels of platelet $\alpha_v\beta_3$. To assess this possibility, we tested the effect of the two anti–$\alpha_v\beta_3$ antibodies, LM609 (which inhibits ligand binding to $\alpha_v\beta_3$) and LM142 (which does not inhibit ligand binding), as well as the effect of these antibodies in combination with 10E5 and c7E3 Fab (Table II). Antibody LM609 (20 μg/ml), as well as its Fab fragment, alone produced slight but significant inhibition of both the thrombin potential and the peak thrombin concentration ($P < 0.001$ for both for 14 exper-

Table VI. Effect of c7E3 Fab on Thrombin–Antithrombin III Complex Formation, Prothrombin Fragment$_{1+2}$ Production, and Release of Platelet-derived Growth Factor and Platelet Factor 4 during the Thrombin Generation Assay

	Control		c7E3 Fab	
	0 Time	10 min	0 Time	10 min
			20 μg/ml	
Thrombin–antithrombin III complexes (ng/ml) ($n = 6$)	3±1*	44,500±8,200	3±0	21,800±1,800‡
Prothrombin fragment$_{1+2}$ (nmol/liter) ($n = 6$)	1±0	1,290±160	1±0	1,030±80§
Platelet-derived growth factor‖ (fmol/ml) ($n = 6$)	50±2	1,180±70	40±2	980±90§
Platelet Factor 4 (ng/ml) ($n = 5$)	4±0	3,370±70	4±0	2,740±120‡

*Mean±SD, ‡$= P < 0.001$ compared with control; §$= P < 0.01$ compared with control; and ‖Triton X-100 (1%) lysis and iso-T6 stimulation (10 μM) released 1,510±250 and 1,330±130 fmol/ml platelet-derived growth factor respectively. Thrombin generation was inhibited by c7E3 Fab by 47±4‡, 47±4‡, 40±4‡, and 48±3%‡ in the experiments with thrombin–antithrombin III complexes, prothrombin fragment$_{1+2}$ production, platelet derived growth factor release, and platelet Factor 4 release, respectively.

Table VII. Effects of c7E3 Fab and 10E5 on Platelet Microparticle Formation during the Thrombin Generation Assay

	Control		c7E3 Fab (20 μg/ml)		10E5 (20 μg/ml)		10E5+LM609 (20 μg/ml)	
Time	$n = 8$		$n = 7$		$n = 3$		$n = 3$	
min	%*	$\times 10^{-9}$/liter‡	%	$\times 10^{-9}$ per liter	%	$\times 10^{-9}$ per liter	%	$\times 10^{-9}$ per liter
0	3±1§	8±3	3±1	9±3	4±1	11±2	4±1	12±1
5	16±2	44±5	3±1‖	6±3‖	9±1π**	27±4π**	9±1π**	25±6π**
10	19±1	46±9	4±1‖	6±3‖	11±1π**	26±2π**	11±1π**	28±5π**
15	23±1	46±7	4±1‖	6±3‖	10±1π**	30±3π**	10±1π**	30±3π**
Thrombin potential inhibition (%)			47±3‖		22±2π**		30±2π**‡‡	

*Expressed as: (100) (number of microparticles)/(number of platelets + microparticles); ‡expressed as absolute number of microparticles per liter; §mean±SD; ‖$P < 0.001$ and π$P < 0.01$ compared with control; **$P < 0.01$ compared with c7E3 Fab; and ‡‡$P < 0.01$ compared with 10E5.

iments using intact LM609). In contrast, LM142 (20 μg/ml) had no observable effect on these parameters. The combination of intact 10E5 (20 μg/ml) and intact LM609 (20 μg/ml) produced significantly more inhibition than 10E5 alone (32 and 38% decrease in thrombin generation in experimental groups A and B, respectively) (Table II), as did the combination of 10E5 Fab and LM609 Fab (41%). The combination of 10E5 and LM142, however, did not produce more inhibition than 10E5 alone. Neither LM609 nor LM142 increased the inhibition of thrombin potential produced by c7E3 Fab alone.

Effect of RGDF peptide and antibodies against GPIIIa. The peptide RGDF, which reacts with both GPIIb/IIIa and the $\alpha_v\beta_3$ vitronectin receptor, inhibited thrombin generation in a dose-response manner (Table I). The maximal effect, which was achieved at ~ 100 μM was similar to that achieved with c7E3 Fab. The antibodies 7H2 and AP3 (both anti–GPIIIa) inhibited thrombin generation in a manner similar to that produced by 10E5, but less than that caused by c7E3 Fab ($P <$ 0.05 compared to control for both antibodies, Table I). In contrast, 7H2 Fab did not show a significant effect on thrombin generation (Table I).

Effect of other antiplatelet agents. Incubation of gel-filtered platelets with aspirin (20 min at 37°C) at 100 μM or 2 mM produced a modest decrease in thrombin potential and peak thrombin level (Table V). Similar inhibition was observed when a normal volunteer ingested 325 mg of aspirin and the results of a sample obtained 2 h later were compared with those from a sample obtained before aspirin ingestion. This dose of aspirin completely inhibited arachidonic acid–induced platelet aggregation at the 2-h time point.

Incubating gel-filtered platelets at 37°C for 20 min with agents able to increase intraplatelet cyclic AMP, including DB-cAMP, 3-MTX, and prostacyclin produced significant inhibition of thrombin generation, but high doses were needed (Table V), and none of these agents produced as much inhibition as antibodies 7E3 and c7E3 Fab.

TAT and F_{1+2}. TAT and F_{1+2} values increased markedly during the thrombin generation assay (Table VI). Preincubation of platelets with 20 μg/ml c7E3 Fab significantly inhibited the production of TAT during the first 10 min, with more than 50% inhibition at the 10-min point. At 15 min, the TAT value in the c7E3 Fab–treated sample was still less than the control, but the difference was not significant (data not shown). Preincubation of platelets with c7E3 Fab significantly reduced F_{1+2} formation throughout the assay, with reductions of 10–21%.

Effect of c7E3 Fab on platelet granule release. The release of the α-granule proteins PDGF and PF4 during the thrombin generation assay was also significantly inhibited by c7E3 Fab (Table VI).

Microparticles induced by exogenous thrombin. The basal level of microparticles in the gel-filtered platelets, measured either as a percentage of platelets + microparticles or as an absolute number, was 4±1% and 9±2 × 10^9 per liter, respectively ($n = 6$). Activation of gel-filtered platelets with 0.25 U/ml thrombin ($n = 6$) caused an increase in microparticles (20±4% and 43±10 × 10^9 per liter, $P < 0.001$ for both). Preincubation of platelets with 20 μg/ml c7E3 Fab inhibited microparticle formation nearly completely (4.0±0.9% and 9.0±5.2 × 10^9 per liter). Preincubation of gel-filtered platelets with 20 μg/ml 10E5, however, only partially reduced microparticle formation (10±5% and 33±15 × 10^9 per liter). Thus, c7E3 Fab reduced microparticle formation induced by a known amount of thrombin to a greater extent than did 10E5.

Table VIII. Effect of Calpeptin (138 μM), c7E3 Fab, and Both on Platelet Microparticle Formation and Thrombin Potential during the Thrombin Generation Test

	Microparticle formation			
Time	Control	Calpeptin	C7E3 Fab	Calpeptin + c7E3 Fab
min	$n = 4$	$n = 4$	$n = 4$	$n = 4$
0	4±1*	3±1	4±2	3±1
15	21±2	8±3‡	4±2§	4±1§
0	9±3‖	9±4	8±3	9±3
15	47±8	17±6‡	10±3§	8±4§
Thrombin potential inhibition (%)		29±10‡	43±7π	46±8π

*Microparticles reported as percent; ‡$P < 0.05$, §$P < 0.01$, and π$P <$ 0.001 compared with control, and ‖microparticles reported as absolute number × 10^{-9} per liter.

Microparticles induced during the thrombin generation assay. In the control samples, microparticles increased ~ 6- to 8-fold during the assay as judged by both methods of analysis (Table VII). The addition of c7E3 Fab inhibited microparticle formation nearly completely (95 and 100%, respectively; $P <$ 0.001 for both), but the addition of 10E5 decreased microparticle formation by only ~ 35 to 50% (Table VII). Combining LM609 with 10E5 produced greater inhibition of thrombin potential, but did not significantly affect microparticle formation.

Effect of calpain inhibition. Since the enzyme calpain has been implicated in microparticle formation (25), we tested the effect of the cell-permeable calpain inhibitor calpeptin (26) on both microparticle formation and thrombin generation (Table VIII). At 138 μM, a dose previously demonstrated to nearly abolish calpain activity in platelets (26), calpeptin significantly inhibited both microparticle formation and thrombin generation, but not as much as c7E3 Fab. Combining calpeptin with c7E3 Fab did not significantly increase the inhibition of thrombin generation.

Effect of c7E3 Fab on thrombin generation of ionophore A23187–treated platelets. In contrast with the results of untreated platelets in the reconstituted system, when the gel-filtered platelets were pretreated with ionophore A23187, c7E3 Fab did not significantly decrease thrombin generation (thrombin potential inhibition 3±2% and thrombin peak concentration inhibition 3±2%; $n = 3$; NS) (Fig. 3). These data suggest that c7E3 Fab cannot inhibit thrombin generation after platelets are activated and microparticles are formed.

Thrombin bound to clots. Clots formed for 30 min from native platelet-free plasma in the absence of gel-filtered platelets bound 210±30 fmol of thrombin ($n = 12$). Assuming complete conversion of fibrinogen to fibrin, the clots contained 2.42 nmol of fibrin monomer, yielding a thrombin-to-fibrin molar ratio of 0.09. Adding gel-filtered platelets to the platelet-free plasma significantly increased the amount of clot-bound thrombin (530±50 fmol, thrombin-to-fibrin molar ratio 0.22, $n = 12$, $P < 0.001$). Incubating the platelets with c7E3 Fab (20 μg/ml) before clotting was initiated decreased clot-bound thrombin by 43% (300±50 fmol, thrombin-to-fibrin molar ratio 0.12, $n = 12$, $P < 0.001$ compared to untreated gel-filtered platelets).

Platelet-rich plasma

To insure that the observed effects of antibody 7E3 on the reconstituted gel-filtered platelet-defibrinated plasma system were not unique to the reagents used in that system, experiments were also conducted using PRP. The overall pattern of thrombin generation in PRP was similar to that of the reconstituted system (Fig. 5), but the lag time, defined as the time until 10 nM thrombin was generated, was longer, averaging 387±58 s (mean±SD; $n = 23$). The shorter lag time in the reconstituted system may reflect slight activation of platelets during gel filtration. As in the reconstituted system, antibody 6D1 (20 μg/ml) had little or no effect on thrombin potential or the peak thrombin concentration, reducing them by only 2±2% and 2±1%, respectively ($n = 4$; NS). As in the reconstituted system, murine 7E3 decreased thrombin generation. When compared to control samples containing 20 μg/ml 6D1, murine 7E3 at 20 μg/ml inhibited thrombin potential by 43±22% and peak thrombin generation by 40±10% ($n = 10$; $P < 0.01$ for each). The lag times were also significantly prolonged (622±99 s; $n =$ 10). The somewhat greater variability in inhibition produced by 7E3 in the PRP system was thought to possibly reflect inter-individual variations in donor plasma; the plasma variations in the reconstituted system were eliminated by using a single batch of pooled plasma.

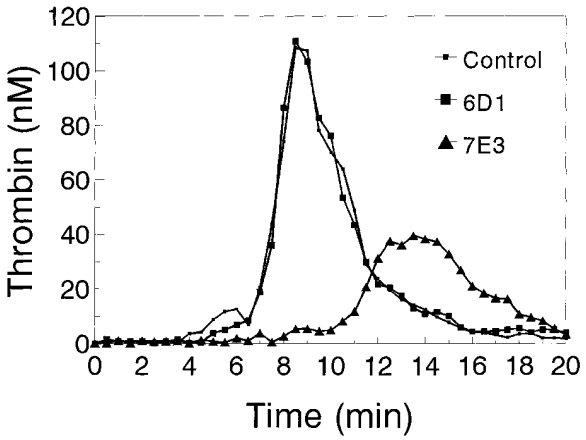

Figure 5. Effects of monoclonal antibodies 6D1 and 7E3 on thrombin generation in the PRP system. Thrombin generation was measured as indicated in Methods using PRP, with or without monoclonal antibodies 6D1 and 7E3 (20 μg/ml). As in the reconstituted system, 6D1 had nearly no effect on thrombin generation, whereas 7E3 decreased thrombin potential by ~ 44%, decreased peak thrombin concentration by ~ 64% and markedly prolonged the lag time. Results are from 1 of 10 similar experiments.

Discussion

Platelets can facilitate fibrin formation initiated either by the extrinsic or intrinsic systems, and platelets activated with thrombin or other agonists are better able to facilitate fibrin formation than unactivated platelets (reviewed in reference 1). Based on analysis of individual reactions in the coagulation "cascade," platelets have been estimated to accelerate thrombin generation by 5–6 orders of magnitude (reviewed in reference 1). There is considerable controversy, however, as to the mechanism(s) responsible for this platelet effect. Proposed mechanisms include: (*a*) Surface exposure of phosphatidylserine and possibly other anionic phospholipids that are ordinarily concentrated in the inner leaflet of the membrane bilayer (27–29); (*b*) surface expression of specific receptors for Factors Va and VIIIa (30, 31); (*c*) microparticle formation, with the concentration of anionic phospholipid receptors for Factors Va or VIIIa, or both, on the surface of the microparticles (32–35), although this mechanism has been questioned (36); (*d*) release of an activated form of Factor V from platelet α-granules (2, 37–39); (*e*) facilitation of Factor VIII activation by thrombin and/or factor Xa (40–42); (*f*) facilitation of contact activation (Factors XII and XI) (1); and (*g*) platelet Factor XI–like activity (1).

Most investigators have suggested that platelets play a dominant role in thrombin generation because: (*a*) fibrin deposition at sites of vascular injury occurs after platelet adhesion and aggregation (reviewed in reference 43); (*b*) fibrin forms in close proximity to the deposited platelets (44); and (*c*) severe thrombocytopenia and an inherited disorder affecting the ability of platelets to support thrombin generation result in pro-

found decreases in fibrin deposition in experimental models (35, 44). Endothelial cells (45) and monocytes (46), however, as well as altered or abnormal erythrocytes (47), can substitute at least in part for platelets in in vitro assays of thrombin generation. Moreover, exposure of tissue factor on the membrane of a cell may provide an adequate surface for the generation of at least small amounts of thrombin (7, 43). Thus, the relative contributions of each of these elements to in vivo thrombin generation remains uncertain. The weight of evidence, however, appears to support a model in which small amounts of thrombin are generated by a mechanism(s) that does not require platelets, but the explosive generation of thrombin that soon follows does rely on platelets (7, 43).

If platelets do make a major contribution to thrombin generation in vivo, one would predict that antiplatelet agents could decrease thrombin generation. This may occur through decreased platelet deposition and/or decreased platelet activation as described above. Moreover, since fibrin can bind thrombin and, in the arterial circulation, fibrin deposition probably initially forms on the platelet framework, decreased platelet deposition may result in decreased fibrin formation, providing a smaller fibrin clot to bind thrombin. Clot-bound thrombin, which is resistant to inactivation by antithrombin III (48), has been implicated in producing: (*a*) resistance to thrombolysis via activation of Factor XIII, leading to fibrin crosslinking and crosslinking of α_2-antiplasmin to fibrin (49); (*b*) prolonged thrombogenicity of blood vessels (50); and (*c*) thrombin-induced smooth muscle cell migration and intimal hyperplasia (5), processes that may contribute to "restenosis" (51). Thus, antiplatelet agents may, in fact, act as anticoagulants, reducing thrombin generation and clot-bound thrombin. Moreover, this anticoagulant effect may contribute to their antithrombotic and possible antirestenosis effects.

Only limited data are available on the effect of antiplatelet agents on thrombin generation. Kyrle et al. (52), Hampton et al. (53), and Szczeklik et al. (54) have reported modest decreases in markers of thrombin generation in plasma and wound fluid from patients ingesting various doses of aspirin. Basic-Micic et al. (55) developed an in vitro assay of platelet aggregation and clot formation using PRP anticoagulated with low concentrations of a low molecular weight heparin. Aspirin treatment prolonged the times to aggregation and clot formation. The PRP from a patient with Glanzmann thrombasthenia, whose platelets lacked GPIIb/IIIa, had very long aggregation and clotting times, supporting a contributory role for the GPIIb/IIIa receptor. Moorehead et al. (56) demonstrated that prostacyclin and the stable prostacyclin derivative carbacyclin prolong the celite-initiated activated clotting time of heparinized whole blood.

We previously demonstrated that aspirin ingestion had a modest inhibitory effect on tissue factor–induced thrombin generation in nonanticoagulated whole blood (10). Both the lag time and peak thrombin generation were affected (lag time, 335±11 s before and 372±13 s after, $P < 0.005$; thrombin peak, 163±8 nM before and 147±6 nM after, $P < 0.05$). The inhibitory effect of aspirin was comparable to adding 0.03 U/ml of heparin to the mixture. No effect of aspirin ingestion was observed, however, using citrated blood or PRP. In the same system, the stable synthetic prostacyclin derivative ilomedine caused both a delay and reduction in tissue factor–induced thrombin generation when added to either unanticoagulated blood or citrated PRP.

Limited data are available on the effect of aspirin on in vivo thrombin generation in active vascular disease and vascular injury. Yasu et al. (57) studied patients with unstable angina and found that patients treated with aspirin had a decrease in TAT complexes during the first 24 h, whereas patients treated with placebo did not. In a brief report of patients undergoing percutaneous coronary artery balloon angioplasty, Andreotti et al. (58) found lower prothrombin F_{1+2} levels post-percutaneous coronary artery balloon angioplasty in patients receiving high dose aspirin compared to patients receiving low dose aspirin.

Agents that block the platelet GPIIb/IIIa receptor, which include monoclonal antibodies and both peptides and peptidomimetics based on the arginine–glycine–aspartic acid (RGD) sequence are more potent inhibitors of platelet aggregation and arterial thrombosis than aspirin (59, 60). Indirect support for the hypothesis that GPIIb/IIIa receptor antagonists can result in a decrease in thrombin formation comes from the studies by Cadroy et al. (61), who showed that at arterial shear rates, blockade of GPIIb/IIIa receptors with murine monoclonal antibody LJ-CP8 nearly abolished ^{125}I-fibrinogen incorporation into thrombi formed on collagen-coated tubing inserted in arteriovenous shunts in baboons. Support for an anticoagulant effect of c7E3 Fab comes from analysis of the activated clotting times in the EPIC study. Moliterno et al. reported that despite receiving similar doses of heparin, patients treated with c7E3 Fab had longer activated clotting times than patients not treated with c7E3 Fab (62).

Studies of the platelets of patients with Glanzmann thrombasthenia have yielded conflicting data with regard to the ability of the platelets to support thrombin generation. Thus, early studies on the coagulation accelerating activity ("Platelet Factor 3") of platelets from Glanzmann thrombasthenia patients gave variable results, with one group obtaining normal values (63), but most obtaining abnormal results (64–67), suggesting that under certain conditions, platelet activation depends on GPIIb/IIIa function, whereas under other conditions, platelet activation is independent of GPIIb/IIIa function. Weiss and Lages found that thrombasthenic platelets supported early thrombin formation after vascular injury as well as normal platelets, but there was a suggestion that late thrombin generation may have been reduced (43). In flow chamber studies, however, thrombasthenic platelets supported fibrin deposition as well or better than normal platelets (44).

The results of our studies support the premise that potent antiplatelet agents may function in vivo as anticoagulants by decreasing thrombin generation at sites of vascular injury. Thus, we were able to show nearly a 50% decrease in thrombin potential with murine 7E3 in both a gel-filtered platelet system and a PRP system when coagulation was initiated with tissue factor, the most likely trigger for thrombin generation in vivo in both physiologic and pathologic states. c7E3 Fab gave results very similar to those of murine 7E3, excluding a significant role for the Fc region of the molecule. Treatment of platelets with c7E3 Fab also decreased platelet aggregate formation, TAT complex formation, F_{1+2} formation, PDGF and PF4 release, platelet microparticle formation, and clot-bound thrombin. Other antiplatelet agents were also capable of decreasing thrombin generation, but they were not as potent as c7E3 Fab. Of note, both murine c7E3 and c7E3 Fab were more potent in inhibiting thrombin generation than antibody 10E5, even though all three antibody preparations block fibrinogen bind-

ing to platelets and platelet aggregation to the same extent. Despite the similarities between 7E3 and 10E5, these antibodies have been shown to differ in their ability to inhibit clot retraction (68, 69), as well as their ability to inhibit platelet interaction with immobilized RGD peptides (23). Moreover, 7E3 has been shown to inhibit $\alpha_v\beta_3$ vitronectin receptor function, whereas 10E5 does not (20). The reduction in thrombin generation observed with the platelets from all three patients with Glanzmann thrombasthenia tested supports a role for GPIIb/IIIa in thrombin generation. Since the reduction in thrombin generation observed with thrombasthenic platelets was similar to that observed with normal platelets treated with antibody 10E5, and since we directly determined that the platelets from all three patients have normal to increased numbers of $\alpha_v\beta_3$ receptors, we considered the possibility that at least part of the difference in behavior between 7E3 and 10E5 may be due to their differences in inhibiting $\alpha_v\beta_3$.

To test this hypothesis, we analyzed the effect of combining 7E3 or 10E5 with LM609 (anti–$\alpha_v\beta_3$), which inhibits $\alpha_v\beta_3$ function, and LM142 (anti–α_v), which does not inhibit $\alpha_v\beta_3$ function. There was slight, but consistent inhibition of thrombin generation by antibody LM609 alone, but none by LM142 alone. LM609 enhanced the inhibition caused by 10E5 but not the inhibition caused by c7E3 Fab. These data suggest that 7E3 inhibits thrombin generation partly by GPIIb/IIIa inhibition, and partly by $\alpha_v\beta_3$ inhibition. We cannot exclude the possibility that other mechanisms are also involved because the combined inhibitory effect of 10E5 and LM609 did not quite equal that produced by 7E3 alone. The RGDF peptide, which inhibits both GPIIb/IIIa receptors and $\alpha_v\beta_3$ vitronectin receptors, gave results similar to those produced by c7E3 Fab.

Although controversy persists, there is considerable evidence linking microparticle formation with the enhanced generation of thrombin produced by platelets (33–36). It has been proposed that microparticles are particularly rich in anionic phospholipids and receptors for Factors Va, VIII(a), and IXa (70). In addition, the patient with a defect in platelet coagulant activity appears to have a decrease in microparticle formation as her primary abnormality (35). Apparently conflicting results of the importance of GPIIb/IIIa in platelet microparticle formation have been reported. Gemmell et al. (71) found that intact GPIIb/IIIa is required for platelet microparticle formation induced by thrombin, that the platelets of a patient with Glanzmann thrombasthenia had markedly reduced microparticle formation in response to thrombin, and that murine monoclonal antibodies 7E3 and A_2A_9 (anti–GPIIb/IIIa) inhibit microparticle formation when platelets are stimulated with thrombin. In sharp contrast, Nomura et al. (72) reported that the platelets of a patient with Glanzmann thrombasthenia produced normal numbers of microparticles in response to thrombin, ionophore A23187, and ADP. Moreover, the microparticles supported prothrombin activation normally. There were significant technical differences between the studies of Gemmel et al. and Nomura et al. with regard to the buffers and platelet preparation procedures, and these may have affected the results. Neither study reported the presence or absence of $\alpha_v\beta_3$ on the patient's platelets, and so variations in surface expression of this receptor may also have contributed to the observed differences. The results of the present study are consistent with those of Gemmel et al. (71) in that c7E3 Fab inhibited microparticle formation. Our data also support a correlation between microparticle formation and thrombin generation since c7E3 Fab inhibited both. Similarly, the calpain inhibitor calpeptin inhibited both microparticle formation and thrombin potential. Moreover, c7E3 Fab did not inhibit thrombin generation when platelets were pretreated with ionophore A23187, an observation consistent with the hypothesis that c7E3 Fab acts proximal to microparticle formation. Additional studies will be required, however, to assess whether the inhibition of microparticle formation by c7E3 Fab is the cause of the decrease in thrombin generation and to define the mechanisms involved.

In conclusion, in addition to its inhibitory effects on platelet aggregation via blockade of GPIIb/IIIa receptors, c7E3 Fab may also inhibit thrombin generation by decreasing the number of platelets in thrombi and by interfering with the platelet-activation events involved in facilitating thrombin generation. The inhibition of thrombin generation by c7E3 Fab may contribute to both its immediate antithrombotic effect and perhaps to its possible effects on long-term vascular restenosis.

Acknowledgments

We thank Suzanne Rivera and Melanie Alvarez for outstanding secretarial assistance.

This work was supported by National Heart, Lung, and Blood Institute grants 19278 and 54469 (B.S. Coller), grant FIS 94/5402 from the Spanish Government and Hospital Clinic Barcelona (J.C. Reverter), and by Program Grant 900-526-192 From the Dutch Organisation for Scientific Research (NWO) (S. Béguin, R. Kumar, and H.C. Hemker).

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