Chimeric 7E3 Prevents Carotid Artery Thrombosis in Cynomolgus Monkeys

William E. Rote, PhD; Mark A. Nedelman, MS; Dun-Xue Mu, MD; Peter J. Manley; Harlan Weisman, MD; Mark R. Cunningham, PhD; Benedict R. Lucchesi, PhD, MD

Background and Purpose We compared the current antithrombotic strategy of antiplatelet therapy with aspirin, and anticoagulant therapy with heparin, with a specific genetically engineered chimeric antibody (c7E3 Fab) directed against the human glycoprotein IIb/IIIa receptor in an animal model of arterial thrombosis.

Methods Anesthetized cynomolgus monkeys (Macaca fascicularis) were instrumented for monitoring of arterial blood pressure, heart rate, and carotid artery flow velocity. Animals were treated with saline (n=6), aspirin (25 mg PO daily for 3 days; n=6), heparin (100 U/kg IV plus infusion adjusted to maintain activated partial thromboplastin time at 2 to 3 times baseline; n=6), aspirin plus heparin (as administered separately, n=6), or c7E3 Fab (0.10 mg/kg IV, n=7; 0.15 mg/kg IV, n=6; 0.20 mg/kg IV, n=6; 0.25 mg/kg IV, n=6). Thrombus formation via anodal electrolytic stimulation (100 μA) to the intimal surface of the right carotid artery was initiated 15 minutes after drug administration and continued for 180 minutes. Electrolytic injury to the left carotid artery began 210 minutes after drug administration and continued for 180 minutes. Whole blood cell counts, glycoprotein IIb/IIIa receptor blockade, ex vivo platelet aggregation, template bleeding time, and activated partial thromboplastin time were assessed at various time points throughout the experimental protocol.

Results Hemodynamic and hematologic parameters were comparable among groups at baseline. Treatment with c7E3 Fab inhibited ex vivo platelet aggregation, increased bleeding time, decreased thrombus weight, and increased time to occlusion in a dose-dependent manner in both vessels. Treatment with aspirin, heparin, or the combination of aspirin plus heparin was ineffective for the prevention of carotid artery thrombosis in this model.

Conclusions Inhibition of the platelet glycoprotein IIb/IIIa receptor with c7E3 Fab was found to be safe and effective for the prevention of primary thrombus formation, whereas treatment with either aspirin or heparin or the combination of the two agents failed to protect against occlusive thrombus formation in cynomolgus monkeys. (Stroke. 1994;25:1223-1233.)

Key Words • antithrombotic therapy • aspirin • platelet aggregation • monkeys

The development and use of antithrombotic drugs have focused on the fundamental differences in the pathogenesis of arterial and venous thrombosis. Antiplatelet agents have been used primarily for the treatment of arterial disease while anticoagulants have been used mostly in venous thromboembolic disorders. Current clinical antithrombotic therapy in acute ischemic stroke and acute myocardial infarction has turned to a dual-arm strategy using both antiplatelet agents (aspirin) and anticoagulants (heparin).1-5 Although this therapeutic combination has led to a reduction of acute thrombotic complications associated with coronary angioplasty and carotid endarterectomy, current research and development is directed toward more potent and specific inhibitors to obtain a further reduction of thrombotic complications or ultimately to prevent them completely.6-8

Platelets play a central role in normal hemostasis, initially by adhering to adhesive glycoproteins in the subendothelium of damaged blood vessels via surface receptors on the platelet and later by aggregating to the adherent platelets and each other via the interaction between fibrinogen and activated surface receptors on platelets.9,10 In the pathological scenario of extensive and/or repeated vessel injury with subsequent exposure of thrombogenic surfaces, platelet aggregation can progress to such a degree as to result in complete thrombotic occlusion of the injured vessel, or large aggregates can break off the original site of injury and result in distal embolization and occlusion of smaller vessels with platelet-fibrin thrombi.11,12

The development of more potent and specific inhibitors of platelets has been directed by the increasing knowledge of platelet activation pathways, especially the role of platelet membrane receptors. Unlike the adhesion function of platelets, platelet aggregation appears to be mediated by a single membrane receptor, the glycoprotein IIb/IIIa (GPIIb/IIIa) receptor.13-14 The GPIIb/IIIa receptor, therefore, has become a logical target for antiplatelet development. Monoclonal antibodies that bind to the platelet GPIIb/IIIa receptor and inhibit the binding of fibrinogen and von Willebrand factor have been shown to inhibit platelet aggregation induced by physiological agonists.15 Synthetic peptides with the tripeptide sequence of arginine-glycine-aspartic acid (RGD) were discovered to bind to several members of the integrin family including GPIIb/IIIa and have been shown to function as antithrombotics in animal models.17,18 Natural peptides (isolated from snake venoms) containing the RGD sequence likewise...
bind to the platelet GPIIb/IIIa receptor, inhibit platelet aggregation, and prevent thrombosis or rethrombosis in animal models.\(^{19,20}\) Mickelson and colleagues,\(^{21}\) as well as others,\(^{22,23}\) have demonstrated that the F(ab')\(_2\) fragment of the monoclonal antibody 7E3 could be used as an effective antithrombotic agent in preclinical animal models; however, preclinical studies have not been reported with the chimeric Fab fragment of the antibody.

A preliminary study\(^ {24}\) in humans indicated that the 7E3-F(ab')\(_2\) resulted in antibody formation against the murine immunoglobulin. The chimeric antibody retains the heavy and light chain variable regions that confer to the molecule its specificity for the GPIIb/IIIa receptor. The constant domains were replaced by human immunoglobulin (Ig) \(G_1\) heavy chain and light chain regions, respectively, thereby resulting in a marked decrease in immunogenicity when administered to humans.\(^ {25}\)

This study was designed to examine the relative efficacy of a genetically engineered monoclonal antibody (c7E3 Fab), developed to block the platelet membrane GPIIb/IIIa receptor, and currently used antithrombotic agents (aspirin, heparin, and the combination of aspirin and heparin) in a nonhuman primate model of carotid artery thrombosis. The primary end point in this study was occlusive thrombus formation resulting from continuous anodal stimulation to the intimal surface of the carotid arteries in cynomolgus monkeys. Systemic arterial blood pressure, heart rate, carotid artery blood flow velocity, blood cell counts, template bleeding time, blockade of platelet GPIIb/IIIa receptors, and ex vivo platelet aggregation were monitored during the study.

**Materials and Methods**

**Animal Investigation**

These studies conform to the **Position of the American Heart Association on Research Animal Use** adopted November 11, 1984, by the American Heart Association. The procedures followed in this study were in accordance with the guidelines of the University of Michigan (Ann Arbor) University Committee on the use and care of animals. Veterinary care was provided by the University of Michigan Unit for Laboratory Animal Medicine. The University of Michigan is accredited by the American Association for Accreditation of Laboratory Animal Care, and the animal care and use program conforms to the standards in *The Guide for Care and Use of Laboratory Animals*, US Department of Health, Education, and Welfare publication NIH 78-23.

**Reagents**

The chimeric Fab fragment (c7E3 Fab) of the murine monoclonal GPIIb/IIIa receptor antibody 7E3, rabbit antimurine 7E3, and 125I-7E3 IgG were supplied by Cenroc Inc. Heparin (porcine) was purchased from Elkins-Sinn, Inc. Sodium citrate, adenosine diphosphate (ADP), acetylsalicylic acid, arachidonic acid, and any reagent used in the laboratory but not specifically mentioned were purchased from Sigma Chemical Co.

**Model of Carotid Occlusion**

The model used in this study is a modification of one developed by our laboratory for the study of experimentally induced coronary artery thrombosis.\(^ {26}\) The experimental procedure results in the formation of a platelet-rich intravascular thrombus at the site of an electrophysiologically induced endothelial lesion in proximity to a distal arterial stenosis. Use of both carotid arteries made it possible to examine dose-response relations as well as the effects of time on efficacy of the interventions. Furthermore, repeated measurements in the same experimental subject serve to reduce variability among animals, thereby simplifying the analysis of the data. In all studies reported here, application of the anodal injury current to the right carotid artery (RCA) was initiated 15 minutes after the animal was stabilized (aspirin-pretreated group) or 15 minutes after drug administration (c7E3 Fab– and heparin-treated groups). The left carotid artery (LCA) underwent the same procedure involving anodal current injury that was commenced 210 minutes after administration of the test compounds. Thus, the elapsed time from the last administration of drug (c7E3 Fab and heparin) was maintained constant in all studies.

**Experimental Protocol**

Male cynomolgus monkeys (*Macaca fascicularis*; 3.4 to 5.1 kg) obtained from Charles River Laboratories were anesthetized with ketamine (10 mg/kg IM) and sodium pentobarbital (30 mg/kg IV). Cannulas were placed into the left femoral artery for monitoring arterial blood pressure (Statham P23 pressure transducer, Gould Inc) and into the right femoral vein for administering intravenous fluids and drugs or obtaining blood samples. Both common carotid arteries were exposed. Standard limb lead II of the electrocardiograph was recorded continuously. A Doppler flow probe (Model 100, Triton Technology) was placed on each common carotid artery proximal to both the point of insertion of the intrarterial electrode and the mechanical constrictor. The mechanical constrictor was constructed of stainless steel in a C shape with a polytetrafluoroethylene (Teflon brand) screw (2-mm diameter) that could be adjusted to control vessel circumference and produce a regional stenosis. The constric tor was adjusted until the pulsatile flow pattern was reduced by 50% without altering the mean blood flow. Blood flow in the carotid vessels was monitored continuously.

Electrolytic injury to the intimal surface of each carotid vessel was accomplished with the use of an intravascular electrode composed of a Teflon-insulated, silver-coated copper wire. Penetration of the vessel wall by the electrode was facilitated by attaching the tip of a 25-gauge hypodermic needle to the uninsulated part of the electrode. Each intrarterial electrode was connected to the positive pole (anode) of a dual-channel stimulator (Grass S88 stimulator and a Grass Constant Current Unit, Model CUC1A, Grass Instrument Co.). The cathode was connected to a distant subcutaneous site. The current delivered to each vessel was monitored continuously on separate ammeters and maintained at 100 \(\mu\)A. The anodal electrode was positioned to have the uninsulated portion in intimate contact with the endothelial surface of the vessel. Proper positioning of the electrodes in the carotid arteries was confirmed by visual inspection at the end of every experiment.

Animals were treated with saline (n=6), aspirin (25 mg PO daily for 3 days, n=6), heparin (100 U/kg IV plus infusion adjusted to maintain activated partial thromboplastin time [aPTT] at 2 to 3 times baseline, n=6), aspirin plus heparin (dosages as before, n=6) or c7E3 Fab (0.10 mg/kg IV, n=7; 0.15 mg/kg IV, n=6; 0.20 mg/kg IV, n=6; 0.25 mg/kg IV, n=6). The experimental protocol is shown diagrammatically in Fig 1. Monkeys were anesthetized, and thrombus formation via electrolytic injury of the endothelium in the RCA was initiated 15 minutes after drug administration and continued for 180 minutes. Stimulation of the LCA began 210 minutes after drug administration. Whole blood cell counts, ex vivo platelet aggregation, aPTT, GPIIb/IIIa receptor blockade, and bleeding time were assessed at various time points throughout the experimental protocol. Blood pressure, heart rate, and carotid artery flow velocity were monitored continuously.

Arterial thrombosis and thrombotic occlusion occurred in response to intimal damage after which the vessel segment was...
ligated, both proximal and distal to the point of injury, and removed without disturbing the intravascular thrombus. The vessel segment was opened along its length, and the intact thrombus mass was lifted off the intimal surface of the vessel. The wet weight of the thrombus mass was determined with an analytic balance. If thrombotic occlusion did not occur, current to the anodal electrode was terminated at 180 minutes, and the vessel was removed as described above.

### Hematologic Measurements

Blood (10 mL) was withdrawn for platelet studies from the femoral vein cannula into a plastic syringe containing 3.2% sodium citrate as the anticoagulant (1:10 citrate to blood [vol/vol]). Blood was taken for platelet aggregation and whole blood cell counts at baseline, 30, 180, and 300 minutes after the administration of the test compound. The platelet count was determined with an H-10 cell counter (Texas International Laboratories, Inc). Platelet-rich plasma (PRP), the supernatant present after centrifugation of anticoagulated whole blood at 1000 rpm for 5 minutes (140g), was diluted with platelet-poor plasma (PPP) to achieve a count of 200,000 platelets per cubic millimeter. PPP was prepared after the administration of the test compound. The platelet count was assessed before and after drug administration. The data were assessed by either paired or group analysis using Student's *t* test when applicable; differences were considered significant if *P* < .05. Incidence of occlusion was compared by Fisher's exact test.

### Quantification of GPIIb/IIIa Receptors

Ex vivo binding of \(^{125}\text{I}\)-labeled m7E3 IgG to platelets was used to quantify the average number of available GPIIb/IIIa binding sites at baseline (predose) and 30, 180, and 300 minutes after treatment. \(^{125}\text{I}\)-labeled m7E3 IgG was incubated at room temperature for 30 minutes with 180 \(\mu\text{L}\) of PRP (standardized to approximately 200,000 platelets per microliter) to yield a final concentration of 0.018 mg/mL m7E3 IgG. Platelet-bound antibody was separated from free antibody by centrifugation of replicate 50-\(\mu\text{L}\) aliquots through 30% sucrose. The number of \(^{125}\text{I}\)-labeled m7E3 IgG molecules bound per platelet was calculated from the platelet count, the percentage of radioactivity in the pellet, and the amount of antibody added. The decrease in GPIIb/IIIa receptor binding sites available for \(^{125}\text{I}\)-labeled m7E3 IgG after treatment was taken as the number of platelet binding sites blocked by the in vivo treatment, as calculated by comparison to the predose determination.

### c7E3 Fab Plasma Concentrations

Free plasma c7E3 Fab concentrations were determined 30 minutes after treatments using an enzyme immunoassay. In brief, microtiter plates were coated with rabbit anti-murine-7E3 IgG antibodies overnight at 4°C. The plates then were washed and blocked with 1% bovine serum albumin and azide in phosphate-buffered saline. Standards of known c7E3 Fab concentration and plasma samples collected from the experiments were added into triplicate wells on the same microtiter plates. After incubation for 1 hour at 37°C, the plates were washed and biotinylated rabbit anti-murine-7E3 IgG antibody was added. The amount of antibody added. The decrease in GPIIb/IIIa receptor binding sites available for \(^{125}\text{I}\)-labeled m7E3 IgG after treatment was taken as the number of platelet binding sites blocked by the in vivo treatment, as calculated by comparison to the predose determination.

### Statistical Analysis

The data are expressed as mean±SEM. Ex vivo platelet aggregation in response to arachidonic acid and to ADP was assessed before and after drug administration. The data were analyzed by either paired or group analysis using Student’s *t* test when applicable; differences were considered significant if *P* < .05. Incidence of occlusion was compared by Fisher’s exact test.

### Results

Forty-nine monkeys were entered into the study. One animal died of undetermined causes during the proce-
duration after the RCA had completed the 180-minute anodal current stimulation period but before the LCA was subjected to electrolytic injury (c7E3 Fab, 0.20 mg/kg IV group).

Heart rates were not different between treatment groups before animals were treated. During the periods of carotid artery anodal current injury (0 to 180 minutes after treatment for the RCA and 210 to 390 minutes after treatment for the LCA), heart rate was not significantly affected in any of the treatment groups at any of the time points and remained within the normal range (100 to 220 beats per minute) for cynomolgus monkeys. Systemic mean arterial blood pressure was elevated in the control (saline) treatment group (101 ± 10 mm Hg for control versus 75 ± 7 mm Hg compared with all other treatment-group baseline values) before the initiation of the electrolytic injury of the RCA. Mean arterial pressure was not affected by any of the treatments during the period of RCA or LCA anodal current injury and was considered normal (58 to 124 mm Hg) for cynomolgus monkeys in all treatment groups. Therefore, none of the treatment regimens produced any significant changes in either of these hemodynamic parameters at any time point in the study.

Baseline values for red blood cells and hematocrit were not significantly different between the treatment groups, although 59% of the animals had red blood cell levels below the normal species range of 5.6 to 6.7 × 10^12/L at the baseline measurement, whereas 92% of the animals at baseline were below the normal species hematocrit range of 31.2% to 40.0%. The observed variances from normal hematologic ranges resulted from the fact that the instrumentation was not calibrated specifically for this species; nevertheless, the hematologic data were evaluated based on the change from baseline values in individual groups and in comparison to changes between groups at the same time point. Baseline values for hemoglobin measurements in the 0.15-mg/kg c7E3 Fab (group 3), 0.20-mg/kg c7E3 Fab (group 4), aspirin (group 6), and aspirin-plus-heparin (group 8) treatment groups were significantly higher than in the other treatment groups, although hemoglobin values within each group were not affected by any of the treatments through 300 minutes of observation. The normal species range for hemoglobin is 10.5 to 13.3 g/dL; 29% of the animals were below this range at baseline, while 65% were above. Significant differences in platelet count were not observed among any of the treatment groups (normal range of 109 to 597 × 10^3/μL). Posttreatment measurements of white blood cells were unchanged from baseline values or were slightly increased but remained within the normal range. Treatment-related effects were not observed in any of the groups in any of these hematology variables measured.

The effects of aspirin and heparin and the different doses of c7E3 Fab on template bleeding time are shown in Fig 3. The lines represent individual animals, the group means are represented as single points (±SEM) adjacent to the lines. Control animals demonstrated bleeding times that were prolonged to approximately 1.5 times baseline values. Monkeys receiving c7E3 Fab exhibited prolonged bleeding times in a dose-dependent manner at 30 minutes (all doses), 180, and 300 minutes (0.15 mg/kg, 0.20 mg/kg, and 0.25 mg/kg, respectively). Bleeding times for animals given the higher doses of the c7E3 Fab (0.20 and 0.25 mg/kg) showed a return toward baseline but remained significantly prolonged 300 minutes after injection. Minor bleeding from surgical sites was noted in animals receiving 0.20 and 0.25 mg/kg c7E3 Fab.

The incidence of complete thrombotic occlusion of the respective carotid arteries is shown in Table 1. Control monkeys exhibited occlusive thrombus formation in all cases. Monkeys treated with aspirin, heparin, or a combination of the two drugs experienced the same incidence of occlusion as saline-treated animals. Inhibition of the platelet GP Ib/IIa receptor with c7E3 Fab was effective for the prevention of acute occlusive thrombus formation. The effect was both dose dependent, as evidenced by the decreasing incidence of thrombosis with higher doses, and time dependent. A significant time interval existed between application of the anodal injury current to each carotid artery in the same animal and the time of administration of c7E3 Fab. Initiation of the injury current in the RCA began 15 minutes after c7E3 Fab administration, compared with initiating the injury in the LCA at 210 minutes after c7E3 Fab administration. The protocol design allowed for the analysis of the time-response characteristics of the antibody. The time dependency for the action of c7E3 Fab was evident in the groups receiving the 0.10- and 0.15-mg/kg doses where the RCA was better protected than the contralateral LCA. The observation was suggestive of a declining action of the chimeric antibody with time. This relation was lost at the higher doses, where c7E3 Fab was equally effective in both arteries despite the elapsed time between the point of drug administration and the later application of the anodal injury current to the LCA.

Carotid artery blood flow velocity in drug-treated monkeys did not differ from that in control animals at the start of anodal current application. Additionally, within-group comparisons demonstrated that at the time current application was begun, blood flow velocity in the RCA was not different from that in the LCA in any of the experimental groups. The effect of each of the treatments on carotid artery blood flow velocity is shown in Fig 3. The lines represent individual animals; the group means are represented as single points (±SEM) adjacent to the lines. Control animals demon-
TABLE 1. Incidence of Occlusive Thrombus Formation

<table>
<thead>
<tr>
<th>Group</th>
<th>Right Carotid Artery</th>
<th>Left Carotid Artery</th>
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</thead>
<tbody>
<tr>
<td>Saline control</td>
<td>6/6 100%</td>
<td>6/6 100%</td>
</tr>
<tr>
<td>Aspirin*</td>
<td>6/6 100%</td>
<td>6/6 100%</td>
</tr>
<tr>
<td>Heparin†</td>
<td>5/6 83%</td>
<td>6/6 100%</td>
</tr>
<tr>
<td>Aspirin + heparin*†</td>
<td>6/6 100%</td>
<td>5/6 83%</td>
</tr>
<tr>
<td>c7E3, 0.10 mg/kg IV</td>
<td>4/7 57%</td>
<td>7/7 100%</td>
</tr>
<tr>
<td>c7E3, 0.15 mg/kg IV</td>
<td>2/6* 33%</td>
<td>4/6 66%</td>
</tr>
<tr>
<td>c7E3, 0.20 mg/kg IV</td>
<td>0/6* 0%</td>
<td>1/5* 20%</td>
</tr>
<tr>
<td>c7E3, 0.25 mg/kg IV</td>
<td>1/6* 16%</td>
<td>0/6* 0%</td>
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</table>

*25 mg PO daily for 3 days.
†100 U/kg IV and infusion to maintain activated partial thromboplastin time at 2 to 3 times baseline.
*P < .05 compared with saline-treated animals, Fisher's exact test.

stratified occlusive thrombus formation by the end of the observation period for each artery (180 minutes), evidenced by the zero flow velocity in both the RCA and LCA. No differences existed between right and left carotid arteries with regard to initial flow velocity or time to occlusion. Within the heparin and aspirin groups, only 2 of the 36 carotid arteries were patent at the 180-minute time point.

Pretreatment with c7E3 Fab was effective in preventing carotid occlusion in this experimental model. The ability of c7E3 to prevent occlusive thrombus formation was characterized by a dose-dependent response. Increasing doses of c7E3 Fab provided a progressive enhancement of protection against thrombotic occlusion in both the RCA and LCA. The protection afforded by c7E3 Fab was clearly superior to that achieved with aspirin, heparin administered individually, or aspirin and heparin combined. The c7E3 treatment groups given 0.20 and 0.25 mg/kg demonstrated a high degree of flow preservation, whereas less protection was obtained (about 50% effectiveness) in monkeys receiving 0.15 mg/kg. The 0.10-mg/kg dose was marginally effective. As was noted for the incidence of occlusion, the blood flow velocity data also suggest the presence of a time response in the animals treated with 0.10, 0.15, and 0.20 mg/kg c7E3 Fab. The effectiveness of c7E3 Fab in preserving carotid artery blood flow velocity was more readily apparent in the RCAs at 180 minutes than in the respective contralateral vessels of the animals receiving 0.10, 0.15, and 0.20 mg/kg of c7E3. The difference became less apparent at the highest dose tested, 0.25 mg/kg.

The time to occlusive thrombus formation is represented in Fig 4 (top) for each treatment group. For those vessels that did not experience zero flow velocity during the period of electrolytic injury, a time of 180 minutes was assigned to allow for a quantitative analysis for the groups. Therefore, a time of 180 minutes for the time to occlusion should be interpreted as a vessel that did not occlude during the period of observation. Significant prolongation in the time to occlusion was noted in the RCAs, but not in the LCAs, in monkeys receiving either 0.10 or 0.15 mg/kg c7E3 Fab. Treatment with either 0.20 or 0.25 mg/kg c7E3 Fab resulted in only two arteries (one in each group, respectively) proceeding to complete thrombotic occlusion with the remaining right and left carotid arteries maintaining blood flow at or near baseline values (Table 1 and Fig 3). Heparin, aspirin, or heparin combined with aspirin failed to prolong the time to occlusive thrombus formation.

Thrombus wet weight was measured 180 minutes after the initiation of anodal current application in arteries that did not occlude or after 30 minutes of zero blood flow velocity in those that proceeded to complete thrombotic occlusion as determined by the Doppler flow probe (Fig 4, bottom). Thrombus weight was reduced in a dose-dependent manner in monkeys treated with the antibody compared with controls. The time dependence of antibody administration was apparent in LCAs, which have greater thrombus weight than RCAs, with the exception of the highest dose, 0.25 mg/kg. No

![Graphs showing carotid artery blood flow velocity (cm/s) at baseline and 180 minutes after anodal current application to the intimal surface of the artery. The data are represented for each individual animal (lines) and as the group means (±SEM) for each treatment group. n = 7 for monkeys receiving 0.10 mg/kg c7E3; n = 6 for the right carotid artery and n = 5 for the left carotid artery for monkeys receiving 0.20 mg/kg c7E3, and n = 6 for all other groups. c indicates right carotid arteries; o, left carotid arteries, and c7E3, chimeric Fab fragment of the 7E3 antibody.](http://stroke.ahajournals.org/)

Fig 3. Graphs showing carotid artery blood flow velocity (cm/s) at baseline and 180 minutes after anodal current application to the intimal surface of the artery. The data are represented for each individual animal (lines) and as the group means (±SEM) for each treatment group. n = 7 for monkeys receiving 0.10 mg/kg c7E3; n = 6 for the right carotid artery and n = 5 for the left carotid artery for monkeys receiving 0.20 mg/kg c7E3, and n = 6 for all other groups. c indicates right carotid arteries; o, left carotid arteries, and c7E3, chimeric Fab fragment of the 7E3 antibody.)
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The mean percentage of receptor blockade was then calculated for each treatment group. There was an increase in receptor blockade with increasing doses of c7E3 Fab. At 30 minutes after injection, the mean percentage of GPIIb/IIIa receptors blocked by c7E3 Fab was highest at the 0.25-mg/kg dose (85%). Receptor blockade decreased over 300 minutes for all c7E3 Fab treatment groups except in animals administered 0.10 mg/kg of c7E3 Fab, which showed an unexplained increase in GPIIb/IIIa blockade at 300 minutes. Receptor blockade continued to be greatest in the 0.25-mg/kg dose group at 300 minutes (65%), although the degree of blockade in this treatment group was not significantly different from the other c7E3 Fab treatment groups. Treatment with aspirin, heparin, and aspirin plus heparin did not have an effect on GPIIb/IIIa receptor blockade; if anything, receptor number appeared to increase in these treatment groups illustrated by negative values of GPIIb/IIIa blockade. The data are summarized in Table 2.

Chimeric 7E3 treatment resulted in a reduction of the number of free IIb/IIIa receptors in a dose-dependent manner. The lowest dose of c7E3 Fab resulted in approximately 50% saturation of the GPIIb/IIIa receptor, whereas the other doses examined resulted in nearly complete receptor blockade and little difference being noted between groups at later time points.

aPTTs were determined at various time points to assess the degree of anticoagulation. The mean aPTT determined at baseline for all of the animals was 57 ±2.6 seconds. Repeated determinations of the aPTT were performed at 5, 60, 180, and 240 minutes after drug administration. No change in aPTT was noted in either the saline- or c7E3 Fab–treated monkeys. Heparin treatment increased aPTT between 3 and 5 times baseline control values and maintained a consistent prolongation throughout the experiment.

Ex vivo platelet aggregation was determined in all animals at baseline and again at 30, 180, and 300 minutes after administration of the antibody. c7E3 Fab inhibited platelet aggregation in a dose-dependent manner as shown in Fig 6. The inhibition of aggregation was similar regardless of the agonist, ADP or arachidonic acid, used to activate the platelets. Inhibition of platelet aggregation paralleled the extension of bleeding time and the percentage of bound GPIIb/IIIa receptors determined to be present on the platelets. No change in ex vivo platelet aggregation was noted in either the

Fig 5. Graph showing the number of unbound glycoprotein IIb/IIIa receptors on platelets taken at serial time points from monkeys treated with chimeric Fab fragment of the 7E3 antibody (c7E3) or saline.

Fig 4. Top, Bar graph showing time to occlusive carotid artery thrombus formation as defined by zero blood flow velocity recorded with a Doppler flow probe applied proximal to the forming thrombus. Bottom, Bar graph showing thrombus weights determined after 30 minutes of zero blood flow velocity or 180 minutes after the initiation of anodal current application in arteries that did not occlude. Arteries that were patent at the end of the period of observation were assigned 180 minutes as the time to occlusion for ease of statistical comparisons. Values are expressed as mean±SEM. n=7 for monkeys receiving 0.10 mg/kg c7E3; n=6 for the right carotid artery and n=5 for the left carotid artery for monkeys receiving 0.20 mg/kg c7E3; and n=6 for all other groups. c7E3 indicates chimeric Fab fragment of the 7E3 antibody.

Reduction in thrombus weight was noted in heparin- or aspirin-treated monkeys.

PRP collected at baseline and 30, 180, and 300 minutes after drug administration was incubated with iodinated m7E3 IgG antibody to determine the percentage of GPIIb/IIIa receptors bound by the chimeric form of the 7E3 antibody. The labeled IgG binds with 1-to-1 stoichiometry and will only bind to receptors that are not occupied by c7E3 Fab, therefore allowing the determination of the free GPIIb/IIIa receptors. The results of this assay are shown in Fig 5. Untreated monkeys had 64 599±3117 GPIIb/IIIa receptors per platelet (mean±SEM, n=31). Measurements of 125I-m7E3 IgG bound per platelet were made in all animals (except control treatment group) at baseline, 30, 180, and 300 minutes after treatment. The degree of receptor blockade at each posttreatment time point was calculated by comparison of the receptor number with the predose determination for each individual animal. The mean percentage of receptor blockade was then calculated for each treatment group. There was an increase in receptor blockade with increasing doses of c7E3 Fab. At 30 minutes after injection, the mean

Fig 6. Bottom, Bar graph showing thrombus formation as defined by zero blood flow velocity recorded with a Doppler flow probe applied proximal to the forming thrombus. Top, Graph showing the number of unbound glycoprotein IIb/IIIa receptors on platelets taken at serial time points from monkeys treated with chimeric Fab fragment of the 7E3 antibody (c7E3) or saline.

right carotid artery

Time after c7E3 Administration (min)

Saline, n=6, mean ± SEM

<table>
<thead>
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<th>Treatment Group</th>
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<th>Left Carotid Artery</th>
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<tbody>
<tr>
<td>Saline, 0.10 mg/kg c7E3</td>
<td>* p&lt;0.05 vs Saline</td>
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<tr>
<td>Heparin, 0.20 mg/kg c7E3</td>
<td>* p&lt;0.05 vs Saline</td>
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</tr>
<tr>
<td>Aspirin, 0.25 mg/kg c7E3</td>
<td>* p&lt;0.05 vs Saline</td>
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</tr>
<tr>
<td>Heparin + Aspirin, 0.25 mg/kg c7E3</td>
<td>* p&lt;0.05 vs Saline</td>
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Time to occlusion (min)

Saline, 0.10 mg/kg c7E3, n=7, mean ± SEM

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<tr>
<td>Heparin + Aspirin, 0.25 mg/kg c7E3</td>
<td>* p&lt;0.05 vs Saline</td>
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</tbody>
</table>

Fig 5. Graph showing the number of unbound glycoprotein IIb/IIIa receptors on platelets taken at serial time points from monkeys treated with chimeric Fab fragment of the 7E3 antibody (c7E3) or saline.
Table 2. Glycoprotein IIb/IIIa Blockade (% Sites Bound per Platelet)

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Baseline</th>
<th>30 min</th>
<th>180 min</th>
<th>300 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline control</td>
<td>0±0</td>
<td>45.1±6.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7E3, 0.10 mg/kg</td>
<td>0±0</td>
<td>80.8±2.1</td>
<td>31.6±9.4</td>
<td>57.4±3.1</td>
</tr>
<tr>
<td>7E3, 0.15 mg/kg</td>
<td>0±0</td>
<td>79.3±5.0</td>
<td>68.7±2.6</td>
<td>62.3±2.8</td>
</tr>
<tr>
<td>7E3, 0.20 mg/kg</td>
<td>0±0</td>
<td>79.3±5.0</td>
<td>71.9±2.6</td>
<td>64.3±5.3</td>
</tr>
<tr>
<td>7E3, 0.25 mg/kg</td>
<td>0±0</td>
<td>84.6±2.2</td>
<td>66.1±6.2</td>
<td>65.0±4.9</td>
</tr>
<tr>
<td>Aspirin*</td>
<td>0±0</td>
<td>2.6±11.6</td>
<td>-38.6±31.4</td>
<td>-5.1±19.6</td>
</tr>
<tr>
<td>Heparin†</td>
<td>0±0</td>
<td>-19.4±15.0</td>
<td>-23.2±15.5</td>
<td>-35.2±26.3</td>
</tr>
<tr>
<td>Aspirin+heparin*†</td>
<td>0±0</td>
<td>-3.4±8.7</td>
<td>-1.5±10.3</td>
<td>-11.5±8.7</td>
</tr>
</tbody>
</table>

*25 mg PO daily for 3 days.
†1100 U/kg IV and infusion to maintain activated partial thromboplastin time at 2 to 3 times baseline.
ND indicates not determined.

Saline- or heparin-treated monkeys, whereas aspirin treatment inhibited aggregation in response to arachidonic acid.

The plasma concentration of c7E3 Fab was determined from samples taken 30 minutes after administration of the antibody; the results are shown in Table 3. Plasma concentration of the antibody increased in a dose-dependent manner. Antibody was present in all samples from animals receiving 0.20 or 0.25 mg/kg, whereas lower doses resulted in only partial detection of antibody.

Discussion

This study sought to establish the relative efficacy of c7E3 Fab–mediated inhibition of the platelet GPIIb/IIIa receptor in an experimental model of carotid artery thrombosis compared with the antithrombotic effects of heparin or aspirin treatment given singly or in combination. The primary end point of the experiments was occlusive carotid artery thrombosis in response to electrolytic induction of vessel wall injury. Thrombus formation was correlated with platelet aggregation, GPIIb/IIIa receptor occupancy, and bleeding time.

Previous experiments with the monoclonal F(ab')2 (mF[ab']2) fragment of the 7E3 antibody in canines demonstrated the inhibition of platelet function in prevention of arterial thrombosis. The present study was conducted in cynomolgus monkeys (Macaca fascicularis) because the affinity of the chimeric form of the 7E3 antibody for the GPIIb/IIIa receptor is greater for platelets in humans and nonhuman primates, relative to the platelets of other mammalian species. Primate models of thrombosis have been shown to replicate the human response to vessel wall injury as manifested by thrombus formation. In addition, our study allowed us to compare the results of experiments in two animal species, primates and canines, using the same model and may allow comparisons to be made with regard to different species and the response to vessel wall injury.

Aspirin (acetylsalicylic acid) is accepted as standard preventive therapy in acute myocardial infarction patients and in patients with transient ischemic attacks or stroke, including those who undergo endarterectomy. Aspirin is an irreversible, noncompetitive inhibitor of cyclooxygenase and prevents platelet aggregation by inhibition of platelet thromboxane synthase. Animals that received aspirin in the present study were treated for 3 consecutive days before vascular injury was initiated. Despite inhibiting ex vivo platelet aggregation in response to 0.65 mmol/L arachidonic acid, aspirin-treated animals were not protected from developing occlusive thrombi. The limited effect of treatment with aspirin in preventing thrombosis may be due to platelets remaining responsive to ADP, platelet-activating factor, and thrombin, which are among the potential endogenous mediators of platelet aggregation and are likely to be generated at the site of severe vessel wall injury. Folts et al have demonstrated that aspirin treatment prevented cyclic flow reductions in their animal model of endothelial- and medial-damaged stenosed arteries resulting in periodic acute platelet thrombosis. However, they also demonstrated that if shear forces were increased by increasing the degree of vessel stenosis, if the vessel was re-injured (exposing additional collagen),
or if catecholamines were infused, the protective effect of aspirin was overcome.38

Meta-analysis of clinical trials of intravenous or subcutaneous heparin in acute myocardial infarction performed during prethrombolysis has indicated a significant reduction in mortality, reinfarction, and stroke in treated patients.39 Heparin is a heterogeneous mucopolysaccharide that accelerates an inhibitory interaction between antithrombin III and a number of circulating anticoagulant proteins, including thrombin (factor IIa) and factor Xa.40 Animals that received heparin in the present study were monitored for adequate dosing by measuring their aPTTs. The targeted aPTT was 2 to 3 times the individual animal baseline value. Heparin may not have been effective in the present model because it has no direct antiplatelet actions and has in fact been reported to have proaggregatory properties.41 Interestingly, the use of aspirin has been reported to prevent heparin-induced platelet aggregation, which may add support for the combination of the two agents in thrombotic diseases.42,43 The use of heparin may also be limited because platelet, fibrin, vascular surfaces, and plasma proteins have been reported to modify the anticoagulant effects of heparin.44

The theory behind the concomitant use of aspirin and heparin to prevent thrombosis is that this combination may have synergistic antithrombotic effects, since they each inhibit thrombotic processes involving different pathways. Huang et al45 have demonstrated efficacy of heparin in vivo in acute myocardial infarction surgery.45-48 On activation, the platelet GPIIb/IIIa receptor undergoes a conformational change that exposes an RGD binding site, which facilitates platelet-platelet interactions with fibrinogen.47 RGD sequences on the fibrinogen alpha chain allow bridging between adjacent activated platelets.46,47 The GPIIb/IIIa receptor represents a crucial site for pharmacologic interaction in thrombus formation because of its position in the final common pathway to platelet aggregation.47 The platelet GPIIb/IIIa complex is the most abundant receptor on the surface of platelets48 and is a member of a family of Arg-Gly-Asp-specific receptors (RGD).50-51 Although the complex is always present on the outer surface of the cell, it undergoes conformational change when platelets are activated.52,53

Preclinical studies have assessed and documented the antithrombotic activity of the mF(ab')2 fragment of the 7E3 antibody in a variety of animal models.21-23,33,54,55 Mickelson et al21 and others53 demonstrated similar prevention of coronary thrombus formation and prevention of rethrombosis after recombinant tissue plasminogen activator55 with the mF(ab')2 fragment of 7E3 in a canine model using anodal electrolytic vessel wall injury. Coller and colleagues22 demonstrated the 7E3 F(ab')2 fragment had antithrombotic effects in a model

### TABLE 3. Plasma Concentration of c7E3 Measured 30 Minutes After Antibody Administration

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma Concentration Mean*</th>
<th>n</th>
<th>Detectable Antibody (% of Samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline control</td>
<td>0.0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>c7E3, 0.10 mg/kg IV</td>
<td>0.108±0.000</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>c7E3, 0.15 mg/kg IV</td>
<td>0.153±0.047</td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td>c7E3, 0.20 mg/kg IV</td>
<td>0.575±0.195</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>c7E3, 0.25 mg/kg IV</td>
<td>1.058±0.270</td>
<td>6</td>
<td>100</td>
</tr>
</tbody>
</table>

*Values are expressed as mean±SEM, μg/mL.
of thrombosis in both dogs and monkeys in which the oscillatory flow pattern in response to vessel wall injury was abolished by inhibition of the platelet GPIIb/IIIa receptor.4 The latter study demonstrated that bleeding time, platelet aggregation, and prevention of thrombus formation correlated with the percentage of GPIIb/IIIa receptors bound by the antibody. Although the aforementioned experiments used a different method for the induction of thrombus formation than the one being presented in this study, the studies have the common feature of vessel wall injury to initiate local platelet reactivity and/or thrombus formation. The two studies are also in agreement with respect to the minimum effective dose of antibody required to prevent thrombus formation. Our results show that the minimum effective dose to abolish in vivo arterial thrombotic occlusion was 0.20 mg/kg IV. This dose of the c7E3 Fab antibody corresponds to an inhibition or occupation of more than 80% of the GPIIb/IIIa receptors and a 90% to 100% inhibition of ex vivo platelet aggregation in response to ADP or arachidonic acid. The findings offer the possibility that quantitation of receptor occupancy by c7E3 Fab may provide a reliable means of assessing the proper dose of the antibody and determination of its pharmacodynamics. The results of the present study confirm those presented recently by Kohmura et al56 who reported on the efficacy of c7E3 Fab in the baboon. The latter authors noted that 0.45 mg/kg of the chimeric murine/human Fab fragment of the monoclonal antibody 7E3 enhanced and sustained arterial recanalization with recombinant tissue plasminogen activator. As was the case in our study, Kohmura et al56 also noted that aspirin (17 mg/kg IV) failed to prevent reocclusion of the femoral artery after successful thrombolysis, an observation that coincides with that reported in the present study.

The ex vivo platelet aggregation studies included the addition of a subaggregatory priming concentration of epinephrine added to the cuvettes containing the PRP and PPP samples, followed by either ADP or arachidonic acid as the aggregatory stimulus. Epinephrine augments ex vivo platelet aggregation induced by a variety of agonists and overcomes the inhibition of several antagonists, including prostacyclin.57-58 Therefore, addition of epinephrine provides a more stringent test for inhibition of platelet aggregation by a test drug.44 Samples from several animals in this study were tested for platelet aggregation to determine the ability of epinephrine to enhance the aggregatory response to ADP and arachidonic acid. Ex vivo platelet aggregation performed without the addition of epinephrine resulted in decreased sensitivity and reproducibility when compared with matched samples in which the agonists were added in the presence of epinephrine.

The purpose of this study was to determine the efficacy of the chimeric Fab fragment of 7E3 as an antiplatelet-antithrombotic in a nonhuman primate model of carotid artery thrombosis. The experimental model involves a deep arterial wall injury leading to the exposure of subendothelial components of the vessel wall, thereby exposing physiological stimuli for subsequent platelet–vessel wall interaction and eventual occlusive thrombus formation. Therefore, the events leading to the initiation and completion of thrombus formation are dependent on endogenous physiological responses to injury and are a realistic reflection of the normal sequence of events involved in arterial thrombosis. The experimental results demonstrate a dose-dependent effect with c7E3 Fab in which the antibody was found to be safe and effective in the prevention of occlusive arterial thrombosis. The results were paralleled by extension of template bleeding time, inhibition of ex vivo platelet aggregation, and blockade of platelet GPIIb/IIIa receptors. In comparative studies, it was noted that neither heparin nor aspirin administered as separate pretreatments or when given in combination could achieve the level of efficacy in preventing occlusive thrombus formation as obtained with the c7E3 Fab antibody. The experimental observations are in keeping with the known mechanisms by which each of the three interventions tested, c7E3 Fab, heparin, and aspirin, affect the various steps in the process leading to intravascular platelet aggregation and subsequent thrombus formation.

Acknowledgments

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