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Antiplatelet Monoclonal F(ab')$_2$ Antibody Directed Against the Platelet GPIIb/IIIa Receptor Complex Prevents Coronary Artery Thrombosis in the Canine Heart

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J. K. Mickelson, P. J. Simpson and B. R. Lucchesi. Antiplatelet Monoclonal F(ab')$_2$ Antibody Directed Against the Platelet GPIIb/IIIa Receptor Complex Prevents Coronary Artery Thrombosis in the Canine Heart. *Journal of Molecular and Cellular Cardiology* (1989) **21**, 393–405. Interactions between platelets with injured vascular endothelium contribute to thrombotic occlusion. A murine monoclonal antibody [7E3 F(ab')$_2$] to the platelet GPIIb/IIIa receptor complex was used to inhibit platelet aggregation in an experimental model of coronary artery thrombosis. Prevention of thrombotic occlusion by 7E3 F(ab')$_2$ (0.8 mg/kg bolus i.v.) was studied in dogs with direct current induced intimal injury (100 μA for 5 h) and critical stenosis of the left circumflex coronary artery (LCCA). Baseline LCCA blood flow (CBF) was similar in 7E3 F(ab')$_2$ and control groups, but decreased in the controls [24 $\pm$ 2 ml/min to 0 $\pm$ 0 ml/min, n = 13 (mean $\pm$S.E.M.)] due to thrombotic occlusion in each case (time to thrombosis 136 $\pm$ 15 min). In the group treated with 7E3 F(ab')$_2$, CBF did not change significantly (27 $\pm$ 3 ml/min to 22 $\pm$ 3 ml/min, n = 6) and thrombotic occlusion did not occur during the 5-h observation period in which intimal injury was produced in the LCCA ($P < 0.001$). Oscillations in CBF preceded thrombosis in the control group, but did not occur with 7E3 F(ab')$_2$ treatment (2.2 $\pm$ 0.7 vs. 0 $\pm$ 0, $P < 0.05$). The thrombus mass recovered from the LCCA 30 min after occlusion was 8.8 $\pm$ 1.3 mg in the controls compared to 2.2 $\pm$ 1.2 mg determined 5 h after administration of 7E3 F(ab')$_2$ ($P < 0.05$). When studied *ex vivo*, before the administration of the test agents, platelets from both groups of dogs aggregated in response to ADP and arachidonic acid. However, after treatment, the *ex vivo* aggregation of platelets from 7E3 F(ab')$_2$ animals was inhibited whereas platelets from the control animals continued to aggregate *ex vivo* throughout the period of the experimental protocol ($P < 0.05$). The labeling of platelets with ^{111}indium showed accumulation of radioactivity within the thrombus and upon the vascular endothelium which was less in 7E3 F(ab')$_2$ treated dogs as compared to the control group ($P < 0.05$). The murine monoclonal antibody 7E3 F(ab')$_2$ did not affect hemodynamic values or the circulating platelet count during the experimental protocol. In conclusion, antibody to platelet GPIIb/IIIa receptors: (1) prevented thrombotic LCCA occlusion, (2) inhibited *ex vivo* platelet aggregation, (3) minimized platelet deposition on injured vascular endothelium and within formed thrombi, and (4) stabilized CBF during 5 h of continuous direct currrent induced intimal injury of the LCCA.

Key Words: Monoclonal antibody; Thrombosis; Coronary artery; Platelets; Coronary artery occlusion; Adhesion promoting glycoprotein receptors.

Introduction

The interaction of blood platelets with the endothelial surface of injured blood vessels as well as with other platelets is associated with the development of serious thromboembolic disorders and is a major factor in coronary artery thrombosis and reocclusion after successful angioplasty and or thrombolytic therapy (DeWood *et al.*, 1980; Harrison *et al.*, 1984; Gold *et al.*, 1986; Bates *et al.*, 1987; Golino *et al.*, 1988). Recent knowledge regarding platelet surface receptors suggest that they participate in both platelet–endothelial cell adhesion and platelet–platelet interaction leading to aggregation (Sakariassen *et al.*, 1986). Platelet adhesion, consisting of a single layer of cells, at the site of vessel injury is essential for hemostasis and offers a minimal threat to the formation of an occlusive thrombus. Conversely, intravascular

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platelet aggregation involves the formation of a platelet–fibrin mass which has the potential to embolize or to form an obstructive lesion. The agonists that are thought to initiate platelet aggregation *in vivo* (adenosine, ADP, epinephrine, collagen and thrombin) are dependent upon the binding of fibrinogen or other ligands to the glycoprotein IIb/IIIa (GPIIb/IIIa) receptor (Coller *et al.*, 1983; Coller, 1985; Coller and Scudder, 1985). The important role of the GPIIb/IIIa receptor has been demonstrated by several studies describing monoclonal antibodies that bind specifically with the receptor complex. The inhibition of the GPIIb/IIIa receptor prevents the binding of fibrinogen and other adhesive glycoproteins to the platelet. The recent development of monoclonal antibodies directed against specific epitopes on the GPIIb/IIIa complex offers an opportunity to explore the use of specific antibodies in the prevention of thrombosis due to *in vivo* platelet aggregation in response to vessel injury. There are *in vitro* and *in vivo* data to demonstrate that 7E3 $F(ab')_2$ fragments of a monoclonal antiplatelet antibody can inhibit platelet function without producing hemorrhage or significant thrombocytopenia (Coller and Scudder, 1985).

In the present study we explored the antithrombotic efficacy of a murine monoclonal antibody [7E3 $F(ab')_2$] that binds to the platelet glycoprotein IIb/IIIa receptor. The results of this study indicate that inhibition of the GPIIb/IIIa receptor complex with a monoclonal antibody results in an alteration of the platelet response to vascular wall injury thereby preventing the formation of occlusive coronary artery thrombosis in the experimental animal. The clinical application of this approach may provide an important adjunctive therapeutic intervention directed against thrombotic reocclusion after successful coronary angioplasty and/or thrombolytic therapy.

Methods

Surgical preparation and instrumentation

Male mongrel dogs, 14 to 20 kg, were selected based on the ability of their platelets to aggregate in response to arachidonic acid and adenosine diphosphate (ADP). The animals were anesthetized with sodium pentobarbital (30 mg/kg, i.v.), intubated and ventilated on room air with positive pressure using a Harvard respirator (Harvard Apparatus, S. Natick, MA) at a volume 30 ml/kg and a frequency of 12 breaths/min. Surgery was performed under aseptic conditions beginning with the placement of cannulas into the left carotid artery and jugular vein for monitoring arterial blood pressure (Statham P23 pressure transducer, Gould Inc., Cardiovascular Products, Oxnard, CA) and administering intravenous fluids. The heart was exposed via a left thoracotomy through the 5th intercostal space. A 2-cm segment of the left circumflex coronary artery (LCCA) was isolated from surrounding tissue by blunt dissection. The artery was instrumented from proximal to distal with an electromagnetic flow probe (Model 501, Carolina Medical Electronics, Inc., King, NC), intracoronary electrode and screw occluder (Fig. 1). The intracoronary electrode was constructed from a 25-gauge hypodermic needle tip attached to a 30-gauge Teflon-insulated silver-coated copper wire. The mechanical occluder was constructed of stainless steel in a C shape with a Teflon screw (2 mm diameter), which could be adjusted to control vessel circumference. The external screw occluder was adjusted to decrease the reactive hyperemic flow (10 s occlusion) by 50 to 70% without affecting basal coronary blood flow. A monopolar epicardial electrode was sutured to the surface of the ventricle in the region of LCCA distribution to monitor ischemic changes. The left atrium was cannulated with polyethylene tubing for administration of ^{111}indium labeled platelets, platelet antibody or saline, and Super Imperse Blue dye. Continuous recordings of blood pressure, limb lead II ECG, epicardial electrogram, and mean and phasic LCCA blood flow were obtained on a Model 7 polygraph (Grass Instrument Co., Quincy, MA).

Protocol

Thirty minutes after the surgical preparation was completed, the experimental protocol was initiated. Platelets labeled with ^{111}indium were administered and 15 min later the animals were given either platelet antibody or an equivalent volume of vehicle (normal

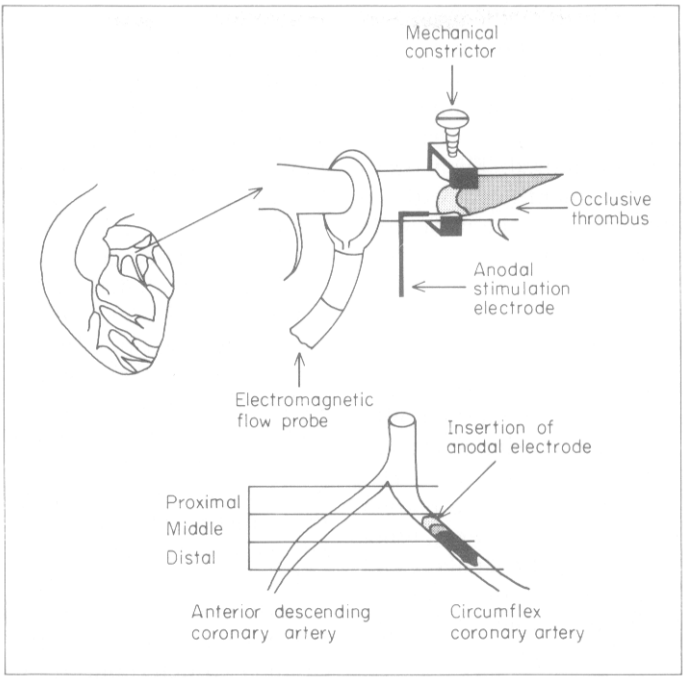

FIGURE 1. Instrumentation of the left circumflex coronary artery. After isolation of the circumflex coronary artery, it was instrumented from proximal to distal with an electromagnetic flow probe, insertion of the intracoronary anodal stimulation electrode, and a mechanical constrictor device for applying an external stenosis to the vessel. The schematic diagram in the lower portion of the figure illustrates the method used to quantitate the accretion of ^{111}indium labeled platelets in the thrombus mass and to calculate the ratio among comparable segments of the left circumflex coronary artery and the left anterior descending coronary artery.

saline) via the left atrium. Finally, 15 min after administration of platelet antibody or saline, a 100 μA continuous anodal direct current stimulation was applied to the intimal surface of the LCCA. The anodal direct current was delivered from a 9 V nickel-cadmium battery. The anode of the battery was in series with a 250 000 Ω potentiometer and the intraluminal coronary artery electrode. The electric circuit was completed by placing the cathode in a subcutaneous site. Prior to occlusion of the artery, spontaneous oscillations in flow occur which have been attributed to either altered vasomotor tone "vasospasm", or formation and dislodgement of partially occlusive platelet thrombi, or perhaps a combination of these phenomenon. The number of oscillations preceding the final occlusive event was recorded.

Each experiment was considered complete 30 min after persistent thrombotic coronary artery occlusion developed or 5 h after initiation of electrical stimulation, whichever occurred first. At the conclusion of the experimental procedure, 10 ml of Super Imperse Blue dye was injected into the left atrium while occluding the LCCA proximal to the area of insertion of the stimulating electrode. In this manner the area subserved by the LCCA, based on the fact that no dye can enter this distribution, was determined *in vivo*. The heart was fibrillated electrically 10 s later and removed quickly. The LCCA was dissected free as far as possible and opened longitudinally; the position of the implanted anodal electrode was verified and the thrombus was removed and weighed. In selected experiments the thrombus was left *in situ* and the vessel was placed in an appropriate fixative and processed for scanning electron microscopy.

The heart was cut from apex to base in 1.0 cm thick sections which were incubated in triphenyltetrazolium chloride (TPT) for 5 min at 37°C. The transverse sections were weighed and traced onto clear plastic sheets: the area stained by Super Imperse Blue dye was the non-risk region (not in the LCCA

distribution), the red stained viable tissue was the risk region (LCCA distribution). Myocardial tissue in the distribution of the LCCA which did not react with tetrazolium and form a red formazan precipitate was considered to be irreversibly injured. The various demarcated areas were quantitated with an Apple IIe microprocessor and the data were expressed as a percent of the total left ventricular weight.

Platelet studies

Platelet counts and *ex vivo* aggregation studies were performed at baseline, 1 h and 5 h after treatment was given. The platelet count was determined with a Haema Count MK-4/HC platelet counting system. Platelet rich plasma (PRP) was prepared from venous blood, using 3.2% sodium citrate as the anticoagulant and the supernate was harvested after centrifugation at 1000 r/min for 5 min (140 × **g**). Platelet poor plasma (PPP) was prepared by centrifuging the remaining blood at 12 000 × **g** for 10 min and discarding the cells. The platelet count of the PRP was determined and then diluted with PPP to achieve a cell count of 200 000/mm^3. Platelet aggregation was determined by established spectrophotometric methods using a four-channel aggregometer (BioData-PAP-4) and recording the increase in light transmission through a stirred suspension of PRP maintained at 37°C (Mills and Roberts, 1967). Aggregation was induced by: ADP 5 μM, ADP 2.5 μM, arachidonic acid (AA) 0.65 mM or 0.325 mM. Epinephrine (550 nM) was used to prime the platelets before stimulation with AA. *In vitro* studies were performed with 7E3 F(ab')$_2$ at concentrations of 10, 20 and 40 μg/ml to compare AA induced aggregation with the above mentioned *ex vivo* studies and to test collagen as an agonist. Aggregation was initiated by the addition of collagen (1 : 10 and 1 : 80 dilution of Ethicon Collagen Dispersion-TD 150) to PRP. Values were expressed as percentage of light transmission standardized to PRP and PPP samples yielding 0% and 100% light transmission, respectively.

Radiolabeling of platelets with 111-indium oxine

Before the thoracotomy, blood (43.5 ml) was withdrawn from the internal jugular cannula into a plastic syringe containing 7.5 ml of 3.2% sodium citrate. The blood was centrifuged at 220 × **g** and the supernatant PRP was removed and the platelets were washed with acidified (pH 6.5) calcium-free Tyrode's buffer containing 0.3 mM EDTA and 0.35% bovine serum albumin as described previously (Thakur *et al.*, 1976). The washed platelets were resuspended gently in 10 ml of Tyrode's buffer and incubated for 15 min at room temperature with 500 μCi ^{111}indium oxine (Diagnostic Isotopes, Inc., NJ). The PRP was centrifuged at 1000 × **g** for 10 min to remove radioactivity not bound to the platelets. The labeled platelets were resuspended in 5 ml of buffer and injected via the left atrial cannula. Samples were taken during the procedure so that labeling efficiency could be determined (Romson *et al.*, 1982).

Fifteen minutes after injecting the ^{111}indium oxine labeled platelets, samples of blood were taken from the internal jugular cannula to determine recovery of the radiolabel. Blood was collected 1 h and 5 h after treatment with either the platelet antibody or normal saline for determining recovery of the radiolabel (Porter-Fink and Eckhauser, 1981). If the artery had occluded, the sample was not obtained.

In the studies conducted with ^{111}indium oxine labeled platelets, the distribution of the radiolabel was determined in the thrombus, LCCA and left anterior descending coronary artery (LAD). A 2 to 3 cm segment of the LAD was dissected free and cut into three segments (proximal, middle and distal) which served as the reference for radiolabeled counts (Fig. 1). In the LCCA the middle segment was the one containing the stimulation electrode. The weighed samples (vessel and thrombus) were counted in a Packard 5320 gamma spectrometer. Radioactivity in each sample was expressed as counts/min/g of tissue. For comparison purposes, in each experiment radioactivity ratios were calculated for similar segments, i.e. mid LCCA/mid LAD.

Antibody administration

Dogs were randomized to receive a single bolus of either normal saline (0.4 ml/kg) or monoclonal platelet antibody 7E3 F(ab')$_2$ (0.8 mg/kg). When ^{111}indium labeled plate-

lets were used, the antibody or vehicle treatment was given 15 min after the labeled platelets were injected, but 15 min before the current was applied to the intracoronary electrode. The antibody was provided by Centocor, Inc. (Malvern, PA) as a sterile, pyrogen free solution.

Detection of residual monoclonal platelet antibody 7E3 F(ab')$_2$

To determine the characteristics of antibody uptake by platelets, samples of plasma were analyzed for the presence of residual antibody at baseline, 1 h and 5 h after administration. Antibody was detected by indirect immunofluorescence analysis in which test cells were incubated in buffer containing test plasma for 30 min at 4°C, and then, after washing, in buffer containing a saturating concentration of fluorescein-conjugated goat anti-mouse immunoglobulin (Tago, Inc., Burlingame, CA) for an additional 30 min at 4°C. Antibody binding to test cells was quantitated by flow cytometry using selective gating of platelets (as determined by log forward angle versus log right angle light scatter) using a Coulter Epics C flow cytometer. The fluorescence intensity of 5000 cells/determination was used as a quantitative measure of antibody binding.

Electron microscopy

Segments of the left circumflex coronary artery were prepared for examination by scanning electron microscopy. The specimens were fixed for 24 h at 4°C in a solution of 1.0% glutaraldehyde (v/v) in 0.1 M cacodylate buffer (pH 7.4). The samples then were dehydrated in a graded series of ethanol solutions, washed in hexamethyl dysilazane and desiccated by critical point drying. The samples were sputter coated with 15 nm of gold spliced paladium (AuPd) and were examined with an AMR 1200 scanning electron microscope.

Statistical analyses

The data are expressed as the mean ± S.E.M. Differences between groups were determined by Student's *t*-test, when applicable, and were considered significant if $P < 0.05$. When indicated, hemodynamic parameters and platelet data were analyzed by two-factor repeated measures analysis of variance (ANOVA). If a significant interaction was present, a Bonferroni multiple comparison method was used to determine the time period(s) at which the treatment means differed significantly ($P < 0.05$). If the time factor main effect was significant, a Newmann–Keuls multiple comparison test was utilized to determine which means were significantly different.

Results

Group characteristics

Nineteen dogs were studied successfully in the electrical induction model of thrombotic coronary artery occlusion. The animals were assigned randomly to two treatment groups: vehicle control (normal saline, $n = 13$) or monoclonal platelet antibody 7E3 F(ab')$_2$ ($n = 6$). There was no difference in body weight between the two groups: control 17.6 ± 0.5 kg (mean ± S.E.M.) vs. antibody 16.5 ± 0.6 kg. There was no significant difference in either the weight of the hearts determined at post mortem (control 149 ± 10 g vs. antibody 139 ± 10 g) or the percentage of the total left ventricle subserved by the left circumflex coronary artery (control 30.4 ± 3.0% vs. antibody 31.4 ± 4.5%). The two groups did not differ with respect to their hemodynamic parameters which were recorded at the beginning of the experimental protocol (heart rate, blood pressure, coronary blood flow).

Left circumflex coronary artery blood flow and coronary artery thrombosis

At the initiation of each experiment, left circumflex coronary blood flow (CBF) did not differ significantly between the two groups: control 23.5 ± 1.5 ml/min vs. antibody 27.3 ± 3.0 ml/min. Over the course of the experimental protocol, CBF was unchanged in the 7E3 antibody treated group and was at a mean value 21.5 ± 3.1 ml/min when recorded 5 h after initiation of the electrolytic lesion in the circumflex coronary artery (Table 1). In the control group CBF decreased progressively over time, as a reflection of the intravascular thrombus which formed in each case. By the 5 h time point, each of the animals in the control group had developed an occlusive intracoronary thrombus and CBF was negligible. The difference in CBF between the two

TABLE 1. Hemodynamic measurements

	Mean arterial pressure (mmHg)		Heart rate (beats/min)		LCCA blood flow (ml/min)	
	Control	7E3	Control	7E3	Control	7E3
Baseline	97 ± 4	119 ± 7	160 ± 6	175 ± 9	24 ± 2	27 ± 3
1 h	100 ± 5	117 ± 7	160 ± 5	164 ± 7	18 ± 2	25 ± 4
2 h	99 ± 6	120 ± 7	158 ± 7	166 ± 9	8 ± 3	25 ± 3[a]
3 h	97 ± 9	119 ± 10	170 ± 20	164 ± 8	8 ± 5	24 ± 3[a]
4 h	105 ± 2	115 ± 7	175 ± 20	167 ± 10	13 ± 9	23 ± 4
5 h	90	118 ± 8	165	163 ± 9	0	22 ± 3

Expressed as mean ± standard error. [a] $P < 0.001$, compared to control at same time point.
n values for control: baseline = 13; 1 h = 13; 2 h = 13; 3 h = 5; 4 h = 1.
n values for 7E3: 6 at all times.

groups nearly achieved statistical significance at the end of the first hour of anodal current stimulation: control 17.5 ± 2.4 ml/min vs. antibody 24.7 ± 3.5 ml/min, $P = 0.054$. The difference in CBF between the two groups was significantly different at subsequent hourly recording: 2 h and 3 h. By 4 h and 5 h, the number of animals in the control was too few to allow for statistical analysis ($P \leq 0.001$ in each case). As the CBF diminished in the control group, there were spontaneous reactive hyperemic responses or oscillations where flow decreased to zero, then abruptly and spontaneously resumed at a rate higher than the preceding flow. The oscillations in the coronary flow did not occur in the antibody treated group (control 2.2 ± 0.7 oscillations vs. antibody 0 ± 0 oscillations, $P < 0.05$).

The time to thrombosis in the control group was 136 ± 15 min ($n = 13$) compared to the antibody group ($n = 6$) in which none of the vessels became occluded during the 300 min (5 h) observation period ($P < 0.001$). At the conclusion of each experiment, the left circumflex coronary artery was opened longitudinally and, when present, the thrombus was recovered intact and weighed. The thrombus weight determined after 30 min of thrombotic occlusion in the control group was 8.8 ± 1.3 mg which was significantly larger than in the 7E3 $F(ab')_2$ antibody group, 2.2 ± 1.1 mg ($P < 0.001$). In the control group thrombotic coronary artery occlusion developed in each animal, while in the antibody treated group small non-occlusive thrombi were found in three arteries, but all arteries were patent after 5 h of anodal current stimulation. It is noteworthy that the non-occlusive thrombi did not appear to disrupt or to diminish CBF. Thus, factors other than the presence of a small intravascular thrombi may interfere with coronary artery blood flow.

Hemodynamic responses

In both the control and 7E3 $F(ab')_2$ antibody treated groups, the main hemodynamic parameters (heart rate and blood pressure) remained essentially unchanged throughout the experimental period (Table 1). The heart rate and mean arterial blood pressure did not differ between the two groups when the number of control animals was large enough to allow for analysis. After the second hour of observation, there were progressively fewer animals remaining in the control group: 3 h ($n = 2$), 4 h ($n = 2$), and 5 h ($n = 1$). The rapid attrition within the control group was due to the fact that experiments were terminated 30 min after blood flow in the circumflex coronary artery had ceased as a result of an occlusive thrombus having formed in response to intimal injury.

Platelet studies

The circulating platelet counts did not change over time and did not differ significantly between the two groups at any time point in the experimental protocol, when the number of control animals was large enough to allow for comparison. The platelet counts in the control and treated group before administration of the antibody were: 453 ± 47 × 10^3/mm^3 ($n = 13$) and 313 ± 25 × 10^3/mm^3 ($n = 6$). One hour after administration of

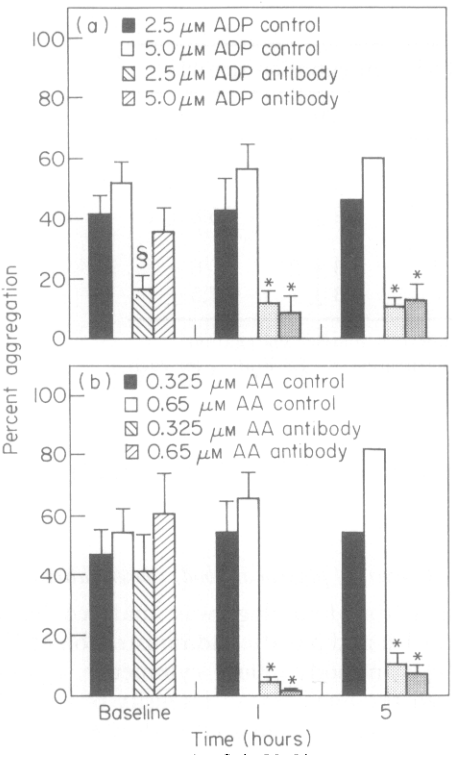

FIGURE 2. (a) Platelet aggregation induced with ADP. Using two concentrations of ADP (2.5 μM and 5.0 μM), platelet aggregation was determined at three time points: baseline, 1 h and 5 h after treatment with diluent or the murine monoclonal 7E3 $F(ab')_2$ antibody. The *ex vivo* responsiveness of platelets to 2.5 μM ADP in those dogs that were subsequently randomized to antibody treatment was lower at baseline than the control platelets (§$P < 0.05$). However, the responsiveness of these same platelets to the higher concentration of ADP (5.0 μM) was similar to that of the platelets from control animals at baseline. Treatment with the monoclonal platelet antibody 7E3 $F(ab')_2$ significantly decreased platelet aggregation compare to baseline and the control group at 1 h (*$P < 0.05$). After 5 h, platelet antibody 7E3 $F(ab')_2$ significantly decreased platelet aggregation compared to baseline (*$P < 0.05$), however only one dog remained in the control group so this comparison could not be made. Data are mean ± S.E.M. (b) Platelet aggregation induced with arachidonic acid. When two concentrations of arachidonic acid (0.325 mM and 0.65 mM) were used with epinephrine primed platelets (550 nM), aggregation was similar at baseline in the two treatment groups. Monoclonal platelet antibody 7E3 $F(ab')_2$ significantly decreased platelet aggregation compared to baseline and to the control group at 1 h (*$P < 0.001$). After 5 h, platelet antibody 7E3 $F(ab')_2$ significantly decreased platelet aggregation compared to baseline (*$P < 0.001$), however, only one dog remained in the control group so this comparison could not be made. Data are mean ± S.E.M.

diluent or the 7E3 $F(ab')_2$ antibody, the respective platelet counts were $397 \pm 63 \times 10^3/mm^3$ ($n = 13$) and $300 \pm 42 \times 10^3/mm^3$ ($n = 6$) (P = N.S.). After 5 h, the platelet count in the 7E3 $F(ab')_2$ antibody treated group was $371 \pm 63 \times 10^3/mm^3$ ($n = 6$).

One hour after vehicle administration in the control group there was no change from the baseline aggregation values [Fig. 2(a)]. After antibody treatment there was a significant decrease in platelet aggregation compared to baseline or to the control group at 1 h, $P < 0.05$ for ADP, $P < 0.001$ for AA [Fig. 2(b)]. After 5 h only one dog remained in the control group, so that statistical comparison between groups could not be performed. However, in the 7E3 $F(ab')_2$ antibody treatment group, significant inhibition of platelet aggregation was present when determined 5 h after administration of the antibody [Fig. 2(a), (b)]. *In vivo* platelet aggregation studies demonstrated that 7E3 $F(ab')_2$ was effective, in a concentration similar to that achieved *in vivo* in preventing platelet aggregation induced by AA and collagen (Table 2).

^{111}*Indium labeled platelet studies*

Platelets were labeled successfully with ^{111}indium and administered to each of six dogs which received 7E3 $F(ab')_2$ antibody and to three dogs which served as controls. Each of the animals provided adequate data with respect to labeling efficiency and *in vivo* radiolabel recovery. A significant decrease in platelet accumulation in the left circumflex coronary artery was observed in the group which was treated with 7E3 $F(ab')_2$ antibody. When the left anterior descending and circumflex coronary arteries from each dog were sectioned into proximal, middle, distal segments, the ratios of radioactivity between the LCCA/LAD segments were much lower in the antibody treatment group when compared to the control group (Fig. 3). Platelets were able to adhere to the surface of the vessel wall to a limited extent in the 7E3 $F(ab')_2$ antibody treated group as evidenced from the presence of the radioactive counts. There was a significant inhibition of further accumulation of platelets by superimposition upon the adherent surface of thrombocytes, thereby impeding the full development of an occlusive thrombus. The ^{111}indium labeled platelets

TABLE 2. *In vitro* platelet aggregation[a]

Agonist	7E3 F(ab')$_2$ antibody concentration (μg/ml)			
	0	10	20	40
Collagen				
1 : 10	38 ± 6[b]	10 ± 2	9 ± 1	9 ± 1
1 : 80	45 ± 3[b]	7.6 ± 2	6.2 ± 1	5.8 ± 1
Arachidonic acid				
0.650 mM	48 ± 8[b]	7 ± 2	4.8 ± 2	6 ± 1
0.325 mM	57 ± 3[b]	4 ± 1	4.8 ± 1	7.5 ± 1

[a] Expressed as mean ± S.E.M.% light transmittance or % platelet aggregation.
[b] $*P < 0.05$ for 0 μg/ml compared to each concentration of 7E3 F(ab')$_2$ for each agonist.
$n = 5$ in each group.

recovered in the few, non-occlusive, adherent thrombi that occurred in the antibody treatment group (225 ± 146 ct/min/g, $n = 6$) suggests that there was a decrease in the number of platelets incorporated into the thrombotic material as assessed by the presence of the radiolabel as compared to the thrombus mass removed from each of the animals in the control group (2100 ± 1200 ct/min/g, $n = 3$; $P < 0.05$).

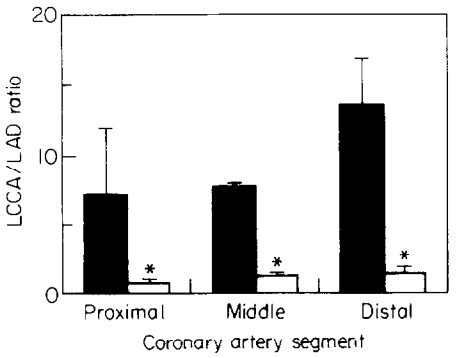

FIGURE 3. The presence of ^{111}indium labeled platelets in the arterial proximal, middle and distal segments of the left anterior descending and left circumflex coronary artery. Homologous platelets were labeled with ^{111}indium and reinjected before the administration of the 7E3 antibody. Anodal current injury of the left circumflex coronary artery was induced in animals which served as vehicle controls (■) and in animals which received the 7E3 F(ab')$_2$ antibody (□). Segments of the left anterior descending coronary artery (LAD) served as a reference. Three segments of each artery were weighed and gamma emission determined as ct/min/g of tissue. A ratio was calculated for purposes of comparison (LCCA/LAD). In the control dogs ($n = 3$), the ratios were higher than in the 7E3 F(ab')$_2$ treated dogs ($n = 6$, $*P < 0.05$ for each segment of artery). Of the three contiguous arterial segments, the segment distal to the placement of the anodal stimulation electrode had the greatest incorporation of ^{111}indium labeled platelets which coincided with the presence and distribution of the thrombus within the vessel.

Analysis of plasma antibody concentrations

Plasma from four dogs was analyzed before and at 1 h and 5 h after administration of 7E3 F(ab')$_2$ antibody. Flow cytometric analysis showed the linear mean intensity of test cells (5000 platelets) incubated with platelet poor plasma at baseline was 0.64 ± 0.08 and increased to 1.03 ± 0.11 at 1 h and 12.03 ± 0.33 at 5 h. There was a significant amount of excess antibody in the plasma over the time course of the experimental protocol with the administered dose of the 7E3 F(ab')$_2$ antibody. The slight increase at 5 h suggests the antibody may not be irreversibly bound to the platelets. The presence of antibody excess in the plasma assured that the antibody concentration would not be limiting and that all available platelet glycoprotein GPIIb/IIIa receptors could be inhibited effectively by binding to the F(ab')$_2$.

Electron microscopy

The luminal surface of the left circumflex coronary artery near the site of insertion of the anodal stimulating electrode was examined by scanning electron microscopy. A scanning electron photomicrograph of a representative circumflex coronary artery, taken from a control animal, shows the presence of an occlusive thrombus mass within the vessel lumen comprised of platelet aggregates, erythrocytes and fibrinous material. The vessel was occluded completely by the thrombotic

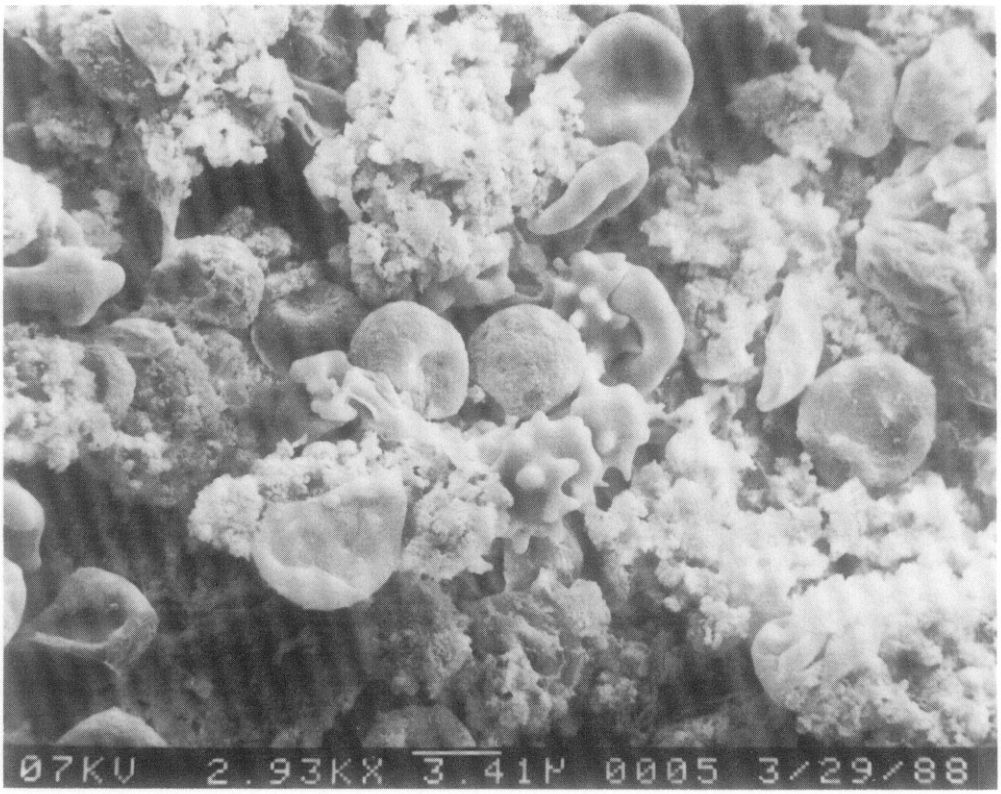

FIGURE 4. A scanning electron photomicrograph of a LCCA from a control animal The region shown is the site at which the anodal stimulation electrode entered the vessel lumen. A view of the luminal surface of the left circumflex coronary artery shows an organized, adherent thrombus within the vessel lumen composed of large aggregates of platelets and erythrocytes enmeshed in a fibrinous matrix. The thrombus is firmly attached to the underlying vessel wall and overlies the point at which the anodal stimulation electrode enters the vessel lumen.

material which is adherent at the site of vessel injury (Fig. 4).

The ability of the 7E3 $F(ab')_2$ antibody to prevent platelet aggregation and subsequent thrombus development on the damaged endothelium is illustrated in Figure 5 which shows relatively few clusters of platelets adherent in the area of the disrupted endothelium with minimal fibrinous material present in the denuded area. Despite the thrombogenic potential of the injured intimal surface, occlusive thrombus formation did not occur in the presence of the 7E3 $F(ab')_2$ antibody.

Discussion

The experimental model of coronary artery thrombosis

The canine model employed in this study possesses anatomic features of coronary artery thrombosis in which an occlusive vascular lesion develops in response to vessel wall intimal injury in a region of high-grade stenosis. The application of an anodal current to the intimal surface of the coronary artery has been shown to be a reliable means of producing a thrombogenic substrate upon which platelets and blood clotting factors will interact and result in the formation of a thrombus mass (Romson *et al.* 1980a, b, 1982). The importance of an *in vivo* model of coronary artery thrombosis becomes apparent in light of the increasing body of data demonstrating that thrombi are composed of platelets and fibrin and that inhibition of the platelet GPIIb/IIIa receptor can prevent platelet aggregation *in vivo* as well as *ex vivo*. The experimental model of intracoronary artery thrombosis, as developed in this laboratory (Romson *et al.*, 1980b), presents a precisely

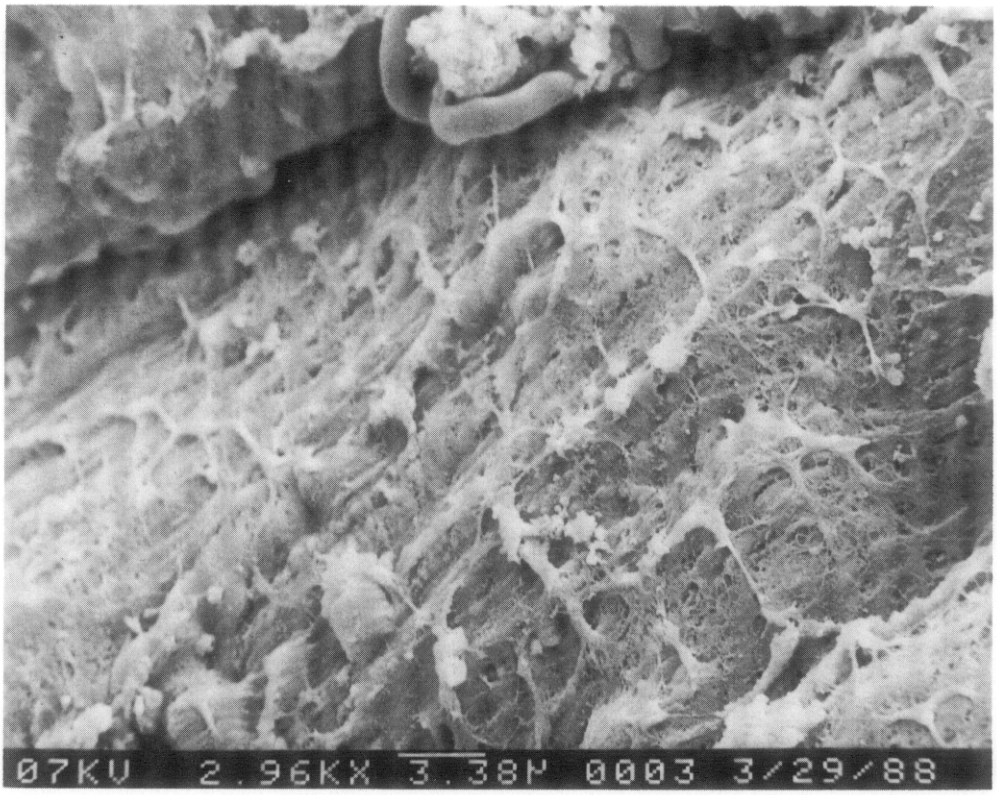

FIGURE 5. A scanning electron photomicrograph of a LCCA from a 7E3 F(ab')$_2$ treated animal. The region shown is the site at which the anodal stimulation electrode entered the vessel lumen. There is an absence of thrombotic material at the site despite the presence of endothelial damage. The endothelium which is denuded remains free of large platelet aggregates and fibrinous material.

defined electrolytic injury resulting in endothelial cell damage in a region of high-grade stenosis. The resulting anatomic substrate provides an *in vivo* environment which will lead to the initiation of cell–cell interactions (platelet–endothelial cell) associated with intravascular thrombus formation.

Selection of the 7E3 F(ab')$_2$ monoclonal antibody

Injury to the surface of the blood vessel exposes components of the arterial media of which fibrillar collagen along with other locally released mediators induce platelet adhesion. Platelet aggregation is superimposed upon the adherent layer of platelets as a result of at least three known mechanisms: metabolites of arachidonic acid, the release of adenosine diphosphate, and/or the pathway involving collagen and thrombin. Each of these pathways may trigger the expression of the platelet receptor GPIIb/IIIa complex. The surface expression of the GPIIb/IIIa receptor complex leads to a binding of fibrinogen, and other adhesive glycoproteins (von Willebrand factor, fibrinogen, fibronectin), to or near the GPIIb/IIIa receptor complex. Platelet aggregation induced by adenosine diphosphate, collagen, epinephrine and thrombin is dependent on the binding of fibrinogen or other ligands to the GPIIb/IIIa receptor complex (Coller *et al.*, 1983). The latter has been identified tentatively as the fibrinogen receptor.

The dimeric structure of fibrinogen allows it to interact simultaneously through the GPIIb/IIIa receptor complex with two platelets thereby serving as a bridging molecule. Coller and colleagues (Coller *et al.*, 1983; Coller, 1985; Coller and Scudder, 1985; Sakariassen *et al.*, 1986) have developed murine monoclonal antibodies which can prevent the binding of fibrinogen to the plate-

let glycoprotein IIb/IIIa receptor complex. Antibody induced inhibition of the GPIIb/IIIa receptor complex has been demonstrated to block the subsequent binding of fibrinogen when platelets are activated (Coller *et al.*, 1983; Hanson *et al.*, 1988). The $F(ab')_2$ fragment which is devoid of the Fc portion of the immunoglobulin molecule, rather than the intact antibody, was employed in the present study in an attempt to circumvent the induction of thrombocytopenia secondary to recognition of the Fc portion of the murine monoclonal antibody by the phagocytic cells of the animal's reticuloendothelial system. The $F(ab')_2$ fragment would have less of a tendency to be cleared by splenic macrophages thereby providing a plasma excess of antibody to interact with available platelet receptor complexes. The continued presence of ^{111}indium labeling in the residual thrombus mass as well as in the circulating pool of platelets throughout the duration of the experimental protocol demonstrates that the platelets which bound to the antibody are not removerd prematurely from the circulation.

Administration of the 7E3 $F(ab')_2$ antibody was not associated with significant changes in the circulating platelet count. A similar observation has been reported by others who have employed the same monoclonal antibody in the canine (Coller and Scudder, 1985; Coller *et al.*, 1985). The antibody has been demonstrated to have a high specificity for platelets and megakaryocytes, thus restricting its effect to these cells (Coller *et al.*, 1983) and to cross react with the GPIIb/IIIa receptor on platelets of primates and dogs (Coller *et al.*, 1986). Nevertheless, the specific inhibition of the GPIIb/IIIa glycoprotein was not associated with evidence of excessive bleeding from the site of the operative incision. The antibody, while interfering with platelet–platelet interactions through the inhibition of fibrinogen binding, may not alter other platelet dependent mechanisms associated with hemostasis. The presence of non-occlusive thrombi attached to the site of intimal injury in the 7E3 $F(ab')_2$ antibody treated animals would be consistent with the notion that platelet adhesion may not be limited entirely. The presence of antibody excess in the plasma would rule out the possibility that a small number of residual unblocked GPIIa/IIIb receptor complexes may have remained functional and would have contributed to the facilitation of hemostasis. Thus other mechanisms such as GPIb and its interaction with von Willebrand factor in the subendothelium might still function so as to promote a limited degree of platelet adhesion. The failure of the platelet GPIIb/IIIa monoclonal antibody to promote excessive bleeding has been reported by other investigators (Coller *et al.*, 1986) and may be related to the presence of alternate receptor sites which can continue to participate in platelet adhesion to the subendothelium.

The prevention of intravascular thrombosis in response to endothelial cell injury

We undertook the present investigation in an effort to define the role of the platelet fibrinogen receptor in thrombus formation *in vivo* and to determine if selective inhibition of the GPIIb/IIIa platelet receptor complex with the 7E3 $F(ab')_2$ antibody could prevent the development of occlusive coronary artery thrombosis and the incorporation of platelets into the resulting thrombus mass. Treatment with a monoclonal platelet antibody, 7E3 $F(ab')_2$(0.8 mg/kg), was effective in preventing occlusive thrombus formation. As a result, the left circumflex coronary artery blood flow remained stable over the duration of the experimental period. The 7E3 $F(ab')_2$ antibody has been shown to be an effective antithrombotic agent in the Folts' model of cyclical blood flow reduction in the canine coronary artery and in monkey carotid arteries (Coller *et al.*, 1986). In both of these models the platelet is thought to interact with damaged vascular endothelium in an area of critical stenosis, adhering and aggregating to form an obstructing thrombus. It is possible that activated platelets release vasospastic substances that cause changes in coronary vascular tone so that obstruction of the vascular lumen could occur with a smaller thrombus mass than would be expected in the absence of an obstructive lesion. Inhibition of arachidonic acid induced aggregation with 7E3 $F(ab')_2$ antibody could not be overcome with epinephrine *in vitro*. The oscillatory coronary flow pattern which characterizes the Folts' model of coronary thrombosis is

enhanced by the systemic infusion of epinephrine. The inhibition of flow reductions by the 7E3 $F(ab')_2$ fragment of antibody 7E3, however, was not reversed by epinephrine (Coller *et* al., 1986). Gold and associates (1988) have demonstrated that the 7E3 $F(ab')_2$ monoclonal antibody accelerated thrombolysis with recombinant tissue plasminogen activator and prevented reocclusion in a canine model in which coronary thrombosis was induced by the injection of stored blood and thrombin directly into the coronary artery. The induced coronary artery thrombi in the control animals underwent lysis with tissue plasminogen activator, but in each instance reoccluded. Thus, as in our studies with 7E3 $F(ab')_2$ antibody, intravascular platelet aggregation could be prevented by selective inhibition of the GPIIb/IIIa receptor.

The 7E3 monoclonal platelet antibody did not exhibit significant hemodynamic effects as suggested by the failure to alter heart rate and blood pressure. In both groups the platelet count was not affected by the experimental protocol. The use of the murine monoclonal 7E3 $F(ab')_2$ fragments did not result in a reduction in the circulating platelet count. A similar benign response to the $F(ab')_2$ fragments has been confirmed in several other models using various animal species (Coller *et al.*, 1986; Gold *et al.*, 1988; Hanson *et al.*, 1988). Coincident with the presence of the 7E3 $F(ab')_2$ on the GPIIb/IIIa receptor is the observation that the *ex vivo* inhibition of ADP or arachidonic acid induced platelet aggregation persisted for 5 h after the antibody had been administered. Others have observed even longer inhibition of platelet aggregation, up to 48 h, with minimal bleeding complications. (Hanson *et al.*, 1988).

The ^{111}indium labeled platelets which subsequently were exposed to antibody did not adhere to the coronary vascular endothelium to the same degree as was observed to occur in the control group of animals. Several methods of vascular injury, including electrically induced deep venous thrombosis and endothelial denudation of the femoral and carotid artery of the dog, are known to cause accumulation of platelets detectable by ^{111}indium labeling (Thakur *et al.*, 1976). The radioactivity ratios in the coronary arterial segments were less in the vessel specimens obtained from antibody treated dogs, thereby suggesting that fewer platelets had adhered to the vessel wall at the site of intimal injury. The resulting thrombus was decreased significantly in overall mass. The ultimate thrombus mass was smaller in the presence of 7E3 $F(ab')_2$ and the concentration of the radiolabel was less in the thrombi recovered from the vessels of animals which received antibody. Because fewer platelets are present, the accumulated mass necessary to obstruct the vessel or the amount of mediators released to evoke the other components of the thrombus (fibrin and neutrophils) are not present. A decrease in the number of platelets incorporated into the thrombus mass might also be associated with a decrease in the local production of fibrinolytic inhibitors which would delay the process of fibrinolysis (24). The development of smaller thrombi may result from retarding or inhibiting those events which lead to platelet accretion on the surface of the thrombus while at the same time permitting unopposed expression of endogenous fibrinolytic mechanisms. It is uncertain whether the local decrease in the number of accumulated platelets and/or the inhibition of aggregation and mediator release account for the observed stabilization of the coronary blood flow in the presence of a nonocclusive thrombus.

Conclusions

The intravenous administration of the murine monoclonal 7E3 $F(ab')_2$ platelet antibody did not produce any adverse effects upon the recorded cardiovascular parameters as evidenced by a failure to alter heart rate, blood pressure, or coronary artery blood flow. In addition the circulating platelet count was unaltered and bleeding or excessive blood loss was not a complication. Whereas the antibody did not achieve a complete prevention of thrombus formation, it did result in a significant decrease in the ultimate size of the thrombus mass thereby preserving coronary artery blood flow.

The 7E3 $F(ab')_2$ platelet antibody may alter the rate of incorporation of functionally intact platelets into the thrombus mass which forms in response to intimal injury in the coronary artery. The latter would result in a

favorable shift in the equilibrium between those factors which promote intravascular thrombus formation and the endogenous local events which favor thrombolysis. Whether or not the immunogenicity of murine monoclonal antibody will preclude its clinical application must be determined. Based upon these observations, it might be speculated that the 7E3 $F(ab')_2$ antibody may serve as an adjunct to other measures directed against intravascular thrombus formation, particularly those associated with coronary artery occlusion after percutaneous transluminal coronary angioplasty or reocclusion after successful thrombolysis as has been suggested by recent experimental studies (Coller *et al.*, 1986; Gold *et al.*, 1988).

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