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Prevention of occlusive coronary artery thrombosis by a murine monoclonal antibody to porcine von Willebrand factor

(platelet thrombus/swine/arterial injury)

DWIGHT A. BELLINGER*†, TIMOTHY C. NICHOLS†‡, MARJORIE S. READ*†, ROBERT L. REDDICK*†, MARY ANN LAMB*†, KENNETH M. BRINKHOUS*†, BRUCE L. EVATT§, AND THOMAS R. GRIGGS*†‡

Departments of *Pathology and ‡Medicine and †Center for Thrombosis and Hemostasis, School of Medicine, University of North Carolina at Chapel Hill, NC 27514; and §Centers for Disease Control, Atlanta, GA 30333

Contributed by Kenneth M. Brinkhous, July 20, 1987

ABSTRACT **A murine monoclonal antibody (mAb) against porcine von Willebrand factor (vWF) induced an antithrombotic state in normal pigs. Thrombosis was induced by a standard procedure of stenosis and mechanical injury of the artery. The mAb was an IgG1 κ that inhibited vWF-induced platelet aggregation at a titer of 1:6250 and bound to immobilized vWF at a maximal dilution of 1:512,000. The antibody did not affect two other vWF functions, platelet adhesion and binding of coagulant factor VIII (factor VIII:C). The antithrombotic state was characterized by a prolonged bleeding time and lack of plasma vWF activity, but with near-normal levels of factor VIII:C and von Willebrand antigen. The circulating Ag·mAb complex demonstrated a multimeric distribution comparable to that of native plasma vWF. Three groups of pigs were studied: group A consisted of nine untreated animals, eight of which developed occlusive coronary thrombosis; group B, four treated animals with a long bleeding time, none of which developed occlusive thrombosis; and group C, two animals with preexisting thrombosis treated with mAb, in which stable blood flow was reestablished. Morphologically, the group B animals showed adherent platelets covering the injured intima but no thrombosis. This mAb is an antithrombotic agent that prevents platelet thrombosis without affecting intrinsic platelet function.**

Thrombus formation in arteries is a multifactorial process. It includes injury to the vessel wall, local alterations in blood flow, platelet adhesion to the intima, and formation of the thrombus proper, with aggregated platelets being a major component. One model for induction of acute coronary arterial thrombosis utilizes constriction of the vessel to produce stenosis, followed by mechanical injury of the wall. An occlusive thrombus develops at the injury site in this stenosis/injury (S/I) model (1–3). A major pathway for recruiting platelets to the buildup of the thrombus appears to involve the plasma von Willebrand factor (vWF), a macromolecular adhesive glycoprotein. vWF is required for platelet adhesion to exposed subendothelium under high wall shear rate conditions, with formation of a monolayer of activated platelets (4, 5). In certain models variable platelet adhesion does occur without vWF, but the platelets appear not to be activated (6, 7). The importance of vWF in causing platelet aggregation is well established when it is induced *in vitro* with ristocetin (8) or botrocetin (9) or with ruminant or pig plasmas with human platelets (10, 11). vWF binds to glycoproteins Ib and IIb/IIIa in the platelet membrane to form interplatelet bridges (12). The role of vWF in platelet thrombus formation has been investigated *in vivo* to only a limited extent. In a study of accelerated atherosclerosis in swine, myocardial infarction evidently secondary to a coronary thrombus occurred in normal pigs but not in pigs with the same degree of atherosclerosis that had von Willebrand disease (vWD) (13). It was recently reported from this laboratory that bleeder pigs with homozygous vWD, lacking plasma vWF, were protected from development of acute coronary thrombosis in the S/I model (3), suggesting an important role of vWF in acute arterial thrombus formation.

In this study we describe a murine monoclonal antibody (mAb) that binds to vWF and, when given to normal pigs, causes a prolonged bleeding time (BT) and depletion of plasma vWF activity without interfering with vWF-dependent platelet adhesion. In the S/I model, the mAb prevented acute coronary thrombosis and caused the reestablishment of stable blood flow in arteries already occluded by thrombi. These data indicate that platelet thrombosis may be prevented or alleviated by altering the reactivity of the vWF molecule without interfering with platelet function. A preliminary report has been published (14).

METHODS

mAb Production. The mAb was prepared as described (15), using a purified porcine vWF as antigen (Ag) (16). Immunoglobulin subtype was determined from cell culture supernatants by using a commercial kit (Boehringer Mannheim). Murine ascites fluid was used as the source of mAb.

Experimental Animals. Normal pigs from the closed colony at the University of North Carolina were used. The standards set forth in "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health publication no. 85-23) were followed.

Assays of vWF and Coagulant Factor VIII (Factor VIII:C). Three functional assays for vWF were performed. The platelet-agglutinating factor (PAggF) assay, termed vWF/PAggF, utilized citrated porcine platelet-poor plasma (PPP) and lyophilized fixed human platelets in a macroscopic platelet agglutination procedure (17, 18). vWF/botrocetin and vWF/ristocetin assays were performed as described (19). Inhibition of vWF-dependent platelet agglutination by mAb was done with each vWF assay procedure, using a 12-well glass titer plate and 50 μl of each reagent. The test mixtures for vWF/PAggF inhibition consisted of imidazole-buffered saline (IBS), pH 7.3 (18), porcine PPP diluted 1:4 with IBS, and serial 1:5 dilutions with IBS of mAb (1:2). After incubation for 5 min at 25°C, human platelets (800,000 per μl) were added and observed for agglutination at 10 min. The mixture

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Abbreviations: Ag, antigen; BT, bleeding time; factor VIII:C, coagulant factor VIII; IBS, imidazole-buffered saline; mAb, monoclonal antibody; PAggF, platelet-agglutinating factor; PPP, platelet-poor plasma; PRP, platelet-rich plasma; S/I, stenosis/injury; vWD, von Willebrand disease; vWF, von Willebrand factor; vWF:Ag, von Willebrand antigen.

for vWF/botrocetin inhibition consisted of lyophilized porcine platelets, porcine PPP, and mAb serially diluted as above. After incubation, botrocetin (40 units/ml) was added. The mixtures for vWF/ristocetin inhibition testing consisted of human platelets, human PPP, and mAb. After incubation, ristocetin (2.4 mg/ml) was added. von Willebrand antigen (vWF:Ag) was determined by Laurell electroimmunoassay (20). Specificity of binding of mAb to porcine vWF was determined by competitive inhibition ELISA (21).

Factor VIII:C activity was assayed by the partial thromboplastin time procedure of Langdell *et al.* (22), using canine hemophilic plasma as substrate. mAb inhibition of factor VIII:C was tested by mixing equal parts of pooled normal porcine plasma and either IBS or serial dilutions of mAb in IBS. The factor VIII:C was determined before and after incubation for 1 hr at 37°C.

Other Tests. Tests for mAb inhibition of platelet aggregation by adenosine diphosphate (ADP; 20 μM), thrombin (2 units/ml), and collagen/$MgCl_2$ (8 mM) were performed in a Payton aggregometer at 37°C, using 0.3 ml of porcine platelet-rich plasma (PRP) (3×10^5 platelets per μl) and 0.1 ml of mAb (1:10) or IBS. After 5-min incubation, 0.1 ml of agonist was added. mAb inhibition of platelet adhesion to deendothelialized porcine artery surface was tested in a Baumgartner *ex vivo* perfusion chamber (23). The ear BT was measured as described by Mertz (24).

S/I Model of Induced Coronary Artery Thrombosis. The Folts system (1, 2) modified by Nichols *et al.* (3) with S/I of porcine coronary arteries was used. Three experimental groups of normal pigs were studied. Group A consisted of nine pigs not treated with mAb. Five of the nine animals served as controls in another study (3). Group B consisted of four animals treated with mAb prior to S/I, and group C (two animals) was treated with mAb after a thrombus had formed. Mean baseline data for the pigs, treated vs. nontreated, were as follows: body weight, 29 vs. 31 kg; platelet count, 4.8 vs. 4.1×10^5 per μl; plasma vWF/PAggF, 0.99 vs. 1.08 units/ml; and BT, 3 vs. 2.25 min. Two intravenous injections of mAb (33 μl/kg) were given with a 30-min interval between doses, after placement of the open clamp and Doppler velocity probe. Platelet count, hematocrit, vWF/PAggF, vWF:Ag, factor VIII:C, and BT were determined prior to mAb infusion, 5 min after infusion, and at 30-min intervals thereafter for the duration of the experiment. In group C, a pattern of cyclic flow reductions was well established after arterial S/I before administration of mAb. Morphometric analysis of the extent of injury and thrombosis was performed for all animals as described (3).

RESULTS

Selection and Some Functional Characteristics of the mAb. Ten clones were screened for their anti-vWF activity, using the PAggF test. One clone, BB3 BD5, with high titer anti-vWF activity was selected for further characterization. It produced an IgG1 κ light chain mAb. The mAb showed binding to immobilized vWF in an ELISA assay at a maximal dilution of 1:512,000.

The specificity of the mAb binding for porcine plasma vWF was shown by competitive inhibition ELISA. Plasmas from normal pigs (1.0 unit of vWF per ml), vWD carrier pigs (0.56 unit/ml), and vWD severe bleeder pigs (<0.01 unit/ml) were used. The relative inhibition of mAb binding caused by the three types of plasmas was dependent on the vWF content of the plasmas. The greatest degree of inhibition was seen with normal plasma, while bleeder plasma, with only a trace of vWF, had a limited effect on antibody binding.

Inhibition of vWF functional activity by the mAb is demonstrated in Table 1. In each of the three tests for vWF activity, the mAb completely inhibited platelet agglutination at maximal dilutions of 1:1250–1:6250. The mAb to porcine vWF demonstrated cross-reactivity with both human and canine vWF. Fig. 1*A* illustrates complete inhibition of vWF platelet-agglutinating activity. Fig. 1*B* illustrates that the mAb caused rapid and complete platelet deagglutination. The mAb had no inhibitory effect on aggregation in PRP induced with the agonists ADP, thrombin, and collagen or on factor VIII:C activity.

Table 1. Inhibition of vWF-dependent platelet agglutination by mAb

Test	Plasma vWF	Platelets	Inhibiting dilutions of mAb
vWF/PAggF	Porcine	Human	1:10–1:6250
vWF/botrocetin	Porcine	Porcine	1:10–1:6250
	Canine	Human	1:10–1:1250
vWF/ristocetin	Human	Human	1:10–1:6250

The effect of the mAb on platelet adhesion to porcine arterial subendothelium is illustrated in Fig. 2. The platelet adhesion index, that is, the percent of arterial surface area covered with adherent platelets, with vWD and normal porcine blood without mAb, was 22% and 79%, respectively. After *in vitro* addition of mAb to normal blood and incubation for 5 min, the value was 73%. With normal blood/mAb mixtures incubated for 30 min, the value was 56%. vWF/PAggF could not be detected in the plasmas from the whole blood/mAb mixtures, while the vWF:Ag and factor VIII:C levels were within normal limits. In another study, whole blood from four normal pigs infused with mAb 30 min earlier (Table 2, group B) was tested in the Baumgartner chamber. The platelet adhesion index was 70%. These studies indicate that the mAb did not inhibit platelet adhesion in this model.

Antithrombotic Effect of mAb. The dose of mAb injected into the test animals was calculated to reduce plasma vWF levels to <5% of normal and to prolong the BT throughout the experimental period. Of the seven treated pigs, all pigs but one were so maintained. Fig. 3*A* demonstrates the effect of injected mAb on plasma vWF/PAggF, BT, vWF:Ag, and factor VIII:C in one animal of group B. Functional plasma vWF was undetectable, while vWF:Ag and factor VIII:C remained above 50% of preinfusion values. The full range

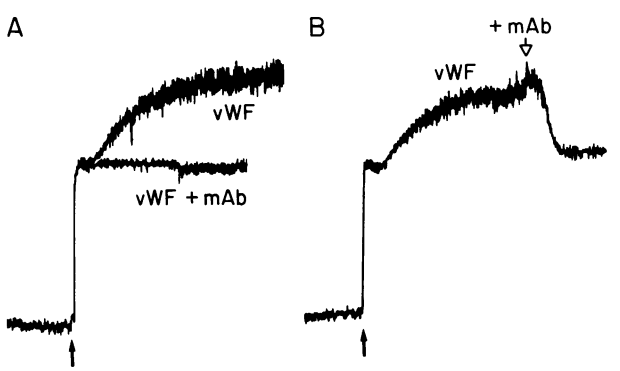

FIG. 1. Aggregometry tracings demonstrating effect of mAb on vWF/PAggF agglutination. A Payton dual-channel aggregometer was used at 37°C, with a stir rate of 10^3 rpm. (*A*) In the upper tracing, 0.2 ml of IBS and 0.1 ml of a suspension of lyophilized human platelets were stirred in the aggregometer for 60 sec. At the arrow, 0.1 ml of porcine PPP and 10 μl of IBS were added simultaneously. In the lower tracing, 10 μl of mAb (1:10) was substituted for IBS. (*B*) At the solid arrow, 0.1 ml of PPP was added. At the open arrow, 10 μl of mAb was added. Immediate complete deagglutination was indicated by decreased light transmission and was verified by phase-contrast microscopy.

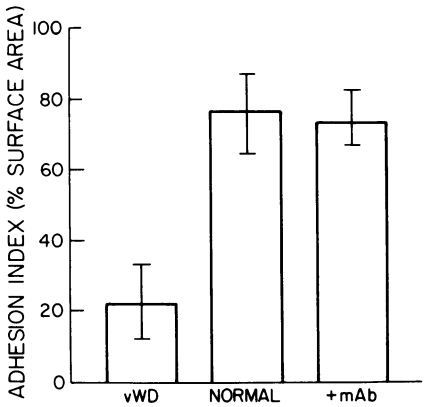

FIG. 2. Effect of mAb on platelet adhesion to subendothelial surface of porcine arteries. The Baumgartner *ex vivo* annular perfusion chamber system (23) was adapted for use with 1-cm segments of everted normal porcine femoral or carotid arteries. Citrated whole porcine blood from either three normal or four vWD animals was perfused across the arterial segment at 130 ml/min, at a shear rate of 1700 sec^{-1} for 5 min, at 37°C. To test the effect of mAb, 50 μl of mAb was added to 55 ml of citrated normal blood, followed by incubation for 5 min at 37°C prior to perfusion. The chamber was flushed with buffer followed by fixation with buffered 4% paraformaldehyde. Plastic-embedded cross sections (2 μm thick) from the midsection of the perfused arterial segments were evaluated for platelet adhesion by light microscopy at 10-μm intervals. The results are expressed as mean percent total surface area of exposed subendothelium covered with adherent platelets, with range of values indicated.

of vWF:Ag multimers was still observed in the plasma (Fig. 4). Fig. 3*B* illustrates the results with one mAb-treated animal in which the BT prolongation was transient and the vWF/PAggF recovered to about 10% of normal during the experiment. The results suggest that the mAb masked the vWF domain(s) required for platelet aggregation and a normal BT and that Ag·mAb complexes remained in circulating plasma.

Table 2 demonstrates the prevention of induced coronary artery thrombosis by mAb administration. Eight of nine animals in the nontreated group, A, developed changes in flow velocity indicative of thrombosis. None of the four animals in the treated group, B, developed occlusive thrombosis, as indicated by lack of change in blood flow velocity. Morphologic data were in accord with the rheologic findings (Fig. 5). Comparison of the mAb-treated and untreated groups indicates statistical significance of the difference (Table 2). Morphometric analysis by light microscopy of the coronary arteries of the treated and untreated animals showed comparable injury to the intima and media. The animal whose responses are depicted in Fig. 3*B*, whose BT returned to normal, developed an occlusive thrombus. This datum along with previous data (3) indicates the importance of a prolonged BT as an indicator of the antithrombotic state.

The effect of administering mAb on established coronary thrombosis was tested in two pigs (group C). In the first pig, two mAb infusions produced total resolution of the cyclic flow reductions despite the presence of stenosis. Fig. 6 illustrates the course of the second pig with established

Table 2. Effect of mAb administration on change in coronary arterial blood flow velocity as an index of thrombosis

Group	No. of animals	mAb treatment	No. of animals with change in flow velocity
A	9	None	8
B	4	Before S/I	0*

*P = 0.007 (Fisher exact test, two-tail) compared to group A.

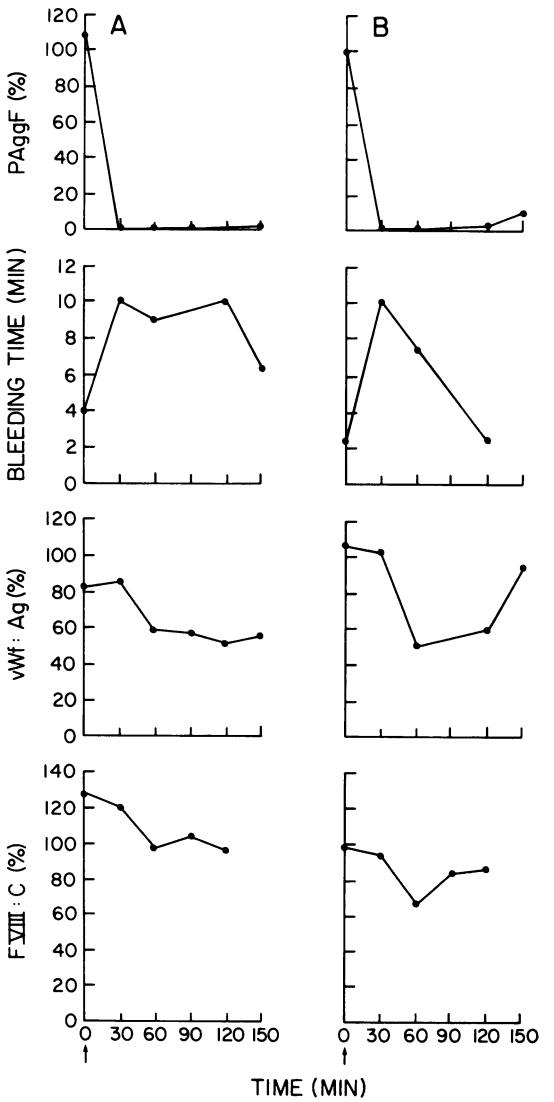

FIG. 3. Effect of administration of mAb to normal pigs on plasma vWF/PAggF, BT, vWF:Ag, and factor VIII:C. Arrows indicate time of mAb injection. (*A*) Illustrative example of one of the four pigs of group B with a persistent prolongation of the BT. (*B*) Data of a pig with transient prolongation of BT.

thrombosis. After mAb infusion, blood flow stabilized. In the succeeding period (not shown), cyclic flow reductions grad-

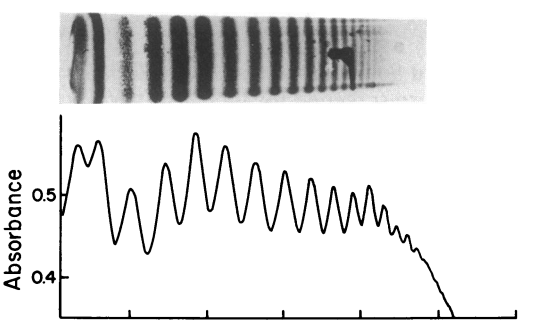

FIG. 4. Densitometer (LKB Ultrascan XL) scan demonstrating multimeric composition of vWF:Ag in a plasma sample 30 min after mAb infusion (see Fig. 3*A*). Sodium dodecyl sulfate gel electrophoresis (Laemmli) was performed by the Aihara *et al.* modification (25) of the Ruggeri–Zimmerman procedure (26). Left to right, low molecular weight to high molecular weight multimers. Each species of multimer observed in the preinfusion plasma sample is represented in this tracing. (Note the tracing is magnified relative to the gel.)

ually resumed. A second infusion of mAb decreased their frequency. After mAb infusion in this group of animals, the BT was prolonged and vWF/PAggF levels were reduced similar to the level shown in Fig. 3*A*. Morphologically, thrombotic material persisted in the coronary arteries of both animals. These data suggest that the mAb caused deaggregation of platelets in the thrombus sufficient to permit reestablishment of stable blood flow.

DISCUSSION

These studies demonstrate that inactivation of plasma vWF in normal pigs by injection of a mAb to porcine vWF protects them from thrombosis and causes a breakup of established thrombi. The mAb is an IgG1 κ that binds specifically to plasma vWF, presumably to an epitope that is coincident to the functional domain responsible for vWF-dependent platelet aggregation. The mAb completely prevented vWF-dependent platelet agglutination induced by ristocetin or botrocetin or in the PAggF test (Table 1). It also caused preformed aggregates to break up (Fig. 1*B*). In the latter case, the mAb apparently can attack the vWF in the interplatelet bridges within the aggregate by forming Ag·mAb complexes, which do not retain the adhesive force of the native vWF. This mAb did not interfere with two other functions of the vWF molecule, platelet adhesion to subendothelium (Fig. 2) and covalent binding of factor VIII:C.

Many anti-vWF mAbs have been described (27). Several of these inhibit platelet adhesion but not ristocetin-induced vWF aggregation of platelets. The majority of these antibodies inhibit both activities. One mAb to human vWF has been described with characteristics similar to mAb (28).

The antithrombotic state caused by injecting the mAb BB5 to normal pigs (Fig. 3) is similar to an mAb-induced vWD-like syndrome described earlier, with loss of plasma vWF activity and a prolonged BT (29). In keeping with the *in vitro* studies of the effect of the mAb on normal plasma, the plasmas of the treated animals retained the ability to induce platelet adhesion in a Baumgartner chamber and retained their associated factor VIII:C activity. In contrast to the loss of functional vWF, the vWF:Ag levels were maintained near normal (Fig. 3).

Densitometer scanning of the gels demonstrating the multimeric structure of the vWF:Ag indicated that the full range of multimers observed in normal plasma persists after treatment with the mAb (Fig. 4). This suggests that the Ag·mAb complex has electrophoretic properties similar to those of the native vWF. While ristocetin and PAggF testing identify only the higher molecular weight vWF multimers, botrocetin recognizes a wide spectrum of multimer sizes (30). These data suggest that the mAb combines with the full spectrum of multimeric forms of vWF.

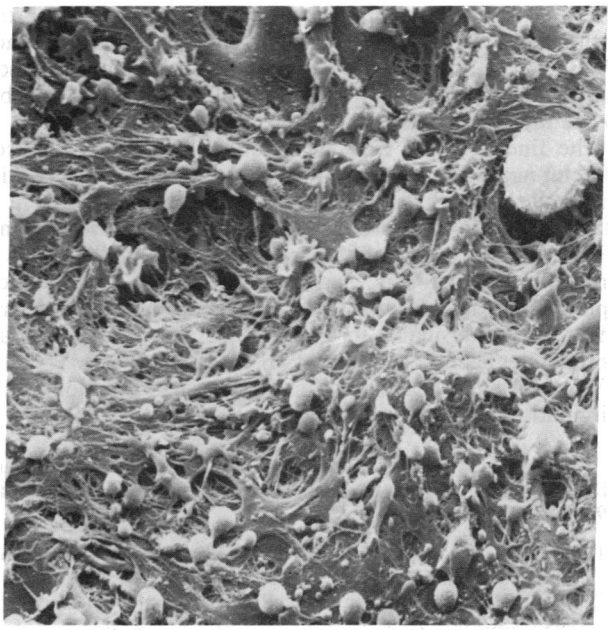

FIG. 5. Scanning electron micrograph of intimal surface of coronary artery segments subjected to S/I after infusion of mAb. Exposed subendothelium is partially covered with adherent platelets, without thrombus formation. (×2500.)

The experimental design for the study of mAb treatment on prevention of thrombosis included maintenance of a prolonged BT throughout the experiment. All of the animals that met this criterion were protected from occlusive thrombosis, which occurred in 8 of the 9 control animals ($P = 0.007$) (Table 2). In the treated animals there was no disturbance in blood flow, which indicated lack of thrombosis. Morphologically, the intimal surface of the damaged coronary artery demonstrated platelet adhesion to exposed subendothelium but gave no evidence of progression to thrombosis (Fig. 5).

In an earlier study demonstrating lack of thrombosis in vWD pigs in the S/I thrombosis model, the persistence of a long BT in transfused animals appeared to be a better indicator of the antithrombotic state than the plasma vWF levels (3). In one animal in the present study, the amount of mAb administered was inadequate to maintain a prolonged BT throughout the experiment (Fig. 3*B*). Thrombosis was induced when the plasma vWF activity increased to about 10% of normal and when the BT was normal.

When a similar thrombosis model in dogs and monkeys was used, a mAb against the platelet receptor glycoprotein IIb/IIIa also caused prevention of arterial thrombosis (31). Fibrinogen binds avidly to glycoprotein IIb/IIIa, and inter-

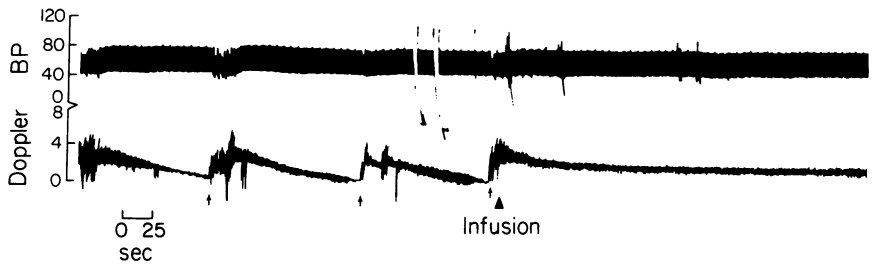

FIG. 6. Restoration of coronary blood flow by infusion of mAb in the porcine S/I thrombosis model. Cyclic reductions in blood flow velocity due to occlusive thromboses within the left anterior descending coronary artery were established and required tapping the vessel to dislodge the thrombus (small arrows). After mAb infusion (large arrowhead), cyclic reductions were abolished for 13 min. Over the next 17 min, cyclic reductions in flow gradually reappeared; a second infusion of mAb again decreased their frequency (not shown). BP, blood pressure in mm Hg (1 mm Hg = 133 Pa); Doppler, blood flow velocity as measured by the Doppler effect on ultrasound in kilohertz.

ference with this binding is probably responsible for the antithrombotic effect. Since this glycoprotein also binds vWF, it too may contribute to the antithrombotic effect. When mAb binds with vWF, the vWF activities lost (Table 1) are those that bind to glycoprotein Ib (12).

The finding of *in vitro* deagglutination of vWF-induced platelet aggregates (Fig. 1*B*) by mAb correlated nicely with the observation that the mAb caused the reestablishment of stable blood flow in coronary arteries occluded with thrombi (Fig. 6). This effect of the mAb, along with its effect in preventing thrombosis, suggests that it may have clinical applications in patients with acute coronary thrombosis. Most drugs designed to prevent platelet thrombosis are antiplatelet agents. In contrast, mAb, acting as it does on the vWF molecule, should not affect the intrinsic functions of the circulating platelets in treated subjects.

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