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Thrombus imaging in a primate model with antibodies specific for an external membrane protein of activated platelets

(radioimmunoscintigraphy/deep venous thrombosis/*Papio cynocephalus*)

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ABSTRACT **The activated platelet is a potential target for the localization of thrombi *in vivo* since, after stimulation and secretion of granule contents, activated platelets are concentrated at sites of blood clot formation. In this study, we used antibodies specific for a membrane protein of activated platelets to detect experimental thrombi in an animal model. PADGEM (platelet activation-dependent granule–external membrane protein), a platelet α-granule membrane protein, is translocated to the plasma membrane during platelet activation and granule secretion. Since PADGEM is internal in unstimulated platelets, polyclonal anti-PADGEM and monoclonal KC4 antibodies do not bind to circulating resting platelets but do interact with activated platelets. Dacron graft material incubated with radiolabeled KC4 or anti-PADGEM antibodies in the presence of thrombin-activated platelet-rich plasma bound most of the antibody. Imaging experiments with ^{123}I-labeled anti-PADGEM in baboons with an external arterial–venous Dacron shunt revealed rapid uptake in the thrombus induced by the Dacron graft; control experiments with ^{123}I-labeled nonimmune IgG exhibited minimal uptake. Deep venous thrombi, formed by using percutaneous balloon catheters to stop blood flow in the femoral vein of baboons, were visualized with ^{123}I-labeled anti-PADGEM. Thrombi were discernible against blood pool background activity without subtraction techniques within 1 hr. No target enhancement was seen with ^{123}I-labeled nonimmune IgG. ^{123}I-labeled anti-PADGEM cleared the blood pool with an initial half-disappearance time of 6 min and did not interfere with hemostasis. These results indicate that radioimmunoscintigraphy with anti-PADGEM antibodies can visualize thrombi in baboon models and is a promising technique for clinical thrombus detection in humans.**

Platelets are anucleate cells that circulate in the blood in a resting form. Upon stimulation that accompanies tissue injury, platelets in the circulation are activated and recruited into the area of injury to participate in thrombus formation. The expression of receptors on the platelet membrane (1), the secretion of granule contents (2), and the extension of pseudopodia (3) are manifestations of platelet activation. Hsu-Lin *et al.* (4) described a platelet protein that is expressed solely on the surface of activated platelets and is not found on the surface of resting platelets. This protein, PADGEM [platelet activation-dependent granule–external membrane protein (5), also referred to as GMP 140 (6)], is an integral membrane protein located in the α-granule membrane of the resting platelet (5, 6). Upon platelet activation, the α-granule contents are secreted with concomitant fusion of the granule membrane into the plasma membrane. PADGEM then becomes a component of the external plasma membrane. Thus, PADGEM serves as a marker of activated platelets. Since activated, but not resting, platelets are incorporated into thrombi at sites of vascular injury, PADGEM might serve as a target for the localization of thrombi *in vivo* by means of specific antibodies.

Other approaches to thrombus imaging have been based upon the incorporation of clot components, including radioactive fibrin (7) or platelets (7–14). These methods require expansion of the thrombus and are complicated by the presence of the radiolabeled agents in both the thrombus and the circulation. Anti-platelet antibodies facilitate the labeling of autologous platelets (15, 16) but are not thrombus-specific, since these antibodies bind to resting platelets in the circulation. In contrast, antibody-targeted thrombus detection using fibrin-specific antibodies (17, 18) is clot-specific, since these antibodies bind to fibrin but not to fibrinogen (19). We have evaluated anti-PADGEM antibodies for antibody-targeted thrombus imaging, exploiting the principle that the target of this antibody, PADGEM, is exposed on activated platelets and that activated platelets are sequestered in thrombi. With *in vitro* and *in vivo* studies employing a baboon (*Papio cynocephalus*) model, we demonstrate the application of anti-PADGEM antibodies for antibody-targeted thrombus imaging.

MATERIALS AND METHODS

Preparation of Immunochemical Reagents. Monoclonal KC4 and polyclonal anti-PADGEM antibodies were produced by immunization of BALB/c mice and New Zealand White rabbits, respectively (4, 20). The polyclonal anti-PADGEM antibodies and KC4 antibody, purified as described (4, 20), migrated as single bands in SDS/PAGE. Iodination was performed with chemical-grade (p, 5n) ^{123}I (Atomic Energy Commission of Canada) or ^{131}I or ^{125}I (New England Nuclear). Anti-PADGEM antibody (100 μg) was incubated with 5 mCi (185 MBq) of ^{123}I, ^{131}I, or ^{125}I for 1 min, and oxidized with chloramine T for 3 min (21). Labeled protein (3–10 μCi/μg) was separated from free iodide on a Sephadex G-50 superfine column equilibrated in Tris-buffered saline (TBS: 0.15 M NaCl/0.02 M Tris·HCl, pH 7.4) containing 0.1% human serum albumin. The radiolabeled anti-PADGEM and KC4 antibodies, evaluated by a RIA (20), bound to thrombin-activated platelets but not to resting platelets. F(ab')$_2$ fragments of anti-PADGEM and nonimmune IgG were prepared by pepsin digestion (22). The F(ab')$_2$

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Abbreviations: PADGEM, platelet activation-dependent granule–external membrane protein; BSA, bovine serum albumin.

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fragments, purified by HPLC on a Beckman chromatograph with a TSK 3000 SW column, were radiolabeled with ^{123}I.

Competition RIA. A solution-phase competition RIA using ^{125}I-labeled PADGEM was used to determine whether antibodies to human PADGEM bind to activated platelets from laboratory animals. Samples containing ^{125}I-labeled PADGEM (35 ng; 20,000 cpm) in 100 μl of TBS with 0.1% bovine serum albumin (BSA) were mixed with 100 μl of a 1:100 dilution of rabbit anti-PADGEM antiserum and serial dilutions of sonicated animal platelets (100 μl) and incubated for 30 min at 23°C. The antigen–antibody complexes were precipitated with 1 ml of 3% (vol/vol) goat anti-rabbit IgG antiserum in TBS containing 25% (wt/vol) PEG 6000 and 0.02% NaN_3. The precipitate, sedimented by centrifugation, was assayed for ^{125}I. Dog, cat, rabbit, rat, guinea pig, baboon, and human platelets were evaluated.

Thrombus Formation on Dacron Grafts *in Vitro*. The binding of polyclonal anti-PADGEM antibody to thrombus formed on Dacron grafts (23) *in vitro* was evaluated. BSA, KC4 antibody, polyclonal anti-PADGEM antibody, and nonimmune rabbit IgG were labeled with ^{131}I (24). Dacron graft material (1.1 × 0.9 cm) was placed in 1.0 ml of platelet-rich plasma (5×10^8 platelets per ml) in the presence of thrombin (1 IU). ^{131}I-labeled KC4 (30 μCi/μg), ^{131}I-labeled anti-PADGEM (30 μCi/μg), ^{131}I-labeled nonimmune IgG (25 μCi/μg), or ^{131}I-labeled BSA (40 μCi/μg), to a total of 20,000 cpm, was immediately added. After 60 min the clot, adherent to the Dacron, was washed and assayed for ^{131}I. Nonspecific binding of radiolabeled protein was determined in parallel experiments using platelet-depleted plasma.

***Ex Vivo* Imaging.** *Ex vivo* imaging experiments were performed with baboons by using an external Ticoflex (DuPont) shunt between the femoral artery and vein. A Dacron graft (6.0 × 0.4 cm) was preclotted by the Sauvage technique (25) and placed in the shunt (26). ^{123}I-labeled anti-PADGEM (800 μCi; 150 μg) was filtered through a 22-μm membrane filter and injected through the venous port in the shunt. Serial 2-min anterior images were obtained for 1 hr with an Ohio Nuclear series 100 Gamma camera with a PDP-11/34 computer in 64 × 64 matrix mode. A low-energy, high-resolution, parallel-hole collimator was used with a 20% window. Serial 1.0-ml blood samples were collected 1, 5, 10, 15, 30, 45, and 60 min after tracer injection and assayed for ^{123}I. Prothrombin time, partial thromboplastin time, platelet count, and platelet aggregation were determined before and 60 min after administration of tracer. The kinetics of tracer uptake by the graft and blood pool were derived from the radionuclide images. These experiments were also performed using ^{123}I-labeled nonimmune IgG (800 μCi; 170 μg) and ^{123}I-labeled $F(ab')_2$ fragments of anti-PADGEM (1.0 mCi; 100 μg) and nonimmune IgG (1.0 mCi; 100 μg).

***In Vivo* Deep Venous Thrombosis Model.** Experimental thrombi were induced in baboons by stasis (27). After a no. 8 French sheath was inserted into a femoral vein, the vascular anatomy was defined by venogram with injection of Renografin. Two 3-mm balloon angioplasty catheters (Mansfield Scientific, Mansfield, MA) were advanced into the femoral vessel under fluoroscopic guidance. A distance of 1–3 cm separated the two balloons. The balloons were inflated, creating venous stasis; after 30 min, the balloons were deflated and the catheters were removed. The extent of the thrombus was confirmed by venography. Twenty-five serial, 6-min anterior images centered over both legs were obtained. ^{123}I-labeled anti-PADGEM or nonimmune IgG (260 μCi; 100 μg) was injected via a superficial basilar vein. Mechanical embolectomy was performed prior to the acquisition of the final image. Venography was then repeated to document the extent of any residual thrombi. Prothrombin time, partial thromboplastin time, platelet count, and platelet aggregation were determined before and 90 min after tracer injection. The radioactivity in the thrombosed and contralateral veins was quantitated.

The organ distribution of the ^{123}I-labeled anti-PADGEM was determined from the radionuclide images 60 min after tracer injection. Radionuclide activity was expressed as a percentage of the injected dose (26).

RESULTS

Anti-PADGEM antibodies specific for activated platelets were used to visualize thrombi in animal models. To study the interaction of these antibodies with thrombi *in vitro*, both monoclonal KC4 and polyclonal anti-PADGEM antibodies were evaluated for their ability to bind to the thrombus formed on Dacron graft material following the addition of thrombin to platelet-rich plasma. The Dacron material exposed to KC4 antibody contained 90 ± 10% of the ^{131}I-labeled KC4 monoclonal antibody. The Dacron material incubated with polyclonal anti-PADGEM antibodies bound 70 ± 12% of the ^{131}I-labeled anti-PADGEM antibodies. In contrast, only 9 ± 2% of the ^{131}I-labeled nonimmune IgG and 1 ± 0.5% of the ^{131}I-labeled BSA were bound to the Dacron material, respectively. Nonspecific binding of all antibodies in plasma depleted of platelets was 4 ± 1%. These results indicate that antibodies directed against PADGEM on activated platelets are specifically incorporated into thrombi on Dacron material.

The KC4 antibody was prepared against human platelets (4). The interaction of this antibody with platelets from humans and laboratory animals was ascertained to select an animal model. KC4 antibody bound to human platelets, but not to baboon, cat, dog, rabbit, rat, guinea pig, or rhesus monkey platelets. For this reason, the interaction of immunoaffinity-purified polyclonal anti-PADGEM antibodies (5) with thrombin-activated platelets of various animal species was evaluated in a solution-phase competition RIA using ^{125}I-labeled PADGEM. The binding of anti-PADGEM antibodies to ^{125}I-labeled PADGEM was completely inhibited by human or baboon platelets at 10^8 per ml. Platelets from rat, rabbit, cat, dog, or guinea pig did not bind to rabbit anti-PADGEM antibodies. On this basis, we used rabbit anti-PADGEM antibodies in baboon models to evaluate the strategy of targeting thrombi *in vivo* with anti-PADGEM antibodies.

Imaging Studies in *ex Vivo* Dacron Shunts. To study the kinetics of platelet uptake on Dacron graft material placed in an external arteriovenous shunt in baboons, radiolabeled anti-PADGEM antibodies were infused through the venous port of the shunt. The uptake of antibody on platelets incorporated into thrombus on the Dacron graft was measured. Infusion of antibody through the venous port of the shunt allowed dilution of the antibody into the baboon circulation prior to the exposure of the thrombus on the graft to the radiolabeled antibody. Thrombi induced by the Dacron graft material in the *ex vivo* shunt were visualized within 10 min after injection of ^{123}I-labeled anti-PADGEM antibodies. Optimal images were obtained at 30 min (Fig. 1*A*). The circulating blood pool radiotracer activity in the distal and proximal tubing of the shunt was negligible compared to the radiotracer activity associated with the Dacron graft. The ratio of graft radioactivity to blood pool radioactivity was 33:1. Five separate baboon experiments yielded similar data (Table 1). On average, the percentage of antibody associated with the thrombus was about 0.1% and the graft/blood pool ratio was 32:1. Parallel baboon experiments using ^{123}I-labeled nonimmune IgG as the radiotracer revealed minimal and inhomogenous uptake by the Dacron graft (Fig. 1*B*). Three separate baboon control experiments yielded similar data, indicating an average percentage of antibody associated with

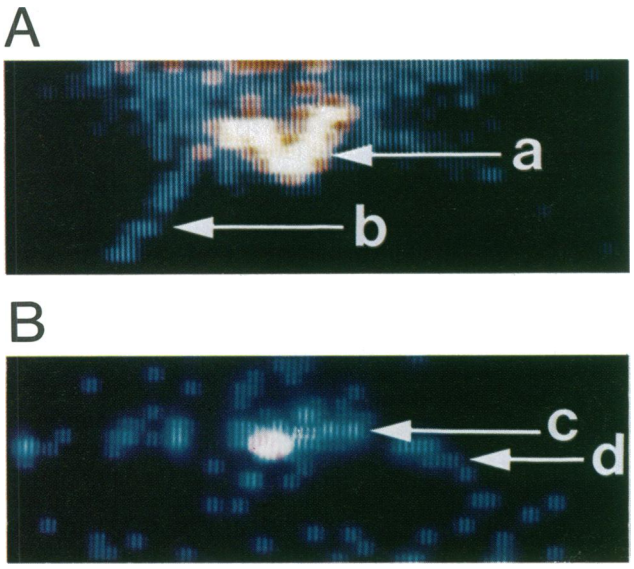

FIG. 1. Radionuclide image of Dacron graft in the *ex vivo* shunt. (*A*) Image of the shunt 30 min after injection of ^{123}I-labeled anti-PADGEM antibodies (800 μCi; 150 μg). The area of uptake correlates with the location of the thrombus in the Dacron graft (a). ^{123}I-labeled anti-PADGEM antibody in the blood pool was visualized in the shunt tubing (b). (*B*) Image of the shunt 30 min after injection of ^{123}I-labeled nonimmune IgG (800 μCi; 150 μg). There was minimal uptake of the ^{123}I-labeled nonimmune IgG by the Dacron graft (c) relative to circulating ^{123}I-labeled nonimmune IgG in the blood pool (d).

the thrombus of about 0.001% and a graft/blood pool ratio of 5:1.

The kinetics of antibody uptake by the graft and blood pool were derived from the radionuclide images (Fig. 2). The ^{123}I-labeled anti-PADGEM uptake by thrombus in the Dacron graft was linear for the initial 10 min and approached maximal levels within 30 min. In contrast, incorporation of ^{123}I-labeled nonimmune IgG into the thrombus increased linearly with time after initial uptake. The ratio of the concentration of anti-PADGEM antibodies in the Dacron graft (target) to the concentration of anti-PADGEM antibodies in the circulating blood pool (nontarget) was 33:1. For ^{123}I-labeled nonimmune IgG, the target/nontarget ratio was 5:1.

When these experiments were repeated using ^{123}I-labeled anti-PADGEM and ^{123}I-labeled nonimmune IgG F(ab')$_2$ fragments, the target/blood pool activity ratios were 31:1 and 3:1, respectively, within 14 min after injection of antibody. These studies indicate that the anti-PADGEM antibodies are bound to thrombus shortly after injection. This uptake is a specific process that allows visualization of the *ex vivo* thrombi by γ scintigraphy without subtraction techniques. Furthermore,

Table 1. Interaction of antibodies with thrombus on Dacron graft

^{123}I-labeled antibody	Exp.	% injected dose on graft	Graft/blood pool tracer ratio
Anti-PADGEM	1	0.01	27
	2	0.04	33
	3	0.09	32
	4	0.21	35
	5	0.09	31
		(0.09 ± 0.05)	(32 ± 2)
Nonimmune IgG	1	0.0020	8
	2	0.0013	5
	3	0.0011	3
		(0.0014 ± 0.0006)	(5 ± 1)

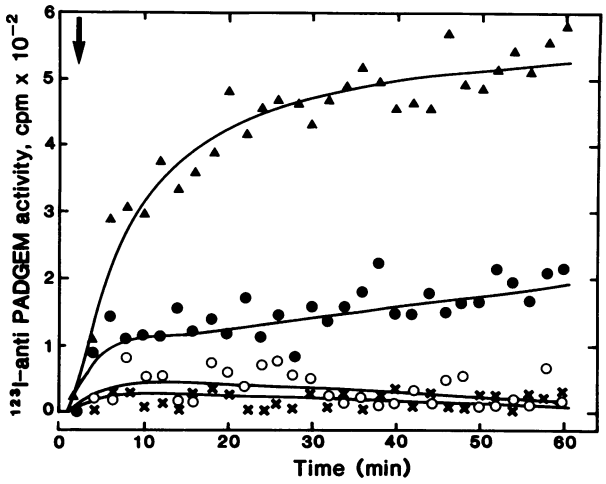

FIG. 2. Kinetics of antibody uptake by thrombus associated with the Dacron shunt. ^{123}I-labeled anti-PADGEM activity associated with the Dacron shunt (▲) is compared with that associated with the circulating blood pool (○). ^{123}I-labeled nonimmune IgG activity associated with the Dacron shunt (●) is compared with that associated with the circulating blood pool (×). The target/blood pool ratio 30 min after ^{123}I-labeled anti-PADGEM infusion was 33:1. The target/blood pool ratio 30 min after infusion of ^{123}I-labeled nonimmune IgG was 5:1.

intact immunoglobulin and IgG F(ab')$_2$ fragments specific for PADGEM demonstrated similar uptake parameters.

***In Vivo* Deep Venous Thrombosis Model.** Thrombus was induced by stasis in the femoral vein of a baboon. The presence of a clot was confirmed by venography (Fig. 3 *A* and *B*). Subsequently, ^{123}I-labeled anti-PADGEM antibodies (100 μg; 260 μCi) were infused through a superficial basilic vein. The thrombus was observable without background subtraction on scintigrams obtained within 15 min after antibody infusion. These images were optimal between 30 and 60 min (Fig. 3*C*). The thrombus/blood pool activity ratios derived from the radionuclide images were 3:1. In one experiment, the clot was successfully removed by thrombectomy; a scintigram obtained after clot removal revealed markedly diminished radioactivity in the area of the thrombus. This 0.4 × 0.2-cm clot contained 0.02% of the injected dose of ^{123}I-labeled anti-PADGEM antibody. In three experiments, the thrombus/blood pool activity ratio was, on average, 3.3 and a clear thrombus image was obtained without blood pool subtraction techniques (Table 2). In two parallel experiments using ^{123}I-labeled nonimmune IgG as the radiotracer, the radionuclide did not concentrate in the region of the thrombus (Fig. 4). The thrombus/blood pool activity ratios derived in these experiments were 1.2:1.

The ^{123}I-labeled anti-PADGEM antibodies cleared the blood pool with an initial half-disappearance time of 6 min. A second phase of antibody clearance exhibited a half-time of about 4 hr. Blood samples drawn 60 min after injection of the radiotracer showed that the circulating blood pool contained 32 ± 3% of the injected dose. Scintigrams obtained 60 min after injection showed the following radionuclide distribution: spleen, 2.5 ± 1%; liver, 21 ± 2%; bladder, 38.9 ± 4%; kidney, 1.8 ± 0.5%. Radioactivity recovered from the urine was free [^{123}I]iodide.

The infusion of anti-PADGEM antibodies did not alter hemostasis. Platelet number; platelet aggregation in response to epinephrine, ADP, or collagen stimulation; and coagulation properties, including the prothrombin time and partial thromboplastin time, were unchanged during the course of the experiments.

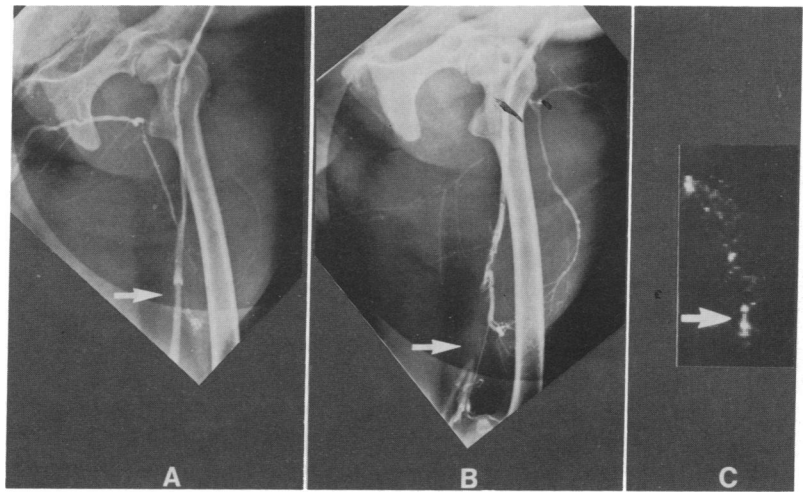

FIG. 3. Venograms and scintigram of *in vivo* venous thrombus with ^{123}I-labeled anti-PADGEM. (*A*) Baseline venogram of the left femoral vein prior to thrombus formation. (*B*) Venogram of the same vein after formation of a thrombus (arrow). (*C*) Scintigram obtained 60 min after infusion of ^{123}I-labeled anti-PADGEM (260 μCi; 100 μg). This anterior view delineates the thrombus (arrow) without any background subtraction.

DISCUSSION

Thrombi form in blood vessels as a component of the normal physiologic response to tissue injury or as part of a pathologic process associated with the disruption of normal regulatory control mechanisms. At present, the definitive diagnosis of thrombosis requires radiographic visualization of the occluded blood vessel by use of a radioopaque contrast agent. A safe and reliable method for the detection and localization of thrombi in patients with thromboembolic disease would greatly facilitate diagnosis. The use of antibodies that bind specifically to a target in the thrombus and that can be labeled to allow detection and quantitation offers one approach to thrombus localization. The ideal target for localization of thrombi (*i*) should be present in the thrombus but not otherwise exposed in any other sites, (*ii*) should be present at a sufficient concentration to facilitate its interaction with the antibody, (*iii*) should be sufficiently exposed to the circulating blood so that the antibody has contact with the target, (*iv*) should be structurally stable over time and should not dissociate from the thrombus, (*v*) must not be obscured by other adherent cells or proteins, and (*vi*) should interact rapidly with the antibody.

With these characteristics in mind, we have evaluated PADGEM as a target molecule for thrombus localization. This platelet protein is internal in resting platelets but is exposed on the platelet surface after platelet activation and secretion (5). Thus, PADGEM is not expressed on the circulating platelets in the blood but is expressed in activated platelets that are incorporated into the thrombus. Because this integral membrane protein remains associated with activated platelets at the site of the thrombus, we have performed experiments in solution and in a baboon model to evaluate PADGEM as a target for thrombus detection.

The monoclonal antibody KC4 is specific for human platelets (4) and does not crossreact with baboon platelets. Therefore, we used immunoaffinity-purified rabbit polyclonal anti-PADGEM antibodies for these studies. These antibodies are specific for PADGEM and bind to baboon and human platelets equivalently. KC4, prepared against thrombin-activated platelets, is directed against a PADGEM epitope that is exposed on intact activated platelets. Anti-PADGEM polyclonal antibodies, prepared against purified PADGEM, contain antibody subpopulations directed against epitopes that are normally buried in the membrane or may be located in the cytosolic compartment. Therefore, it was anticipated that a fraction of the anti-PADGEM antibodies would not bind to activated platelets. Indeed, almost all of the KC4 antibody was associated with the clot, whereas only 70% of the anti-PADGEM was bound to the clot. In the absence of a monoclonal antibody that bound to baboon platelets, the polyclonal monospecific anti-PADGEM antibody reagent represented an option for use in an animal model.

Table 2. Interaction of antibodies with deep venous thrombus

^{123}I-labeled antibody	Exp.	Thrombus/blood pool tracer ratio
Anti-PADGEM	1	3
	2	4
	3	3
		(3.3 ± 0.4)
Nonimmune IgG	1	1.5
	2	0.9
		(1.2 ± 0.3)

Angiography is currently the major clinical method of thrombus detection and localization. However, this diagnostic procedure requires cannulation and the injection of contrast material into the vessel. Alternative methods have been based on the principle that radiolabeled components of the clot, such as ^{125}I-labeled fibrinogen (7) or ^{111}In-oxine-labeled platelets (7–14), can be incorporated into the developing thrombus. Some of the labeled fibrinogen is converted to fibrin and incorporated into the growing clot (7). In scintigraphy using ^{111}In-oxine-labeled platelets, a subpopulation of the labeled platelets in the circulation becomes incorporated into the thrombus (7–14). These methods have allowed detection of ventricular thrombi (10), venous thrombosis (7, 14), and coronary artery thrombosis (9). Both methods are compromised by the small amount of radiotracer that becomes associated with the thrombus relative to the large amount of radiotracer that persists within the circulation. Because of the small signal (thrombus) superimposed on a high background (blood), only large thrombi are easily detected.

Oster *et al.* (15) employed a radiolabeled anti-platelet monoclonal antibody for thrombus detection (see also ref. 28). This antibody, directed against the platelet membrane glycoprotein complex gpIIb–IIIa, binds to resting and activated platelets. Another anti-platelet monoclonal antibody has also been employed for thrombus imaging (16). In both cases, the platelet target is not specific for platelets incorporated in thrombi but is associated with the circulating platelets as well. Nonetheless, the use of an anti-platelet antibody facilitates association of radiolabel with the platelet, a practical improvement over ^{111}In-oxine-labeled platelets (11). However, platelet antibodies that alter platelet function may cause undesired antithrombotic or bleeding complications (29).

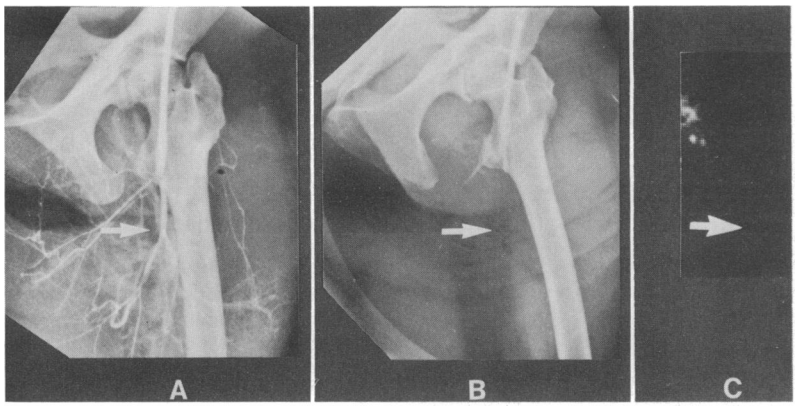

FIG. 4. Venograms and scintigram of an *in vivo* venous thrombus with ^{123}I-labeled nonimmune IgG. (*A*) Venogram of the left femoral vein prior to thrombus formation. (*B*) Venogram obtained after a thrombus (arrow) was formed. (*C*) Scintigram obtained 60 min after infusion of ^{123}I-labeled nonimmune IgG (260 μCi; 100 μg). The thrombus was not visualized.

Thrombi are composed of activated platelets and crosslinked fibrin strands and usually also include trapped erythrocytes. Plasma does not contain significant fibrin but only its precursor, fibrinogen. Thus, fibrin is a potential target for thrombus imaging using such reagents as anti-fibrin antibodies. Haber and colleagues (19, 30) described the preparation of anti-fibrin antibodies that bind to fibrin but not to fibrinogen. The abundance of fibrin in the thrombus makes this an excellent target (17). However, small amounts of fibrin do circulate in the plasma, and the preference of these antibodies for soluble fibrin over crosslinked fibrin may complicate the use of this antibody for thrombus imaging and detection.

We have evaluated PADGEM as a target for platelet imaging. Although this protein has been found within venous endothelial cells (31), it is neither exposed on circulating, resting platelets nor present in a soluble form in plasma. We have shown that anti-PADGEM antibodies are sequestered at the site of thrombus formation in baboons in both an *ex vivo* shunt model of thrombosis and an *in vivo* venous thrombosis model. The kinetics of antibody uptake in the vicinity of the thrombus is rapid, allowing visualization within 10 min and optimal imaging within 1 hr. Because the target antigen does not circulate in the blood, the radiolabel in the blood is rapidly cleared, leading to low background blood pool activity and concentration of radioactivity at the site of the thrombus. This obviates the need for subtraction techniques to detect and localize the thrombus. Moreover, this antibody does not inhibit normal hemostasis. These initial results suggest that PADGEM may be a useful platelet target for the imaging of thrombi *in vivo*. Furthermore, anti-PADGEM antibodies may prove useful for clot lysis, based on a strategy of antibody-targeted fibrinolysis with an antibody–fibrinolytic enzyme conjugate (32, 33).

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