Studies With a Monoclonal Antibody Against Activated Platelets: Evidence That a Secreted 53,000-Molecular Weight Lysosome-like Granule Protein Is Exposed on the Surface of Activated Platelets in the Circulation

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To define the role of activated platelets we have attempted to prepare monoclonal antibodies specific for activated platelets. The IgG2b antibody of one of the clones, designated 2.28, was studied in more detail. Native platelets from normal individuals bound 650 ± 2.28 molecules/platelet, whereas thrombin-activated platelets bound 12,600 molecules/platelet with high affinity (4.6 nmol/L). Immunoelectrophoretic analysis revealed that 2.28 reacted with a 53,000-mol wt protein. Immunocytochemistry showed that the antigen is located in a special subclass of platelet granules in unstimulated platelets and is exposed on the surface of thrombin-activated platelets. Double-labeling studies with immunogold labels disclosed simultaneous localization of 2.28 binding sites and cathepsin D in the same granules both in megakaryocytes and endothelial cells, thereby indicating that the antigen may be localized in lysosomes. By using flow cytometry, in vivo platelet activation was studied in patients undergoing cardiac surgery with cardiopulmonary bypass. Increased numbers of platelets that expressed the 2.28 antigen on their surface were observed after extracorporeal perfusion. The percentage of 2.28-positive platelets in the circulation was 3.9% ± 2.7% (SD) in controls (n = 20), 5.5% ± 3.0% in patients (n = 10) before cardiopulmonary bypass surgery, 24.6% ± 13.5% after the bypass, and 8.5% in two patients with acute deep venous thrombosis. These data indicate that 2.28 may serve as a useful probe of in vitro and in vivo platelet activation.

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CTIVATION OF HUMAN BLOOD platelets is associated with changes in platelet morphology, biochemistry, and membrane composition. The events result in binding or increased surface expression of several endogenous platelet proteins and plasma proteins. A number of the biologic activities of platelets in hemostasis and thrombosis have been attributed to these changes in membrane composition. Stimulated platelets bind von Willebrand factor, fibronectin, fibronectin, and thrombospondin, interactions involved in platelet adhesion and aggregation. Other changes in the platelet membrane such as the exposure of a procoagulant surface that promotes prothrombin and intrinsic factor X activation and the expression of binding sites for high-molecular weight (mol wt) kininogen, factor Xla, factor XIIIa, activated protein C, and plasminogen may be important for the role of activated platelets in coagulation and fibrinolysis.

Blood tests reflecting activation of platelets are potentially useful in understanding the physiological role of activated platelets and may be useful in evaluating patients with thrombotic diseases. To develop methods for the detection of activated platelets in the circulation, we used the hybridoma technique to produce murine monoclonal antibodies (MoAbs) that react with activated platelets but do not react with resting platelets. This report describes the characteristics of one of the most potent MoAbs.

METHODS

Platelet preparation. Freshly fixed platelets were prepared by collecting blood by venipuncture in 3 vol of paraformaldehyde (final concentration, 1% wt/vol) in phosphate-buffered saline (PBS). The fixed platelets were washed twice and resuspended in PBS.

Thrombin-activated platelets were prepared by collecting blood in 0.1 vol 135 mmol/L trisodium citrate. The platelets were washed three times in 5 mmol/L EDTA-PBS, and afterwards thrombin, to a final concentration of 5 U/mL, was added to the platelet suspension and incubated without stirring for two minutes. The activated platelets were washed (for the preparation of MoAbs) or fixed and washed as described earlier (for immunofluorescence microscopy, flow cytometry, and binding studies).

Antibody production. BALB/c mice were immunized subcutaneously on day 1 with 5 × 106 thrombin-activated washed platelets, suspended in 300 μL Tris-buffered saline and 1 mmol/L EDTA, and mixed with complete Freund's adjuvant and with activated platelets without Freund's adjuvant on days 21 and 28. Mice were boosted with 10 × 108 activated platelets on day 35, three days before removal of the spleens. Spleen cells were fused according to Köhler and Millstein with cells of the mouse myeloma cell line X63.Ag 8.653, a hypoxanthine phosphoribosyl transferase-deficient line that synthesizes no immunoglobulin. Four hybridoma supernatants (2.2, 2.28, 2.45, and 2.47) were identified that bound to thrombin-activated platelets but did not react with resting platelets as assessed by fluorescence microscopy. These hybridomas were subcloned by limiting dilution. Ascitic fluid rich in 2.28 antibody was prepared by intraperitoneal injection of pristane-treated BALB/c mice with the hybridoma cell line 2.28.

Antibody purification. Ascites was applied to a 0.9 × 12.0-cm column of protein A-Sepharose CL-4B that had been equilibrated with 0.1 mol/L sodium phosphate buffer, pH 8.0. The column was eluted with the phosphate buffer until the optical density of the eluate returned to base line, after which elution was accomplished with 0.1 mol/L acetic acid and 0.15 mol/L NaCl, pH 2.5. Protein elution was monitored by optical density at 280 nm. Fractions were pooled and dialyzed against PBS, pH 7.3. This purification yielded an antibody preparation that was >95% pure as assessed by sodium dodecyl sulfate-polycrylamide gel electrophoresis (SDS-PAGE).

Antibody iodination. The purified MoAb 2.28 was radiolabeled with 125I by using the chloramine-T method. Noncovalently linked 125I was removed by dialysis overnight at 4°C against 0.05 mol/L dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).
Tris-HCl and 0.1 mol/L NaCl, pH 7.0. The specific activity was 530 cpm/ng, and greater than 96% of the radioactivity was precipitated by 10% trichloroacetic acid.

**Immunocytotoxicity.** Immunofluorescence studies were performed on paraformaldehyde-fixed resting platelets, fixed thrombin-activated platelets, and sections of about 200 nm of platelets and endothelial cells as described. In some studies we used a rabbit antihuman placental cathepsin D, an antibody that has been characterized before.

Platelet aggregation. Platelet aggregation studies were performed at 37°C in a Payton dual-channel aggregation module (Payton Associates Inc, Buffalo) with 5 μmol/L adenosine diphosphate (ADP) (Dade Diagnostics Inc, Aquada, PR) 5 μmol/L epinephrine (Dade), 1.5 mmol/L arachidonic acid (Bio-Data Corp, Hatboro, PA), 10 μmol/L l-onophore A23187 (Boehringer GmbH, Mannheim, FRG), and 1 mg/mL ristocetin (H. Lüdecke & Co, Copenhagen). The aggregation studies were performed in platelet-rich plasma (0.5 mL, 250,000 platelets/L) after preincubation with 10 to 40 μL 2.28 ascites for 15 minutes at 37°C.

Electrophoretic analysis of platelet antigen recognized by 2.28. Human blood platelets isolated from 100 mL blood were washed three times and suspended in 2 mL of 5 mmol/L EDTA-PBS. Secretion of proteins was induced by adding 10 units thrombin (Sigma Chemical Co, St Louis) at 37°C. After two minutes 20 units of hirudin (Sigma) was added. The suspension was then centrifuged, and the supernatant was used for further electrophoretic analysis.

One-dimensional SDS slab gel electrophoresis was performed according to Laemmli with minor modifications using nitrocellulose sheets (Schleicher & Schuell, FRG, BA 85, 0.45 μm) and electrophoresis for 15 hours at 12 V/cm. Immediately after transfer, the nitrocellulose sheets were immersed in 5% bovine serum albumin in Tris-buffered saline (0.15 mol/L NaCl, 10 mmol/L Tris-HCl, pH 7.4) for 16 hours at 4°C to saturate additional protein-binding sites. The sheets were rinsed six times in Tris-buffered saline and 0.1% Tween 20 and incubated for two hours at 20°C with the radiolabeled monoclonal antibody 2.28 (0.5 μg/mL diluted 1:100 in Tris-buffered saline with 5% bovine serum albumin). The sheets were then washed in Tris-buffered saline and 0.1% Tween 20 (six changes in one hour) and dried between cellophane sheets (Bio-Rad Laboratories, Richmond, CA). Autoradiography of the dried gels was performed at 70°C by using Kodak X-Omat AR5 (Eastman Kodak Co, Rochester, NY) x-ray film and DuPont Cronex Lightening Plus intensifying screens (DuPont Instruments, Wilmington, DE). A mixture of low-mol wt proteins (Pharmacia Fine Chemicals, Uppsala, Sweden) and high-mol wt proteins (Bio-Rad) was analyzed on every gel as mol wt markers. For blocking experiments, the sheets were incubated with 125I-2.28 and an excess (200-fold) of nonradiolabeled 2.28 or nonspecific mouse ascites from X63.Ag 8.653 myeloma cell lines.

Electrophoretic analysis of the endothelial cell component recognized by 2.28. Human vascular endothelial cells were isolated from umbilical veins and cultured in 75-cm² flasks as described by Jaffe et al with some minor modifications. The cells were identified by their typical characteristics. Cells from the second passage were cultured, and after the cells had reached confluency, they were washed twice with PBS and incubated for 24 hours with leucine-depleted Ham's F-10 medium (Flow Laboratories, Irvine, UK) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), amphotericin B (5 μg/mL), ultraser (2% vol/vol final concentration), and 250 μCi 1H-leucine (Amersham Corp, Buckinghamshire, England). The cells were washed twice with PBS and solubilized for one hour at 4°C with 10 mmol/L triethanolamine and 150 mmol/L NaCl buffer, pH 7.8, containing 1% (vol/vol) nonidet NP-40 (Sigma), and the following protease inhibitors: 4 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L d-amino benzamidine, 0.2 mg/mL soybean trypsin inhibitor, 2 mmol/L iodoacetamide, 2 mmol/L l-aminocaproic acid, and 5 mmol/L NaCl EDTA. The lysate was centrifuged for five minutes (10,000 g) at room temperature to remove undissolved material.

The cell lysate (1 mL from approximately 10⁶ cells) was passed over a C18-Sepharose 4B (Pharmacia) column (0.6 x 3 cm) to which 2.1 mg of normal goat IgG had been covalently coupled according to the manufacturer's instructions. Protein A-Sepharose CL4B (Pharmacia) was rehydrated in 0.1 mol/L phosphate buffer, pH 8.0, supplemented with 0.05% (vol/vol) Tween 20, 0.02% (wt/vol) NaN₃, and 0.1% (wt/vol) bovine serum albumin. After the Sepharose had been washed twice with the buffer, approximately 3 mg of protein A-Sepharose was incubated overnight at 4°C with either 50 μL normal mouse serum or 50 μL of 2.28 ascites fluid. The Sepharose beads were incubated for three hours at room temperature with the flow-through fraction of the cell lysate. The beads were washed eight times by centrifugation with 10 mmol/L triethanolamine and 500 mmol/L KCl buffer, pH 7.8, containing 1% (vol/vol) NP40 and then twice with PBS. The bound protein was extracted from the Sepharose beads by boiling for five minutes in the presence of 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 0.025% bromphenol blue, and 5% 2-mercaptoethanol in 0.125 mol/L Tris-HCl, pH 6.8. The extract was analyzed by SDS-PAGE in slab gels (3% to 30%). After electrophoresis gels were treated with EN²HANCE (New England Nuclear, Boston) according to the manufacturer's instructions and dried, and subsequently an autoradiogram was made.

**Binding of 125I-antibody to platelets.** Binding of 125I-2.28 to paraformaldehyde-fixed unstimulated and thrombin-stimulated platelets was studied by adding varying concentrations of 125I-2.28 to the washed platelet suspensions in such a way that the final platelet

![Fig 1. Immunofluorescent staining for 2.28 binding sites in 200-nm cryosections of endothelial cells. The labeling reveals a punctate, intracellular pattern, which indicates localization in granules. (Original magnification x 1,500; current magnification x 1,125; bar, 10 μm.)](from www.bloodjournal.org by guest on August 15, 2017. For personal use only.)
concentration was $10^4$ platelets/mL and the final volume 120 $\mu$L. After an incubation for 120 minutes at 20°C, a 100-$\mu$L aliquot was layered on top of 50 $\mu$L phthalate and centrifuged at 12,000 g for two minutes. After removing the supernatant, centrifuge tube tips were cut off and the bound radioactivity determined in a gamma counter. Nonspecific binding was determined by adding a 200-fold excess of unlabeled antibody to the incubation mixtures. This value was subtracted from the bound counts per minute to yield total specific bound counts per minute. The binding assays were performed in duplicate. The binding affinity of antibody 2.28 and the total number of antibody molecules bound per platelets were calculated by using the HYPER computer program as described by Cleland. 13

Flow cytofluorometry. Measurements were carried out in a cytofluorograph 50-H (Ortho Instruments, Westwood, MA) using laser light of 488 nm at an intensity of 250 mW. Fluorescence and scatter signals were standardized daily by using fluorescent monodisperse carboxymethylated microspheres (diameter, 2.04 $\mu$m ± 0.02 SD; Polysciences, Inc, Warrington, PA). Size was measured by using the blue forward scatter, and gating was set such that 90% of the particles in a sample were accepted for fluorescence analysis. Freshly paraformaldehyde fixed unstimulated platelets and fixed thrombin-activated platelets (4 x $10^4$ platelets in 200 $\mu$L EDTA-PBS) were incubated with 2 $\mu$g purified 2.28 (in 200 $\mu$L EDTA-PBS) for two hours at 20°C. After incubation the platelets were washed three times and incubated with 50 $\mu$L of diluted (1:50) fluorescein isothiocyanate (FITC)-labeled goat antimouse (GAM) IgG (Central Laboratory of Blood transfusion, Amsterdam) for 30 minutes at 20°C. The platelets were then washed twice with PBS and resuspended in 1 mL PBS. A total of $10^7$ platelets of each sample was analyzed at a rate of 2,000 platelets per second. A control specimen containing the FITC-coupled antibody with a purified aspecific MoAb and a control specimen containing 2.28 without the FITC-coupled antibody served as negative controls. Flow cytofluorometry results were compared with visual fluorescence microscopy and found to be concordant. Twenty healthy controls and 12 patients were studied.

Fig 2. Localization of 2.28 binding sites by electron microscopic immunocytochemistry with colloidal gold particles in unstimulated (A to D) and thrombin-stimulated (E) human blood platelets. (A to D) Label is present in granules with moderate to low electron density but not in the cytoplasmic matrix, mitochondria, or on the cell membrane. (E) In stimulated platelets gold particles are present in the surface-connected canalicular system. A few particles can be seen on the platelet plasma membrane at the right bottom. Colloidal gold particles, 8 nm. (Original magnifications: A x44,000, B x95,000, C x81,500; D x40,000; E x41,000; current magnifications: A x33,000, B x71,250, C x48,125, D x30,000, E x30,750; bar, 0.5 $\mu$m.)
Other methods. Measurement of \( \beta \)-thromboglobulin was performed by using a commercially available radioimmunoassay (Radiochemical Centre, Amersham, UK).

Patient studies. Ten patients were studied who underwent surgery for coronary bypass grafting (n = 8) or mitral valve replacement (n = 2). The mean age of the patients was 64 years. Surgery was carried out under moderate hypothermic conditions (29° to 27°C). A Bentley Bos bubble oxygenator was used, and the mean duration of extracorporeal circulation was 92 minutes with a range of 64 to 148 minutes. Heparin, 300 U/kg, was administered initially, and 100 U/kg was given each hour of extracorporeal perfusion. Protamine sulfate was administered at termination of the bypass in a ratio of 1 mg/100 U of heparin given. A blood sample was drawn via a catheter immediately before perfusion and 20 minutes after cessation of bypass.

Two patients were studied with acute deep venous thrombosis. The diagnosis was made by impedance plethysmography. Blood samples were drawn at presentation by venipuncture.

Twenty normal individuals (aged 22 to 48) volunteered as controls. None of the controls had used any medication for at least 14 days before the investigation.

RESULTS

Using immunofluorescence as a screening test, we identified four MoAbs that bound to thrombin-activated platelets but did not react with resting platelets. The antibodies, designated 2.2, 2.28, 2.45, and 2.47, were shown to be IgG1, IgG2b, IgG1, and IgM respectively by Ouchterlony immunodiffusion analysis. Ascites fluid obtained from mice injected with cells obtained from cell line 2.28 was used for purification of 2.28 by protein A-Sepharose affinity chromatography. Purified 2.28 did not agglutinate platelets, nor did it inhibit ADP-, epinephrine-, thrombin-, collagen-, ristocetin-, or ionophore A23187-induced platelet aggregation.

Immunocytochemical localization. Immunofluorescence studies on cryosections of washed platelets (data not shown) and endothelial cells (Fig 1) revealed a dotlike labeling pattern for 2.28, with no fluorescence of the cell membrane, thereby suggesting that the antigen was localized in granules. To visualize the 2.28 antigen, we used protein A-colloidal gold for immunoelectron microscopy. These studies confirmed that the label was localized in specific platelet granules (Fig 2A to 2D) in unstimulated platelets. The intragranular distribution of the label was diffuse. The label was found in granules with moderate to low electron density but not in the cytoplasmic matrix, mitochondria, or on the platelet membrane. \( \alpha \)-Granules were negative. In platelets stimulated with thrombin before fixation, the label was found in the surface-connected canalicular system and on the platelet membrane (Fig 2E). The label was also present in specific granules of endothelial cells. The distribution of the label suggested that the antigen was localized in lysosomes. To prove this localization we investigated simultaneously with a double-labeling technique the distribution of the 2.28 antigen and the lysosomal enzyme cathepsin D. Figure 3 shows that both labels were present in the same granules both in megakaryocytes and in endothelial cells.

Identification of the antigen recognized by 2.28. The supernatant of thrombin-activated platelets was subjected to electrophoresis in a polyacrylamide gel after solubilization in SDS, subsequently blotted on nitrocellulose, and incubated with radiolabeled 2.28. Autoradiography (Fig 4) revealed that \( ^{125} \)I-2.28 bound to a protein with an apparent mol wt of 53,000. A minor radiolabeled protein with a mol wt of 40,000 was also present. It is unknown thus far whether the minor band represents a cleavage product induced by thrombin activation of the platelet or another form of the protein.

![Simultaneous electron microscopic immunocytochemical localization of 2.28 binding sites (6-nm gold particles) and cathepsin D (10-nm gold particles) in the same granule in (A) megakaryocytes and (B) endothelial cells. (Original magnifications: A ×53,000, B ×31,000; current magnifications: A ×45,050, B ×26,350; bar, 0.5 \( \mu \)m.)](image)
Fig 4. Analysis of platelet and endothelial proteins that bind to 2.28. (Lane A) Autoradiogram of proteins secreted by thrombin-stimulated platelets, subsequently electrophoresed in 3% to 30% polyacrylamide gel, blotted, and incubated with \(^{125}\text{I}-2.28\). Lane A shows a major band of 63,000 and a minor band of 40,000. Lane B and C show blocking experiments. The binding of \(^{125}\text{I}-2.28\) could be blocked by an excess (200-fold) of nonradiolabeled 2.28 (lane B). and nonspecific mouse ascites had no effect (lane C). Lane D shows the \(^{3}\text{H}\)-leucine-labeled extract of endothelial cells immunoprecipitated by 2.28. and lane E shows the control using a nonspecific mouse IgG monoclonal antibody.

These data were obtained by electrophoresis under nonreducing conditions. When the experiments were performed under reducing conditions, \(^{125}\text{I}-2.28\) did not bind, presumably because the 2.28-binding site was disrupted by \(\beta\)-mercaptoethanol.

We also used another approach to identify the component recognized by 2.28. Cultures of human endothelial cells were labeled with \(^{3}\text{H}\)-leucine, and the endothelial proteins were solubilized and immunoprecipitated. The immunoprecipitate was solubilized in SDS, electrophoresed in a polyacrylamide gel under reducing conditions, and visualized by fluorography. Figures 4D and E show that 2.28 recognizes a protein with a mol wt of 53,000 that is synthesized by human endothelial cells. Immunoprecipitation studies with the medium of endothelial cells revealed that the antigen was not excreted in the medium.

**Binding of \(^{125}\text{I}-2.28\) to platelets.** Binding studies indicated that there was a minimal specific binding of \(^{125}\text{I}-2.28\) to fixed unstimulated platelets. \(^{125}\text{I}-2.28\) bound to fixed thrombin-activated platelets, and time course studies (data not shown) indicated that equilibrium was established after two hours’ incubation. Binding was saturable, and analysis (Fig 5) of the binding data indicated that \(^{125}\text{I}-2.28\) bound with high affinity (4.6 nmol/L). Fixed unstimulated platelets bound 650 \(^{125}\text{I}-2.28\) molecules per platelet, and fixed thrombin-activated platelets bound 12,600 \(^{125}\text{I}-2.28\) molecules per platelet.

**Analysis of activated platelets by flow cytofluorometry.** We studied the number of circulating 2.28-positive platelets by flow cytofluorometry. Figure 6 shows representative fluorescence profiles and cumulative distribution curves of unstimulated and thrombin-activated platelets labeled with 2.28-FITC-GAM.

The percentage of 2.28-positive cells was calculated from cumulative distribution curves. Each sample was analyzed in three ways, which resulted in three cumulative curves: (a) a curve (+2.28, +FITC) showing the fluorescence of cells labeled with 2.28 and FITC-GAM, (b) a curve ( −2.28, +FITC) showing the fluorescence of cells obtained by addition of a nonspecific MoAb and FITC-GAM, and (c) a curve showing the fluorescence obtained by the addition of 2.28 alone. Both latter curves represent basic fluorescences, and since these curves were similar in our studies, it was not necessary to correct the data for the aspecific fluorescence caused by FITC-GAM alone. The percentage of 2.28-positive cells in a sample was calculated as the distance (percentage) between the point on the second curve ( −2.28, +FITC) at the 99th percentile and the point on the first curve (+2.28, +FITC) in the same channel (Fig 6D).
The percentage of 2.28-positive platelets in the circulation was 3.9% ± 2.7% (SD) in healthy controls (n = 20). In vitro stimulation with 5 units thrombin resulted in 76.1% ± 6.4% (SD) 2.28-positive platelets (n = 7). Patients undergoing cardiopulmonary bypass surgery (n = 10) had 5.5% ± 3.0% (SD) 2.28-positive platelets immediately before extracorporeal perfusion and 24.6% ± 13.5% (SD) after the bypass. There was a correlation of .82 with the plasma levels of β-thromboglobulin that increased from 78.9 ± 32.7 (SD) ng/mL before to 645.9 ± 318.0 (SD) ng/mL after extracorporeal perfusion. No correlation was found between the number of 2.28-positive cells and the time of bypass. Two patients with acute deep venous thrombosis had increased levels of 2.28-positive platelets of 7% and 10% respectively.

DISCUSSION

In the present study we characterized a murine MoAb, designated 2.28, that binds to a protein expressed on the surface of human blood platelets after activation. The IgG2b antibody identifies a protein with a mol wt of 53,000 that is also present in granules of endothelial cells, and by using immunoprecipitation studies of endothelial cell cultures we could demonstrate that the protein is synthesized by human endothelial cells. Double-labeling experiments showed that the protein, recognized by 2.28, and cathepsin D were localized in the same granules, thereby indicating that the protein is localized in lysosomes. The identity and function of the reactive antigen is unknown thus far. Its mol wt does not agree with the characteristics of the few proteinases of platelet lysosomes described previously in the literature.

A mol wt of 33,000 has been reported for platelet cathepsin D, 60,000 for cathepsin E, 40,000 for trypsin, 32,000 for a chymotrypsinlike enzyme, and 26,000 for platelet elastase. The function of this protein could not be clarified by using the antibody 2.28. The antibody did not affect platelet aggregation induced by several stimuli.

The binding of radiolabeled 2.28 to thrombin-activated platelets was saturable. Fixed resting platelets bound ~650 molecules of 2.28 per platelet, and fixed thrombin-activated platelets bound ~12,600 molecules per platelet. The low level of binding in native platelets may be due to a low number of binding sites for 2.28 on the total population of native platelets or may be due to heterogeneity of the platelet population, that is, a high number of binding sites on a small number of platelets. To study these two hypotheses we used flow cytometry, a technique that enables measurement of the expression of binding sites for an antibody in each cell separately. The results of the immunofluorescence analysis indicated that immediately fixed native platelets were heterogeneous, a small subpopulation of platelets (4%) expressed the antigen on the surface, and the rest of the platelets did not. This small percentage 2.28-positive platelets may reflect a small number of nonresting platelets present in the circulation in normal subjects. These platelets may have undergone the secretion reaction and may be responsible for the basic levels of platelet-specific proteins like β-thromboglobulin in plasma. On the other hand, we also observed a heterogeneity in platelets treated with thrombin in vitro. Some platelets did not express the antigen on the surface after stimulation. Further studies must be carried out to characterize these subpopulations, and flow cytometric sorting will provide a means to these studies.

Five other antiplatelet MoAbs that react preferentially with activated platelets have been described recently. KC1,26 and S1232 identify a platelet α-granule membrane protein of mol wt 140,000 that is expressed on the platelet plasma membrane during degradation. This protein may be a component of the internal α-granule membrane that is fused with the plasma membrane during activation. An antibody described in preliminary form, Pn,35 may be directed against the same protein. The number of binding sites on stimulated platelets of these antibodies, 13,400, 9,600, and 11,200 molecules per platelet respectively, is in the same range as our antibody 2.28: 12,600 molecules per platelet. The other characteristics of 2.28, however, indicate that 2.28 identifies a different protein. Two antibodies whose binding to platelets is not dependent on platelet secretion recognize changes in the conformation or microenvironment of the membrane glycoprotein IIb-IIIa complex. Antibody 7E326 binds both to resting and activated platelets; the rate of binding, however, is increased after platelet activation. PAC141 recognizes an epitope on the IIb-IIIa complex that is exposed after platelet activation.

Interest in the role and detection of activated platelets is mainly based on the hypothesis that activated platelets may have an important role in arterial disease and in sudden clinical events such as myocardial infarction and stroke that accompany it. Many tests of platelet function have been devised to test this hypothesis; a direct test for the detection
of activated platelets in the circulation, however, is lacking thus far. MoAbs specific for activated platelets are of potential value in this aspect.

We used flow cytofluorometric analysis to test the hypothesis that activated platelets in vivo may be detected with antibody 2.28. Cardiopulmonary bypass was chosen as a model since it has been shown that platelet activation occurs during this procedure and that afterwards degranulated platelets are present in the circulation.42-44 The number of platelets that expressed the 2.28 antigen on their surface was significantly increased after extracorporeal perfusion. There was a correlation with the β-thromboglobulin levels in plasma, and this supports the assumption that the number of 2.28-positive platelets represent platelets that are not in a resting state. We cannot exclude the possibility that some molecules on the platelets that are recognized by 2.28 are leukocyte derived. During cardiopulmonary bypass degranulation of these cells occurs,43 and we observed 2.28 antigen localized in granules in monocytes (data not presented). A small increase in the number of 2.28-positive platelets was observed before extracorporeal perfusion in the patients as compared with the controls. This may have been caused by the fact that the first blood sample in the patients was taken via a catheter after the beginning of the operation, immediately before the extracorporeal perfusion.

Recently George et al46 studied the binding of antibody S12 to platelets in patients after cardiac surgery with cardiopulmonary bypass. In these patients they found no increased expression of the α-granule membrane protein GMP-140, the protein to which S12 binds. In contrast we found secretion of both α-granules and lysosomal granules in our patients. It seems unlikely that differences in the time of extracorporeal perfusion (average, 74 minutes ± 92 minutes in our study), as suggested by George et al, explain the differences in secretion in these and other studies.42-46

To evaluate the usefulness of the test in thrombosis we studied two patients with acute deep venous thrombosis. Both had an increased number of 2.28-positive platelets in the circulation. Further studies in patients with clinical conditions that are known to be associated with a high risk of thromboembolic complications and studies on the effects of antithrombotic agents are needed to confirm the potency of our test. Once this test or a combination of platelet function tests and classic risk factors has been shown to be able to detect persons who will develop a myocardial infarction or stroke in the future, our ability to diagnose and treat patients with thrombotic vascular diseases will be enhanced.

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REFERENCES

22. Gueze HJ, Slot JW: The subcellular localization of immunoglobulin in mouse plasma cells as studied with immunoferitin
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