A monoclonal anti-human platelet antibody, TP82, is described, which caused irreversible aggregation of platelets in association with the release of adenosine triphosphate or $[^{3}^{3}]$C serotonin, and which inhibited ristocetin-induced agglutination. Immunofluorescence assay showed that the antibody binds to platelets, megakaryocytes, and common acute lymphoblastic leukemia cells. The antibody (IgG,) immunoprecipitated a polypeptide of 23,000 daltons with an isoelectric point of about 7.0. The aggregation induced by the purified antibody and/or F(ab')2 fragments occurred in platelet-rich plasma and with washed platelets, but not with formalin-fixed washed platelets. TP82-induced aggregation was completely inhibited by disodium ethylenediaminetetraacetate, dilitazem, W-7, PGE1, and several metabolic inhibitors. At a concentration of apyrase or CP/CPK, which inhibited adenosine 5-diphosphate-induced aggregation, TP82-induced aggregation was only partially affected. Thrombin was not required for the antibody-mediated effects, since two thrombin inhibitors failed to block the reaction. The antibody, at least at a high concentration, induced platelet aggregation by a mechanism almost independent of thromboxane A2 formation, since cyclooxygenase inhibitors had little inhibitory effect on aggregation. TP82 monoclonal antibody is a new platelet-aggregating substance that interacts with a low-molecular-weight binding site on the platelet membrane.

In this article, we report a monoclonal antibody, designated TP82, that causes irreversible aggregation of human platelets and release of adenosine triphosphate (ATP) or $[^{14}]$C serotonin. The relevance of this antibody to platelet physiology was studied with respect to both stimulatory and inhibitory effects on normal platelet function.

**MATERIALS AND METHODS**

**Reagents**

Bovine serum albumin (fraction V), pepsin, Nonidet P-40 (NP-40), phenylmethylsulfonyl fluoride, 2-mercaptoethanol, luciferin-luciferase, lactoperoxidase (70 to 100 U/mg of protein), phosphocreatine, creatine phosphokinase (from rabbit muscle, type I), potato apyrase, iodoacetate, antymycin A, ristocetin, imipramine, dibutyryl cyclic adenosine monophosphate (dbcAMP), acetylsalicylic acid (aspirin), indomethacin, theophylline, and hirudin were obtained from Sigma Chemical Co, St Louis. Disodium ethylenediaminetetraacetate (EDTA) was obtained from Nakarai Chemical Co, Kyoto, Japan. Amoiphone (pH range of 3.5 to 10.0) was purchased from LKB, Bromma, Sweden. W-7 was purchased from Rikaken Co, Ltd, Nagoya, Japan. TMB-8 was made by the method of Malagodi and Chiosi. PGE1 and OKY-1581 were gifts from Ono Pharmaceutical Co, Osaka, Japan. Triton X-100 and acrylamide were purchased from Wako Pure Chemical Industries, Ltd, Osaka, Japan. Glutaraldehyde (25% aqueous solution) was purchased from Taab Laboratories, Reading, England. Human fibrinogen (grade L) from Kabi, Stockholm, Sweden, was treated with diisopropyl fluoro-phosphate (DFP, Sigma) as described by Mustard et al5 to inactivate any coagulant activity present. Equine tendon collagen (1 mg of native collagen fibrils per milliliter suspended in isotonic glucose solution, pH 2.7) was obtained from Horm Chemie, Munich, West Germany. Heparin was obtained from Novo Co, Copenhagen. DEAE-Sepharose A50, Sephadex G150, and cyanogen bromide-activated Sepharose 4B were obtained from Pharmacia Fine Chemicals, Dorval, Quebec. Goat anti-mouse IgG serum and fluorescein isothiocyanate-conjugated sheep anti-mouse IgG were purchased from Cappel Laboratories, Inc, Malvern, Pa.

**Production of TP82 Monoclonal Antibody**

TP82 monoclonal antibody (murine ascites) was produced by H.M. The procedures used for its production were as follows:
Washed human platelets were prepared as described by Mustard et al., except that the final washing was in 0.15 mol/L phosphate-buffered saline (PBS), 1 mmol/L EDTA, pH 7.4. BALB/c mice were immunized with 10⁶ washed platelets without any adjuvant by two intraperitoneal injections three weeks apart. Spleen cells were harvested three days after the last injection and were fused with cells of the X63.Ag8.653 murine myeloma cell line by the method of Galfre et al. After fusion, the cells were suspended in hypoxanthine-aminopterin-thymidine (HTA) medium and dispensed into the wells of a Microtest II plate (Falcon Plastics, Oxnard, Calif) at a density of 3 x 10⁶ cells per well. The hybridoma cells from wells containing antiplatelet antibodies were subcloned twice by limiting dilution in 96-well plates on spleen cells used as the feeder layer. Supernatants were screened for reactivity with human washed platelets by mixed passive hemagglutination assay. The clones were expanded and finally grown in ascitic fluid in BALB/c mice by intraperitoneal injection. About three weeks later, the ascitic fluid was collected. The monoclonality of the TP82 antibody was confirmed by two-dimensional gel electrophoresis as described below. The original clone has remained stable in culture for longer than 12 months.

**Antibody Purification and F(ab')₂ Fragment Preparation**

Ascitic fluid containing the monoclonal antibody was heated to 56 °C for ten minutes and then centrifuged at 1,100 g for ten minutes at 22 °C. The supernatant was diluted 1:1 with PBS (pH 7.2), precipitated with ammonium sulfate (50% final saturation) at 4 °C for 24 hours, centrifuged at 30,000 g for one hour, suspended in PBS solution (pH 7.2) and dialyzed at 4 °C overnight against 0.1 mol/L Tris-Cl buffer, pH 6.5. Two-dimensional electrophoresis was performed according to the method described by Lowsy et al. Monoclonal antibody TP80 (IgG₂, specific for platelet GPIb/IIIa) was produced and purified by the same method as for TP82 antibody. TP80 inhibited platelet aggregation and ATP release induced by adenosine 5'-diphosphate (ADP), epinephrine, collagen, and thrombin, but not by ristocetin (unpublished data). Monoclonal antibody IC-1 A (IgG₂, anti-HLA-A,B,C common determinant) and TP80 were used as a negative control for platelet activation.

**Indirect Immunofluorescence Assays**

Washed human platelets were prepared as described. Mononuclear cells from heparinized aspirates of bone marrow and peripheral blood were obtained by Ficoll-Hypaque density centrifugation. The cells were adjusted to 10⁶/mL in PBS (pH 7.2) containing 1% bovine serum albumin (BSA), 0.1% sodium azide, and 2 mmol/L EDTA (PBS-1% BSA). One hundred microliters of cell suspension (10⁵) was incubated with an equal amount of 1/100 diluted hybridoma ascitic fluid or purified intact IgG (100 μg/mL) in a Fisher tube for 30 minutes at 22 °C. Anti-Ia monoclonal antibody ascitic fluid and nonimmune mouse IgG (Cappel) were used as a negative control. The cells were resuspended in 100 μL of 1/20 diluted fluorescein isothiocyanate-conjugated sheep anti-mouse IgG serum and were incubated for an additional 30 minutes at 4 °C. The cells were washed three times and resuspended in PBS-1% BSA for analysis by conventional immunofluorescence microscopy (Nikon, Tokyo) or by a fluorescence-activated cell sorter (FACS II, Becton Dickinson Co, Mountain View, Calif).

**Immunoprecipitation Studies of Solubilized Iodinated Platelets**

Washed human platelets were prepared as described by Mustard et al., except that the final washing was in 0.15 mol/L PBS and 1 mmol/L EDTA, pH 7.4. One millicurie of Na₁₂⁵I (New England Nuclear [NEN], Boston) was added to a 150-μL suspension of 3 x 10⁶ washed platelets in phosphate buffer following by 0.15 mol/L sodium phosphate, pH 7.0, and 50 μL of lactoperoxidase solution (2 mg/mL in PBS). Twenty microliters of freshly prepared hydrogen peroxide solution (0.03% in PBS) was added to the cell suspension, which was then mixed vigorously and incubated at 30°C for four minutes. An additional 20 μL of hydrogen peroxide solution was added, and the mixture was incubated for ten minutes at room temperature. Five milliliters of cold PBS containing 0.02% sodium azide and 2 mmol/L potassium iodide was added, and the platelets were washed three times in PBS and 1 mmol/L EDTA. The final platelet pellet was resuspended in 0.3 mL of the following lysis buffer--0.5% NP-40, 10 mmol/L Tris-HCl, pH 7.6, 1 mmol/L MgCl₂, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 0.02% sodium azide— and centrifuged at 10,000 g for 30 minutes. The supernatant was incubated overnight at 4 °C with 5 μL of TP82 ascites. TP80 ascites, or control ascites obtained from an X63.Ag8.653 myeloma cell line. The immunoadsorbent was prepared by incubating 100 μL of anti-mouse IgG antibody (5 mg/mL) with an equal volume of packed protein A-Sepharose CL-4B for 60 minutes at 4 °C and was used for subsequent immunoprecipitation after washing four times with NET buffer (0.15 mol/L NaCl, 5 mmol/L EDTA, 50 mmol/L Tris-HCl, pH 7.5) to remove the unbound antibody. The antibody-bound cell lysate was incubated for 60 minutes at 4 °C in 500 μL of 10% anti-mouse IgG-coupled protein A-Sepharose CL-4B suspended in NET buffer supplemented with 0.5% NP-40 and 0.1% BSA. The immunoprecipitate was washed three times with 0.5 mol/L NaCl, 5 mmol/L EDTA, 50 mmol/L Tris-HCl, pH 7.5, and 0.5% NP-40 and twice with NET buffer containing 0.5% NP-40. The radiolabeled antigen was dissociated from antibody and protein A-Sepharose by boiling in 100 μL of the following sampling buffer—2.3% sodium dodecyl sulfate (SDS), 62.5 mmol/L Tris, 10% glycerol (wt/vol), with or without 5% (vol/vol) 2-mercaptoethanol, pH 6.8—and applied to 10% SDS-polyacrylamide gel according to Laemmli's method. Two-dimensional gel electrophoresis was performed according to the method described by Jones. The first dimensional separation by nonequilibrium pH gradient gel electrophoresis (NEPHGE) was performed in 2.5-mm x 130-mm cylindrical gels containing 9.2 mol/L urea, 4% acrylamide, 2% NP-40, and 2% ampholine (pH 3.5 to 10.0). The samples were electrophoresed at 500 V for six hours. After equilibration in SDS sampling buffer for two hours, the NEPHGE gels were embedded in 1% agarose on top of a slab gel with
Platelet Preparation

Blood from healthy volunteers was collected in 3.8% wt/vol sodium citrate (9 vol of blood for 1 vol of sodium citrate). Platelet-rich plasma (PRP) was obtained by centrifuging at 120 g for ten minutes. Platelet-poor plasma (PPP) was obtained by centrifuging the remaining blood at 2,000 g for 15 minutes. Platelet counts were performed with a Coulter counter, model C (Counter Electronics, Inc, Hialeah, Fla). Platelet counts of PRP varied from 30 × 10^9 to 50 × 10^9/µL during these studies unless otherwise noted. Washed platelets (WP) were prepared by a modification of Mustard's method as follows: PRP was centrifuged at 1,000 g for ten minutes and the platelet pellet was resuspended in washing buffer (113 mmol/L NaCl, 4.3 mmol/L K2HPO4, 4.3 mmol/L NaH2PO4, 22.4 mmol/L NaN3, 3 mg of apyrase per dL, 1 mg of glucose per mL, 1 mmol/L EDTA, pH 6.5). The washing procedure was repeated three times, and the final platelet pellet was resuspended in Ca2+-free Tyrode's buffer with BSA (0.2%) and glucose (1 mg/mL) to achieve a final count of 30 × 10^9/µL. When trace amounts of 125I-fibrinogen were added to the PRP, 99.9% of the fibrinogen originally present in the PRP was removed by this washing procedure. Formalin-fixed washed platelets (FPW) were prepared as described by Allain et al and were stored at 4°C in the presence of 0.05% sodium azide until needed. The FPW were washed three times with phosphate buffer, pH 6.5, and resuspended in fresh PPP to a final concentration of 30 × 10^9/µL. All procedures were carried out at room temperature.

Platelet Aggregation and ATP Secretion

Platelet aggregation and ATP secretion were measured on a Chronolog Lumiaggregometer (Chronolog Corp. Havertown, Pa). The instrument was calibrated for zero light transmission with a platelet suspension and with PPP or Tyrode's buffer for 100% transmission. The reaction mixture containing 0.45 mL of PRP and 50 µL of luciferin-luciferase solution (40 mg/mL) was preincubated at 37°C for three minutes. The reaction was initiated by adding, with stirring (1,100 rpm), of aggregating agents according to the method of Feinmann et al.

Release of Serotonin (5-HT)

Serotonin release was measured according to the method of Nishikawa and Hidaka with a minor modification as follows: 100 parts of citrated PRP were preincubated with one part of [14C] serotonin creatinine sulfate (50 to 60 mCi/mmol, Amersham Corp., Arlington Heights, Ill) diluted in 2% ethanol (10 µCi/mL) at 37°C for 30 minutes and then at room temperature for 30 minutes. Under these conditions, 88 ± 3% of the radioactivity was taken up by the platelets. In the study of the release reaction of WP, PRP preincubated with [14C] serotonin was centrifuged at 1,000 g for ten minutes, washed three times as described, and resuspended with Ca2+-free Tyrode's buffer containing BSA (0.2%) and glucose (1 mg/mL). One micromole imipramine was added to PRP or WP just before stimulation to prevent the reuptake of secreted [14C] serotonin. Five-tenths-milliliter samples were preincubated with stirring for three minutes at 37°C and then 10 µL of TP82, TP80, or 1C6-1A was added (control). The release reaction was stopped by adding 0.2 mL of 0.1% ice-cold glutaraldehyde. After centrifugation for ten minutes at 2,000 g, aliquots of the supernatant were removed for determination of radioactivity by liquid scintillation counting. The results were expressed as the percent release of the total [14C] serotonin in the platelets.

Lactic Dehydrogenase Loss

Samples of citrated PRP (500 µL) were stirred at 37°C and exposed to TP82 antibody, collagen, or saline alone. After seven minutes, platelet samples were removed and centrifuged, and lactic dehydrogenase (LDH) activity in the supernatant was determined according to the method of Wroblewski and La Due. The LDH activity was expressed as a percentage of the total LDH determined by solubilization with 0.5% (wt/vol) Triton X-100.

Thromboxane Synthesis

Platelet counts were adjusted to 3 × 10^9/mL by PPP. Four-hundred-ninety-microliter samples of PRP were incubated with 10 µL of TP82 (600 µg/mL), collagen (100 µg/mL), or saline under continuous stirring (1,100 rpm) in a Chronolog Lumiaaggregometer. After seven minutes, the reaction was stopped with 12.5 µL of ice-cold buffer containing 250 mmol/L Tris-EDTA and 0.1 mmol/L indomethacin. The precipitate was removed by centrifugation and the amount of TXB2 in the clear supernatant was determined by radioimmunoassay as described by Jaffe et al. using TXB2 antibody (Seragen Co, Boston) and [3H]TXB2 (100 to 150 Ci/mmol, NEN).

Patients

One female patient with Bernard-Soulier syndrome (BSS) (T.M.) had prolonged bleeding time and enlarged platelets that showed defective adhesion to the subendothelium, agglutination with ristocetin, and lacked membrane glycoprotein Ib. Her platelet count was between 4 × 10^9 and 8 × 10^9/µL. PRP was obtained from this patient (and from appropriate control subjects) by gravity sedimentation at room temperature for two to three hours. The platelet counts of the controls were adjusted to the patient's platelet count by gravity sedimentation. Two patients with Glanzmann's thrombasthenia (M.A. and H.W.) had normal platelet counts, markedly prolonged bleeding time, decreased or absent clot retraction, low α-granule fibrinogen content, and lack of platelet aggregation in response to ADP, epinephrine, or collagen. By indirect immunofluorescence, the patient's platelets were negative for fibrinogen by antibody (Seragen Co. Boston) and [3H]TXB2 (100 to 150 Ci/mmol, NEN).

RESULTS

Specificity and Characterization of TP82 Monoclonal Antibody

Monoclonal antibody, designated TP82, was shown to be IgG1, κ light chain in isotype, by Ouchterlony immunodiffusion analysis. Indirect immunofluorescence assay showed that TP82 exhibited binding to platelets, to large cells with nuclear and cytoplasmic characteristics of megakaryocytes, and to about 10% of bone marrow cells. It was not possible to determine whether the small fluorescence-positive cells in the
bone marrow belonged to a specific cell lineage or were megakaryocyte precursors. The platelets of one patient with BSS and two patients with thrombasthenia were as intensely fluorescent as the control platelets. No peripheral blood cells except platelets and only a small percentage of granulocytes were bound to the antibody. Among leukemic cells and cell lines that we have investigated so far, the antibody reacted with leukemic cells from patients with common ALL (9/9 patients, 100%). The distribution on the other hematopoietic cells and non-hematopoietic cells is under investigation.

Electrophoresis of radiolabeled antigen gave the pattern shown in Fig 1A (one-dimensional separation, reduced). TP82 monoclonal antibody immunoprecipitated a polypeptide of 23,000 daltons (reduced state) and 24,000 daltons (unreduced state). With two-dimensional separation in the reduced state, TP82 antibody immunoprecipitated a single polypeptide of 23,000 daltons with an isoelectric point of about 7.0 (Fig 1B).

Platelet Aggregation and Release Reaction

Dose-dependent platelet aggregation and release of ATP or [14C] serotonin induced by TP82 antibody in PRP are shown in Fig 2. TP82-induced aggregation was associated with a transient decrease in light transmission and a lag period that was prolonged in inverse proportion to antibody concentration. During the lag period, neither ATP release (Fig 2) nor [14C] serotonin release (data not shown) occurred. In most experiments, at a concentration of 12 μg/mL, all of the platelets were present in one or a few large aggregates (superaggregation

Fig 1. Autoradiograph of radiolabeled platelet membrane proteins that bind to TP82 monoclonal antibody. Platelet membrane was iodinated by the lactoperoxidase technique, solubilized in 0.5% NP-40, and immunoprecipitated as described in the Materials and Methods section. The immunoprecipitate was solubilized in SDS, reduced with 2-mercaptoethanol, and electrophoresed in 10% polyacrylamide gel. (A) One-dimensional electrophoresis. Lane 1: Control obtained with ascites from X63.Ag8.653 myeloma cell lines. No immunoprecipitate is found. Lane 2: Control obtained with TP80 ascites. Lane 2 contains the immunoprecipitate in reduced state showing two bands of mol wt of 132,000 and 114,000. In unreduced state, TP80 immunoprecipitated two polypeptide of mol wt of 142,000 and 99,000 (data not shown). Lane 3 and Lane 4: Immunoprecipitate obtained from TP82. Lane 3 contains the immunoprecipitate in reduced state, showing a single band of mol wt of 23,000. Lane 4 contains the immunoprecipitate in unreduced state, showing a single band of mol wt of about 24,000. (B) Two-dimensional electrophoresis. Immunoprecipitate with monoclonal antibody TP82 in reduced state, showing a band of mol wt of 23,000 with isoelectric point (pl) about 7.0.

Fig 2. TP82-induced platelet aggregation and secretion of ATP or [14C] serotonin in a dose-dependent manner. Reaction mixture containing 0.45 mL of PRP and 50 μL of luciferin-luciferase solution (40 mg/mL) were preincubated at 37 °C for three minutes, and the reaction was initiated by addition of TP82. The final concentration of antibody is (a) 12 μg/mL, (b) 2.4 μg/mL, (c) 0.6 μg/mL, (d) 0.3 μg/mL. ATP release induced by TP82 (12 μg/mL) in WP, expressed as percentage (mean ± SD) of that releasable by thrombin, was 84 ± 5% (n = 6). The numbers in the boxes represent the percentages of [14C] serotonin release from prelabeled PRP as described in the Materials and Methods section. Values are means for three experiments.
small aggregates formed at lower concentrations (0.3 to 1.2 μg/mL). The minimal concentration of the antibody required for aggregation varied with platelets from person to person but remained within a range of 0.3 to 1.2 μg/mL. In the absence of stirring, TP82-induced aggregation did not occur and ATP release was almost undetectable. However, [14C] serotonin release induced by antibody (12 μg/mL) after seven minutes was 11.2% ± 2.1% (mean ± SD, n = 6) without stirring. Neither monoclonal antibody 1C6-1A (200 μg/mL) nor TP80 (200 μg/mL), whether in PRP or WP, caused platelet aggregation (data not shown) or release of [14C] serotonin (0.44% ± 0.10%, 0.50% ± 0.12%, respectively, mean ± SD, n = 3).

Platelets washed three times were aggregated by TP82 but not by ADP (10 μmol/L) or epinephrine (20 μmol/L). In WP, exogenous fibrinogen (500 μg/mL) shortened the time required for half maximal aggregation, but the maximal aggregation after seven minutes or the percentage secretion of [14C] serotonin was not influenced (Table 1).

To determine whether these effects were due to direct stimulation by the antibody or by way of an Fc receptor, we obtained F(ab')2 fragments of TP82 by pepsin digestion as described in the Materials and Methods section. Purified F(ab')2 fragments, whether in PRP or WP, induced platelet aggregation (Fig 3) and release reaction (data not shown). The pattern of aggregation induced by purified F(ab')2 fragments was similar to that of intact IgG, whereas pFc fragments (200 μg/mL) were not effective.

The results of an aggregation study using PRP from one patient with BSS and two patients with Glanzmann's thrombasthenia are shown in Fig 4. TP82-induced aggregation was within normal limits in PRP with BSS. The change in light transmission was slight (5% to 15%) after TP82 was added to PRP from two patients with thrombasthenia. No aggregates were seen in either gross or microscopic examinations of the cuvette contents. Despite the lack of aggregation, the platelets still changed shape, as evidenced by the transient decrease in light transmission.

Effect of TP82 on TXB2 Synthesis and LDH Loss

TXB2 synthesis by TP82 (12 μg/mL) was much greater than that of collagen (2 μg/mL) (Table 2). In
MONOCLONAL ANTIBODY AGAINST PLATELET

Inhibition of Platelet Aggregation and Secretion

The effects of agents that inhibit platelet aggregation and the release reaction were examined (Table 3). A calcium chelator, EDTA (5 mmol/L); Ca\(^{2+}\) blockers, diltiazem\(^{40}\) (0.5 mmol/L) and TMB-8\(^{24,41}\) (0.5 mmol/L); a calmodulin inhibitor, W-7\(^{34}\) (100 μmol/L); and agents that inhibit platelet function by way of cyclic AMP (cAMP) accumulation, PGE\(_1\) (1 μmol/L), dbcAMP (3 mmol/L), and theophyllin (6 mmol/L) inhibited TP82 (12 μg/mL)-induced aggregation completely. However, serotonin release was not completely blocked by diltiazem (6%), TMB-8 (24%), or W-7 (14%), although agents that increase intracellular cAMP blocked it completely. In our experiments, these inhibitors at the concentrations shown in Table 3 induced leakage of small amounts of \(^{14}\)C serotonin (5% to 18%) without any stimulants. Neither LDH nor ATP release was induced by these inhibitors (data not shown). A similar effect induced by TMB-8 was reported by Murer and Siojo.\(^{42}\) Iodoacetate (0.5 mmol/L) plus antimycin A (2 μmol/L) completely inhibited the platelet aggregation and the release reaction. However, both glycolysis and mitochondrial energy production must be blocked to inhibit platelet activation, because neither iodoacetate nor antimycin A was effective alone. TP82-induced platelet aggregation and release reaction did not occur with FWP (data not shown). At a concentration of apyrase (2 mg/mL) or CP/CPK (3 mmol/L: 15 U/mL) that inhibited ADP-induced aggregation completely, TP82-induced aggregation was partially affected. Thrombin inhibitors such as heparin (5 U/mL) or hirudin (2 U/mL) did not impair the reaction.

TP82-induced aggregation was accompanied by TXB\(_2\) formation similar to the case of collagen-induced aggregation (Table 2). In a preliminary experiment at a low concentration (0.6 to 1.2 μg/mL), TP82-induced platelet aggregation and the release reaction after seven minutes were almost entirely blocked by both cyclooxygenase inhibitors, such as aspirin (100 μg/mL) and indomethacin (20 μg/mL), and a TXA\(_2\) synthetase inhibitor (OKY-1581,\(^{43}\) 500 μmol/L). However, both aggregation and release reaction induced by TP82 at a concentration of 12 μg/mL were partially affected by these inhibitors (Table 3).

Inhibition of Ristocetin-Induced Agglutination by TP82

When PRP was preincubated with TP82 (purified intact IgG or F(ab')\(_2\) fragments) and EDTA (5 mmol/L) for two minutes, ristocetin-induced agglutination was inhibited by the antibody in a dose-dependent manner (Fig 5A). Moreover, the deaggregation occurred when TP82 was added at any time during the course of ristocetin-induced agglutination (Fig 5B). Like saline, nonimmune mouse IgG (1 mg/mL), monoclonal antibody 1C6-1A (200 μg/mL), and TP80 (200 μg/mL) showed no inhibitory effect (data not shown).

### Table 3. Effect of Inhibitors on TP82-Induced Aggregation and Serotonin Release

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Incubation Time (min)</th>
<th>Aggregation*</th>
<th>Release of Serotonin†</th>
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<tr>
<td></td>
<td></td>
<td>3</td>
<td>++++</td>
<td>100</td>
</tr>
<tr>
<td>PGE(_1)</td>
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<tr>
<td>dbcAMP</td>
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<td>0</td>
</tr>
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<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>2</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Diltiazem</td>
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<td>0</td>
<td>6</td>
</tr>
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<td>TMB-8</td>
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<td>5</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>W-7</td>
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<td>0</td>
<td>14</td>
</tr>
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</tr>
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<tr>
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</table>

Saline was used for dissolving all inhibitors except aspirin, indomethacin, and antimycin A. These three inhibitors were dissolved in 99.9% ethanol and diluted with saline (under 0.1% ethanol in final solution).

Four hundred ninety microliters of PRP was preincubated at 37 °C with 10 μL of inhibitors for indicated times, respectively, before TP82 antibody addition (12 μg/mL in final).

*Degree of aggregation without inhibitors is expressed as ++++. Aggregation is recorded on a scale of 0 to ++++.†Means for three experiments.
Because F(ab')2 fragments of this antibody induced platelet aggregation in the same fashion as intact IgG (Fig 3), as well as the release reaction (data not shown), the possibility that antibody bound to a platelet acts as an immune complex for another platelet and induces aggregation by way of Fc receptors appears to be remote. Another possibility, namely, that the cross-linking of the protein by divalent F(ab')2 fragments produces nonspecific membrane alterations that lead to platelet activation, may be discounted because neither control monoclonal antibody 1C6-1A nor TP80-induced platelet aggregation and release reaction. In unpublished studies, the purified Fab fragments (100 μg/mL) do not induce platelet aggregation nor release reaction, suggesting that bivalence of TP82 is required for the induction of aggregation and release reaction. Although TP82 immunoprecipitated a polypeptide of 23,000 daltons with an isoelectric point of about 7.0 (Fig 1), TP82-induced platelet activation does not necessarily indicate that 23K membrane protein is normally involved in vivo activation. Confirmation that TP82 leads directly to platelet activation by binding to the 23K membrane protein in vivo cannot be obtained until we can find the agent that combines with the 23K membrane protein, effectively blocking the activation induced by TP82.

There are two fundamental questions regarding the relevance of TP82 to the physiology of the platelets. First, there is the matter of whether or not any relationship exists between 23K membrane protein and receptors to a number of physiologic stimuli, such as the von Willebrand factor (vWF), thrombin, ADP, and collagen. Second, there is the question of whether or not any similarities exist in the mechanism of platelet activation between TP82 and other stimulants. Ristocetin-induced platelet agglutination was inhibited by TP82 in a dose-dependent manner but not by control monoclonal antibodies 1C6-1A or TP80 (Fig 5A and B), suggesting that the inhibition of agglutination results from the antibody’s inhibition of vWF binding to platelets rather than from nonspecific effects. The formation of large aggregates seems to be affected by TP82, since the antibody had little effect on the initial slope of aggregation (Fig 5A). This makes this antibody very different in behavior to GPI-specific monoclonal antibodies ANS13 and 6D1.4 GPIb is considered to function as the surface receptor for vWF.45 It has been reported that GPIbβ (mol wt 22,000, pl 6.0 to 7.0, according to the report of Clementson et al46) is difficult to iodinate by the lactoperoxidase method, and that this glycoprotein was found to be either absent or present in only trace amounts detectable after long exposure times in the unreduced state.46 Because the 23K membrane protein band was detected in both reduced and unreduced samples by the lactoperoxidase technique (Fig 1), it might not have been GPIbβ, the localization of which in two-dimensional electrophoresis was similar to that of 23K membrane protein. Moreover, TP82-induced...
aggregation was within normal limits in PRP with BSS (Fig 4), and the platelets of this patient were as intensely fluorescent as the control platelets, suggesting that 23K membrane protein is not a subtraction of GPIb or other glycoproteins that are deficient in BSS platelets. Grant et al reported that ADP inhibited ristocetin-induced agglutination in normal EDTA-treated citrated PRP. Moore et al showed that agglutination of human platelets by ristocetin and vWF was inhibited by heat-aggregated human IgG, although the structures involved in the platelet receptors for aggregated IgG and ristocetin-vWF appear to be different. These observations may support the possibility that TP82 indirectly masks the surface structure that mediates the ristocetin-induced, vWF-dependent platelet agglutination and thus interferes with subsequent platelet agglutination. The possibility that 23K membrane protein is a part of, or sterically related to, the receptor of aggregated IgG (Fc receptor) or one of the unknown binding sites (receptors) of physiological aggregating substances such as collagen or ADP, remains to be determined. The binding assays to establish whether Fab or F(ab')2 fragments of TP82 may block the binding of these stimulants, including vWF, to human platelets, is under investigation.

Extracellular fibrinogen was not a prerequisite for TP82-induced aggregation, although it shortened the time required for half maximal aggregation (Table 1). Most evidence suggests that fibrinogen-binding sites on the platelet membrane are, or are closely associated with, GPIIb/IIIa, in which platelets from patients with Glanzmann’s thrombasthenia are deficient. TP82-induced platelet aggregation did not occur in PRP from patients with thrombocytopenia but was associated with a transient decrease in light transmission (Fig 4). These data suggest that GPIIb/IIIa plays an important role in TP82-induced platelet aggregation, as in the case of aggregation induced by ADP, thrombin, or collagen.

The studies using several inhibitors to clarify any similarity in the mechanism of platelet activation between TP82 and other aggregating substances (Table 3), suggested that (a) TP82-induced platelet activation may involve an intracellular Ca2+ flux; (b) intracellular increase in cAMP prevents this activation; (c) released ADP does not play an essential role in this aggregation, but has a synergistic effect on it; and (d) TP82-induced platelet aggregation, at least at a concentration of 12 µg/mL, proceeds by a pathway different from that involved in TXB2 synthesis, but that the cyclooxygenase pathway is likely to be involved in the activation at low concentrations (0.6 to 1.2 µg/mL). The effect of the antibody is reminiscent of that produced by thrombin, since aggregation induced by thrombin was not inhibited at a high concentration (400 nmol) by indomethacin, but at a lower concentration (60 nmol). However, the TP82-induced aggregation and release reaction does not require thrombin participation because thrombin inhibitors (hirudin and heparin) do not impair the reaction (Table 3).

By indirect immunofluorescence assay, TP82 reacted not only with platelets and megakaryocytes, but also with common ALL cells, suggesting that platelets and common ALL cells share a cell surface component. Other investigators have produced monoclonal antibodies designated CALL1,12 DU-ALL-1,13 and BA2,32 which recognize antigens similar to 23K membrane protein in terms of molecular weight or distribution. However, the isoelectric point from two-dimensional electrophoresis or the effects on platelet function were not shown. Coprecipitation and antigen-blocking studies may establish whether TP82 and these three monoclonal antibodies recognize the same molecule.

In conclusion, we have described a monoclonal anti-human platelet antibody, designated TP82, that recognized a 23K membrane protein found on platelets, megakaryocytes, and common ALL cells; that caused irreversible platelet aggregation and release; and that inhibited ristocetin-induced agglutination. Recently, Thiragarajan et al briefly reported a monoclonal antibody, designated B1.12, that recognized a polypeptide of 21,000 daltons and induced platelet aggregation. Jennings et al showed that a monoclonal antibody (Fab fragments) specific for GPIIb/IIIa initiated platelet activation followed by platelet aggregation, with an observed time course for [3H]serotonin secretion identical to that observed with divalent antibody. Although our observations and the above reports are consistent with the idea that certain types of autoantibodies or alloantibodies may induce platelet activation, it remains to be proven whether any of these antibodies occurring naturally in vivo react with the 23K membrane protein. Support for this idea may be obtained by determining whether or not TP82 has decreased reactivity with platelets from patients with immune thrombocytopenia (ie, ITP), as shown by Varon and Karpatkin using a monoclonal antibody 3B2 specific for GPIIb-IIIa complex.

Further studies with the TP82 antibody should make it possible to (a) undertake a detailed in vitro investigation into the mechanism of platelet activation induced by anti-human platelet antibody in vivo; (b) interpret details of platelet activation (ie, Ca2+ mobilization or platelet protein phosphorylation) occurring before the initiation of platelet aggregation or release reaction because of the lag period observed in TP82-
induced aggregation; and (c) biochemically identify the 23K membrane protein using affinity chromatography, thus providing insights into platelet physiology.

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