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# A monoclonal anti-platelet antibody with decreased reactivity for autoimmune thrombocytopenic platelets

(platelet autoimmune antigens/membrane glycoproteins IIb and IIIa/anti-glycoprotein IIb monoclonal antibody)

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**ABSTRACT** **Two monoclonal anti-platelet antibodies, 3B2 and 8G11, have been raised that are specific for normal human platelets. 3B2 is unique in that it has decreased reactivity for platelets from 16 patients with autoimmune thrombocytopenic purpura [mean platelet count, 65,000 ± 6,000 (SEM)]. With 8G11 in an enzyme-linked immunosorbent assay, the mean of the ratios of patient platelet OD to control platelet OD was 0.95 ± 0.07, whereas with 3B2, the mean of the ratios of patient platelet OD to control OD was 0.24 ± 0.04, $P < 0.001$. With 3B2 the mean of the OD ratios of five patients with autoimmune thrombocytopenic purpura in remission (>150,000 platelets per $mm^3$) compared to controls was 0.80 ± 0.14. 3B2 did not react with platelets from a patient with Glanzmann's thrombasthenia, in which membranes lack glycoproteins IIb and IIIa (GPIIb and GPIIIa). Platelet membranes were run on crossed immunoelectrophoresis against a rabbit polyclonal anti-human platelet membrane antibody with $^{125}I$-labeled purified 3B2 in an intermediate spacer gel. 3B2 reacted with the GPIIb–GPIIIa–$Ca^{2+}$ complex in the presence of excess $Ca^{2+}$ and with GPIIb alone in the presence of excess EGTA. When Triton X-100-solubilized platelet membranes were immunoprecipitated with 3B2 plus rabbit anti-mouse IgG, reduced, and run on NaDodSO$_4$/polyacrylamide gel electrophoresis, a single protein band was obtained with a molecular weight of 120,000 (the molecular weight of GPIIb). Thus, the reactivity of monoclonal antibody 3B2 with GPIIb or the GPIIb–GPIIIa–$Ca^{2+}$ complex appears to be inhibited by the presence of autoantibody on platelets.**

Autoimmune thrombocytopenic purpura (autoimmune TP) is a disorder of increased peripheral platelet destruction due to autoantibody directed against platelets. Although considerable information has been accumulated with respect to the nature of the antibody and its presence on platelets (1), little is known regarding the platelet antigen(s) to which the autoantibodie(s) are directed. The purpose of this study was to investigate platelets for antigenic determinants to which autoantibodies are directed. This was approached by the use of hybridoma monoclonal antibodies directed against human platelets. One such hybridoma antibody, 3B2, was produced that reacted with normal platelets as well as platelets of autoimmune patients with TP in remission but reacted poorly with platelets of thrombocytopenic autoimmune TP patients. This hybridoma antibody was shown to react with glycoprotein (GP) IIb and with the platelet membrane GPIIb–GPIIIa–$Ca^{2+}$ complex (2–4). The present communication documents these observations and suggests that reactivity of monoclonal antibody 3B2 with GPIIb or the GPIIb–GPIIIa complex is inhibited by the presence of autoantibody on platelets.

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## MATERIALS AND METHODS

**Cells.** Human platelets were isolated from EDTA-anticoagulated blood and washed in 1% ammonium oxalate followed by human–Ringer–EDTA solution as described (5). Mononuclear leukocytes were prepared from defibrinated blood (to eliminate platelet contamination) by centrifugation in Ficoll/Hypaque (6) (Sigma). Erythrocytes were prepared from the lower fraction of the Ficoll/Hypaque separation for mononuclear cells and washed in the human–Ringer solution. Human melanoma cell lines Rubesco, Schneider, and M-450 were obtained at confluence from F. Valentine (New York University Medical Center).

**Monoclonal Antibody Production and Purification.** BALB/c female mice were injected three times intraperitoneally with $10^8$ washed platelets, with a 1-wk interval between each injection. A mouse with demonstrable anti-platelet antibodies [titer, >1:1,000 by enzyme-linked immunosorbent assay (ELISA)] was boosted by intravenous injection of $10^8$ platelets and sacrificed 4 days later. The splenocytes were fused with P3U1 myeloma cells, kindly supplied by V. Nussensweig (New York University Medical Center), by a modification of the method of Davidson and Gerald (7). The cells were incubated in a humidified, 7.5% $CO_2$ incubator environment at 37°C for 24 hr, and the medium was supplemented with 0.1 mM hypoxanthine/0.4 μM aminopterin/16 μM thymidine (Sigma) to select hybridized cells. Screening of the supernatants for anti-human platelet antibody revealed positive reactions for 28 of 768 wells. Positive clones were transferred to 2-ml microtiter wells of a 24-well microtiter plate (Costar, Cambridge, MA). Two positive clones, 8G11 and 3B2, were specific for normal human platelets in that they reacted with 15 different normal platelet preparations but did not react with 6 different mononuclear cell preparations, 2 erythrocyte preparations, or 3 human melanoma cell lines. 3B2 was unique in that it reacted poorly with platelets of autoimmune TP patients with thrombocytopenia but normally with platelets of autoimmune TP patients in remission. Therefore, 8G11 and 3B2 were selected for further study. They were subcloned by limiting dilution and injected into pristane-primed (Aldrich) BALB/c mice to obtain ascitic fluid.

Ascitic fluid from three mice was pooled, treated with 50% saturated ammonium sulfate, dissolved in and dialyzed against 0.01 M Tris/0.02 M NaCl, pH 8.6, then applied to a DEAE-52 ion-exchange column, and eluted with a 0.02–0.2 M NaCl gradient in 0.01 M Tris buffer (pH 8.6). Ouchterlony immunodiffusion with rabbit anti-mouse heavy chain (Bethesda Research Laboratories) and light chain antisera (Litton Bionetics) revealed 8G11 to have an IgG γ2a heavy chain and κ light chain;

Abbreviations: GP, glycoproteins; ELISA, enzyme-linked immunosorbent assay; CIE, crossed immunoelectrophoresis; TP, thrombocytopenic purpura.

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and 3B2 to have an IgG γ1 heavy chain and κ light chain.

**Solid-Phase ELISA for Screening of Anti-Platelet Antibody.** Cell suspension (50 μl; $10^6$–$10^7$ cells) was applied to polyvinyl chloride U-shaped microtiter wells (Dynatech, Alexandria, VA), covered tightly with Parafilm, and stored at 4°C until tested (generally 1–3 wk). Fifty microliters of culture supernatant or ascites fluid was applied to each well for 30 min. The cells were washed and then treated with rabbit anti-mouse IgG conjugated to β-galactosidase for 2 hr at room temperature by using the hybridoma screening kit of Bethesda Research Laboratories. Binding of the anti-mouse IgG–β-galactosidase conjugate was determined by addition of the substrate *p*-nitrophenyl-β-D-galactoside for 30 min, followed by quenching with 0.5 M sodium carbonate. Optical density was read at 405 nm. Each sample was tested in duplicate. Platelets of patients and normal subjects were of the same storage duration. Several control platelet preparations (3–10) were run at the same time as the patient's platelet population.

**Iodination Procedures.** Purified mouse IgG (0.5–1.0 mg/ml) was labeled with $Na^{125}I$ (Amersham; 15 mCi/μg; 1 Ci = 37 GBq) by the lactoperoxidase method (8). The specific activity of a typical iodination was $1 \times 10^9$ cpm/mg of IgG.

**Binding of Monoclonal Antibody to Platelets.** Iodinated monoclonal antibody binding to washed human platelets was performed by a modification of the procedure of McEver *et al.* (9). Platelets ($10^8$) were suspended in 1 ml of phosphate-buffered saline and incubated with nonimmune human IgG (50 μg/ml) (Miles) at room temperature for 15 min. Increasing quantities of $^{125}I$-labeled monoclonal IgG were then added in a total volume of 20 μl for 30 min at room temperature; 400 μl of each incubation was then layered on 0.5 ml of a 1:1 (vol/vol) mixture of silicon oils 550 and 556 (specific gravity, 1.034; Contour Chemical, North Reading, MA) in a 1.7-ml microcentrifuge tube (Denville Scientific, Denville, NJ) and centrifuged for 2 min at $1,200 \times g$ at room temperature in a microcentrifuge (Beckman). The supernatant was carefully aspirated and assayed for unbound radioactivity. The tips of the tubes were cut off and assayed for bound radioactivity. Nonspecific binding was determined by addition of 100-fold excess of nonradioactive purified monoclonal antibody.

**Crossed Immunoelectrophoresis (CIE).** CIE was carried out by procedures described previously with platelet membranes dissolved in 1% Triton X-100 containing 0.07 M Tris, 0.02 M sodium barbital buffer (pH 8.6), 10 mM benzamidine, 100 μg of soybean trypsin inhibitor per ml with or without EGTA (3, 10), and platelet membranes enriched for GPIIb and GPIIIa (11). The rabbit polyclonal anti-human platelet membrane antibody R4 was prepared from platelet membrane preparations dissolved in 1% Triton/5 mM EGTA. This resulted in the production of antibodies with greater precipitating activity for the separated GPIIb and GPIIIa glycoproteins than in previous antibody preparations (3, 10) and antibodies that reacted with the combined GPIIb–GPIIIa–$Ca^{2+}$ complex. Labeled monoclonal antibody was used as a spacer gel with the assumption that the radioactive antibody would bind to the platelet membrane and comigrate with the glycoprotein to which it is attached, precipitating at equivalence with the polyclonal rabbit anti-human antibody applied during the second dimension. CIE slides were washed, pressed, stained with Coomassie blue, and exposed to Kodak x-ray film at −80°C as described (3).

**Immunoprecipitation of the Hybridoma Antibody with Its Platelet Membrane Epitope.** Twenty-five microliters of platelet membrane extract (50 μg) was incubated with 50 μg of purified monoclonal 3B2 IgG or nonimmune mouse IgG (Sigma) for 1 hr. Rabbit anti-mouse IgG (Sigma; 25 μl) was then added and incubated for an additional 2 hr with constant gentle agitation. The immunoprecipitate was centrifuged at $12,000 \times g$ for 4 min in a microcentrifuge, washed once with a buffer containing 150 mM NaCl, 50 mM Tris·HCl (pH 7.4), 2.5% Triton X-100, 50 units of trasylol per ml, and 0.2% azide, and then was boiled in 10% $NaDodSO_4$ for 10 min. Aliquots were electrophoresed on 7.5% $NaDodSO_4$/polyacrylamide gels by the method of Laemmli (12). The gels were then stained by silver stain (13).

**Platelet Aggregation Studies.** An aggregometer (Bio-Data, Willow Grove, PA) was used for aggregation studies with platelet-rich plasma anticoagulated with 0.38% sodium citrate (14). ADP (Sigma) was used at 2 μM; epinephrine hydrochloride (Parke, Davis), at 20 μM; collagen (Hormone-Chemie, Munich), at 0.5 μg/ml; and ristocetin (Bio-Data), at 1.2 mg/ml. Platelet-rich plasma was incubated with 80 μg of monoclonal antibody or nonimmune mouse IgG per ml for 5 min at 37°C prior to the addition of platelet-aggregating agent.

## RESULTS

**Reactivity of Hybridoma Antibodies 8G11 and 3B2 with Autoimmune TP Platelets and Normal Platelets.** Fig. 1 shows the ratio of OD readings obtained for each hybridoma antibody for autoimmune TP platelets divided by the mean of 3–10 ODs obtained for control platelets tested at the same time. Thus, for hybridoma antibody 8G11, the reactivity for 16 autoimmune TP platelet preparations of thrombocytopenic patients [mean platelet count, 65,000 ± 6,000 (SEM)] was similar to the reactivity for control platelets, [ratio of 0.99 ± 0.08 (SEM) (Fig. 1, first column)]. However, hybridoma antibody 3B2 showed considerably less reactivity for autoimmune TP platelets compared to control platelets [ratio of 0.24 ± 0.04 (Fig. 1, second column); $P < 0.001$, matched Student test]. Furthermore, the ratio of only 1 of the 16 autoimmune TP platelet preparations over-

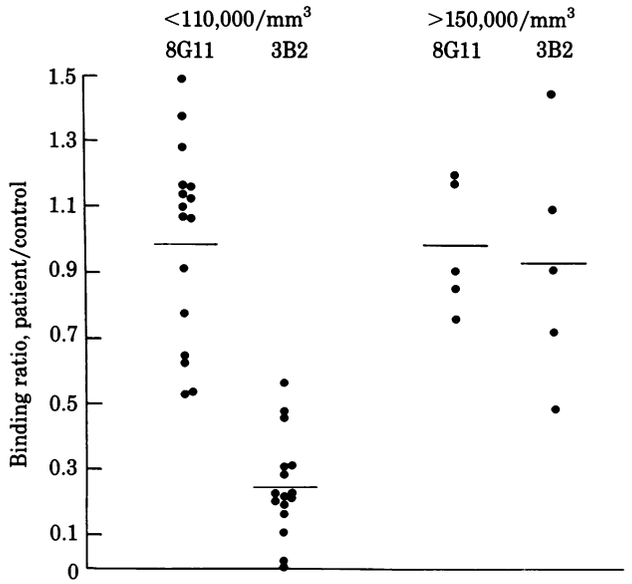


FIG. 1. Binding of anti-platelet monoclonal antibodies 3B2 and 8G11; binding ratio of platelets of patients with autoimmune TP (<110,000 platelets per $mm^3$) (*Left*) and of patients who had recovered from thrombocytopenia (>150,000 platelets per $mm^3$) (*Right*) to normal platelets. Washed platelets from normal and autoimmune TP patients were incubated with antibodies, and the degree of binding was determined by OD reading with ELISA. The binding of each antibody to patient's platelets divided by the binding to control platelets (mean of 3–10 control platelet preparations) is plotted in the diagram. Note the poor binding of 3B2 to platelets of thrombocytopenic autoimmune TP platelets (*Left*). Bars indicate mean values.

lapped with the ratios obtained for monoclonal antibody 8G11. Five patients with autoimmune TP who had recovered from thrombocytopenia had similar monoclonal antibody sensitivity for their platelets, [ratio of 0.98 ± 0.06 for antibody 8G11 (Fig. 1, third column) and ratio of 0.93 ± 0.20 for antibody 3B2 (Fig. 1, fourth column)]. Similar reactivities for both antibodies were also noted for five control thrombocytopenic patients without autoimmune TP (mean platelet count, 69,000 ± 10,000; ratio of 0.9 ± 0.20). Data not shown.

**Reactivity of Monoclonal Antibodies 8G11 and 3B2 with Glanzmann's Thrombasthenic Platelets.** The platelets of patient M.M. with severe Glanzmann's thrombasthenia reacted normally with monoclonal antibody 8G11 (OD ratio of 0.67) but did not react at all with antibody 3B2 (OD ratio of 0.003).

**Direct Binding of Monoclonal Antibody 3B2 to Washed Platelets.** Purified $^{125}$I-labeled 3B2 IgG was used for binding experiments. Saturation binding was noted at ≈4 μg/ml (Fig. 2; typical of four different experiments). Binding could be inhibited with nonradioactive hybridoma antibody 3B2 at 100-fold the concentration of the $^{125}$I-labeled antibody. A Scatchard plot analysis revealed 15,000 antibody binding sites per platelet with a $K_d$ of 8 μM (Fig. 2 *Inset*).

**Characterization of the Platelet Membrane Antigens That React with Monoclonal Antibody 3B2 by CIE and Autoradiography.** Fig. 3*a* demonstrates the CIE obtained with a platelet membrane preparation run in the presence of sufficient $CaCl_2$ (2 mM) to keep the GPIIb–GPIIIa–$Ca^{2+}$ complex intact. Fig. 3*b* shows the CIE obtained in the presence of sufficient EGTA (5 mM) to dissociate the complex (3). Fig. 3 *c* and *d* show the autoradiograms obtained when $^{125}$I-labeled monoclonal antibody 3B2 ($2 \times 10^6$ cpm/ml of agarose) was placed in an intermediate spacer gel. Note that antibody 3B2 comigrates with the GPIIb–GPIIIa–$Ca^{2+}$ complex in the presence of $CaCl_2$ and with GPIIb alone in the presence of EGTA. Similar results were obtained with four different experiments. Control experiments were simultaneously run with nonimmune mouse IgG in the intermediate spacer gel ($2.5 \times 10^6$ cpm/ml) and with antibody 8G11 in the spacer gel ($0.5 \times 10^6$ cpm/ml). No radioactivity was associated with any of the immunoprecipitates (data not shown).

**Apparent Molecular Weight of the Platelet Membrane Antigen That Reacts with Monoclonal Antibody 3B2.** When platelet preparations were enriched in GPIIb and GPIIIa (11) and run on CIE with $^{125}$I-labeled monoclonal antibody 3B2 in the spacer gel, similar observations were obtained for the reactivity of antibody 3B2 with GPIIb (data not shown). The EGTA preparation was treated with antibody 3B2 and immunoprecipitated with rabbit anti-mouse IgG. The immunoprecipitate was then collected, washed, boiled in $NaDodSO_4$, reduced, and run on 7.5% $NaDodSO_4$/polyacrylamide gel electrophoresis. Fig. 4 shows the electrophoretic pattern obtained after the use of a silver stain. A prominent band of $M_r$ 120,000 was obtained when compared to standard protein markers; this is the apparent molecular weight of GPIIb. The heavier band noted at $M_r$ 55,000 and the faint band at $M_r$ 25,000 refer to the heavy and light chains of IgG. When 8G11 was used as monoclonal antibody, no protein band could be identified.

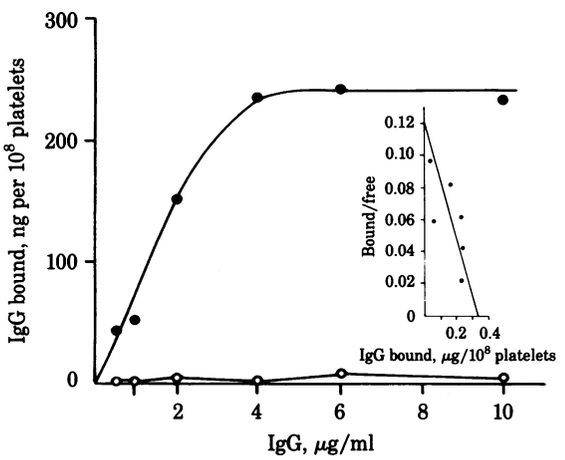


FIG. 2. Direct binding of antibody 3B2 to normal platelets. Purified 3B2 IgG was isotopically labeled with $^{125}$I, incubated with washed platelets, applied to a mixture of oils, and centrifuged to separate bound from unbound labeled antibody. Symbols refer to 3B2 monoclonal antibody bound per platelet in the absence (●) and presence (○) of 100-fold nonradioactive 3B2. A Scatchard plot is given in the *Inset*.

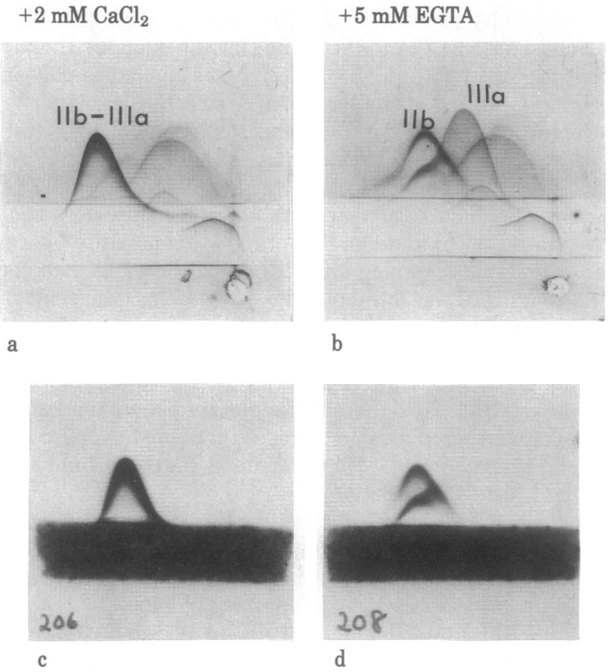


FIG. 3. CIE of human platelet membranes, demonstrating the reactivity of antibody 3B2 with the platelet membrane major antigen GPIIb–GPIIIa–$Ca^{2+}$ complex (*a* and *c*) and with GPIIb (*b* and *d*). Human platelet membrane (25 μg) was electrophoresed in the first dimension of 2.5 hr at 150 V. The agar above the electrophoresed strip was removed and repoured with agar containing polyclonal rabbit anti-human platelet membrane antibody R4 (100 μl/ml). After the gel hardened, an intermediate space was cut out and filled with agar containing $^{125}$I-labeled hybridoma antibody 3B2. The gel was then run in the second dimension for 18 hr at 55 V and processed for Coomassie blue staining (*a* and *b*) and autoradiography (*c* and *d*). (*a* and *c*) Membranes processed in the presence of 2 mM $CaCl_2$. (*b* and *d*) Membranes incubated in the presence of 5 mM EGTA for 15 min at room temperature, allowing dissociation of the complex into glycoproteins GPIIb and GPIIIa. Note the reactivity of $^{125}$I-labeled 3B2 with the GPIIb–GPIIIa–$Ca^{2+}$ complex and with GPIIb.

**Platelet Aggregation Studies.** Neither monoclonal antibody, 3B2, nor 8G11 had any effect on platelet aggregation induced by ADP, epinephrine, collagen, or ristocetin (four experiments; data not shown).

## DISCUSSION

Two monoclonal anti-platelet antibodies have been developed, 3B2 and 8G11, that are specific for normal human platelets. The specificity of 3B2 has been identified. On CIE, 3B2 was shown to react with GPIIb, whether this glycoprotein was separate or complexed with GPIIIa. 3B2 is unique in that it reacts poorly with platelets of at least 15 of 16 chronic thrombocy-

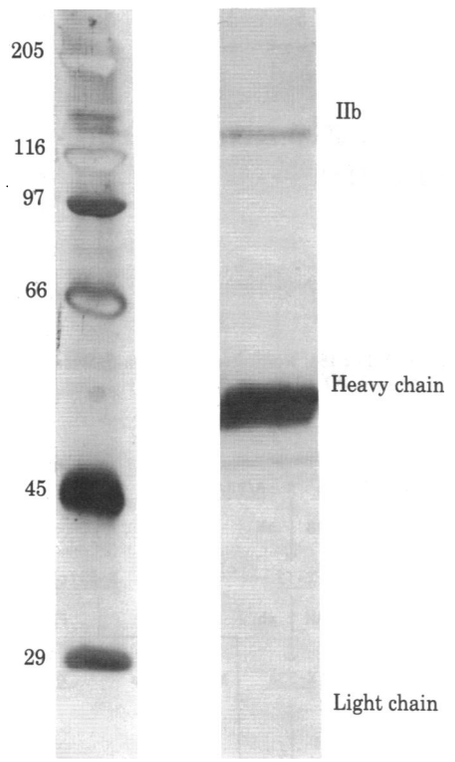


FIG. 4. Immunoprecipitation of solubilized platelet membrane preparation with antibody 3B2. A 0.1% Triton X-100-solubilized platelet membrane preparation was treated with purified antibody 3B2, followed by rabbit anti-mouse IgG. The precipitate was centrifuged, washed, boiled in 10% $NaDodSO_4$, reduced with 10% mercaptoethanol, and then applied to a 7.5% $NaDodSO_4$/polyacrylamide gel. Standard protein markers ($M_r$s are shown $\times 10^{-3}$) were also applied to the gel and stained with silver stain. A prominent band of $M_r$ 120,000 was obtained, along with the heavy chain ($M_r$ 55,000) and faintly visible light chain ($M_r$ 25,000) of IgG. The other faint bands noted were not consistently observed.

topenic patients with autoimmune TP; however, it does react with platelets of such patients in remission. 3B2 also does not react with the platelets of a patient with severe Glanzmann's thrombasthenia, a platelet membrane disorder in which glycoproteins GPIIb and GPIIIa are missing. Thus, platelets of thrombocytopenic patients with autoimmune TP, which are known to have bound autoimmune anti-platelet antibody, do not react with a hybridoma anti-platelet antibody with epitope specificity for GPIIb. These data are compatible with the concepts that bound autoimmune anti-platelet antibody directly or indirectly blocks the binding of 3B2 to platelets and that GPIIb or the GPIIb–GPIIIa–$Ca^{2+}$ complex or a location sterically adjacent to GPIIb contains the antigenic determinant(s) against which autoimmune anti-platelet antibody is directed. A second possibility is that autoimmune anti-platelet antibody might cleave the GPIIb–GPIIIa–$Ca^{2+}$ complex from the platelet membrane. However, this was not the case with CIE platelet membrane preparations from two thrombocytopenic autoimmune TP patients (data not shown).

Support for the former concept may be obtained from the recent report of van Leeuwen *et al.* (15), who observed binding of serum and platelet eluates of autoimmune TP patients to normal platelets. When Glanzmann's thrombasthenic platelets were used, binding could not be demonstrated with 14 of 42 positive-reacting sera and 35 of 42 positive-reacting eluates. Two recent abstracts also support this concept. Woods *et al.* (16) studied the ability of plasma from autoimmune TP patients to react with a Triton X-100-solubilized platelet extract enriched with the GPIIb–GPIIIa–$Ca^{2+}$ complex by adsorption with a monoclonal antibody specific for the complex. Plasma from 5 of 59 autoimmune TP patients reacted with this complex. Beardsley *et al.* (17) studied the ability of affinity-purified anti-platelet antibody (serum adsorbed to platelets and eluted with diethyl ether) to bind to $NaDodSO_4$-solubilized platelet proteins separated on polyacrylamide gels and transferred to nitrocellulose paper. Sera from 7 of 13 children with chronic autoimmune TP reacted with a $M_r$ 100,000 band, which comigrated with GPIIIa. Sera from all patients, as well as controls, also reacted nonspecifically with a $M_r$ 200,000 band.

Our observations on intact platelets and the preliminary reports of others using platelet extracts (16, 17) support the concept that GPIIb or the GPIIb–GPIIIa–$Ca^{2+}$ complex may contain antigenic determinants to which autoimmune anti-platelet antibody is directed in autoimmune TP patients. This appears to be the case in 94% of patients tested by the hybridoma technique. The lower incidence reported by others may reflect a lower sensitivity of their assay procedure.

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