Further Characterization of the Thrombasthenia-related Idiotype OG. Antiidiotype Defines a Novel Epitope(s) Shared by Fibrinogen Bβ Chain, Vitronectin, and von Willebrand Factor and Required for Binding to β_3

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Summary

A patient (OG) with Glanzmann thrombasthenia became refractory to platelet transfusion after the production of an immunoglobulin G (IgG) isoantibody (Ab1) specific for the integrin subunit β_3. To determine the frequency at which the OG idiotype is found in the general population and in immune-mediated disease states, we developed a rabbit polyclonal antibody (Ab2) specific for affinity-purified OG anti-β_3 Fab. The binding of Ab2 to Ab1 is inhibited by purified $\alpha_{IIb}\beta_3$. Ab2 also binds to IgG specific for $\alpha_{IIb}\beta_3$ obtained from one nonrelated Glanzmann thrombasthenia patient ES who has developed isoantibodies of similar specificity. On the other hand, Ab2 does not recognize $\alpha_{IIb}\beta_3$-specific antibodies produced by two Glanzmann thrombasthenia patients, AF and LUC, who have developed isoantibodies with specificities distinct from that of the OG isoantibody. Moreover, Ab2 does not recognize $\alpha_{IIb}\beta_3$-specific antibodies developed by three representative patients with (autoimmune) thrombocytopenic purpura or six representative patients with alloimmune thrombocytopenias, nor does it bind to IgG from any of 13 nonimmunized individuals. We have found that Ab2 also binds to selected protein ligands of $\alpha_{IIb}\beta_3$ namely, fibrinogen, vitronectin, and von Willebrand factor, but not to other protein ligands or control proteins, such a fibronectin, type I collagen, and albumin. The epitope(s) recognized by Ab2 on each adhesive protein are either very similar or identical since each protein can inhibit the binding of Ab2 to any of the other proteins. The epitope on fibrinogen recognized by Ab2 resides in the Bβ chain, and is likely contained within the first 42 amino acids from the NH_2 terminus. Since OG IgG inhibits fibrinogen binding to $\alpha_{IIb}\beta_3$, the specificity of the OG idiotype defines a novel binding motif for the integrin $\alpha_{IIb}\beta_3$ that is shared by fibrinogen, vitronectin, and von Willebrand factor, but distinct from previously described RGD-containing sites on the fibrinogen, Aα chain or the fibrinogen γ chain COOH-terminal decapeptide site. Our findings reported here represent an excellent example of molecular mimicry in which an antigen-selected, IgG inhibitor of $\alpha_{IIb}\beta_3$ function shares a novel recognition sequence common to three physiologic protein ligands of that receptor.

The integrin $\alpha_{IIb}\beta_3$ is probably the most immunogenic protein complex on the surface of human platelets. This generalization is based on the fact that it is the most frequently identified target of human autoantibodies in immune-mediated thrombocytopenia and immune-mediated platelet dysfunction (1) and that $\alpha_{IIb}\beta_3$ bears no less than five amino acid polymorphisms that are responsible for a majority of cases of platelet-specific alloimmunization among Caucasians (1). In addition, patients with the inherited abnormality of $\alpha_{IIb}\beta_3$ known as Glanzmann thrombasthenia (GT)[1] tend to develop anti-$\alpha_{IIb}\beta_3$ antibodies (isoantibodies) after receiving blood transfusions to correct bleeding diatheses. Some of the isoantibodies react solely with the $\alpha_{IIb}\beta_3$ complex (2), others are specific for either the α_{IIb} (3) or the β_3 subunits (4, 5).

[1] *Abbreviations used in this paper:* AP, alkaline phosphatase; GT, Glanzmann thrombasthenia; Vn, vitronectin; vWf, von Willebrand factor.

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Although GT isoantibodies are not the class of anti-$\alpha_{IIb}\beta_3$ antibodies most frequently encountered by the serology laboratory, they are of significant clinical and scientific interest since they can lead to a severe state of refractoriness to platelet transfusion therapy and because they generally recognize epitopes on $\alpha_{IIb}\beta_3$ that are involved in the adhesive function of this integrin.

We have recently defined the OG idiotype (Ishida, F., Y. Gruel, E. Brojer, D. J. Nugent, and T. J. Kunicki, manuscript submitted for publication) associated with IgG antibodies specific for the integrin β_3 subunit produced by such a GT patient (OG) who suffered from persistent and often serious bleeding episodes as a result of his disease (6). The frequency and severity of bleeding episodes in OG were far above the norm for this disease, presumably because this high titered, IgG antibody which proved to be in vitro an effective inhibitor of transfused platelets and thereby rendered therapeutic platelet intervention ineffective (4, 7).

It is our contention that the characterization of the OG idiotype will lead to a better understanding of the mechanisms involved in immunization against the integrin $\alpha_{IIb}\beta_3$, both in the case of OG and in the case of other individuals, with or without GT. In this report, we use a highly specific, polyclonal anti-OG idiotype reagent (Ab2) to determine the distribution of antibodies bearing the OG idiotype among individuals immunized against $\alpha_{IIb}\beta_3$ and normal volunteers. In addition, we provide evidence that Ab2 binds to epitopes common to three adhesive proteins recognized by $\alpha_{IIb}\beta_3$, fibrinogen, vitronectin (Vn), and von Willebrand factor (vWf).

Materials and Methods

Human IgG. The clinical history and laboratory findings of patient OG have been described (4, 7) (Ishida et al., manuscript submitted for publication). In Western blot assays and an antigen capture enzyme-linked immunoabsorbant assay, we consistently detect IgG in OG plasma that binds to free β_3 (4). This IgG (Ab1) completely blocks fibrinogen binding to $\alpha_{IIb}\beta_3$ and thus platelet aggregation induced by ADP, thrombin, or other physiologic agonists (4, 7). Other patients contributing to this study include three unrelated individuals with GT: patient ES, described by Coller et al. (2), has developed an IgG isoantibody that binds predominantly to the $\alpha_{IIb}\beta_3$ complex and does not cross-react with $\alpha_v\beta_3$; IgG from patient LUC (8) is predominantly reactive with α_{IIb} and, consequently, does not cross-react with $\alpha_v\beta_3$; and IgG from patient AF (5) binds equally well to both $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ and has been found to recognize an epitope(s) localized within $\beta_3$324-422 (9). Additional patients include six individuals who have developed plasma IgG $\alpha_{IIb}\beta_3$-specific alloantibodies, namely, three with anti-Pl^{A1} antibodies (10, 11), GAS, KRO, and WHA; two with anti-Pen^b (12, 13) alloantibodies, YuA and TRU; and one with anti-Pen^a antibodies, YuB (12); and three individuals diagnosed to have chronic (autoimmune) thrombocytopenic purpura who have developed β_3-specific autoantibodies, RA, GER, and BER (14, 15).

Comparative Binding of Human IgG Antibodies. Established procedures were used for purification of $\alpha_{IIb}\beta_3$ (16) or $\alpha_v\beta_3$ (17). The amount of IgG in plasma or purified IgG fractions which binds to $\alpha_v\beta_3$ or $\alpha_{IIb}\beta_3$ integrins was measured by ELISA. Briefly, 100 μl of 0.05 M sodium carbonate buffer, pH 9.6, containing 2 μg/ml of purified $\alpha_v\beta_3$ or $\alpha_{IIb}\beta_3$ were added to each well of a microtiter plate, and plates were incubated at 4°C overnight to permit maximal adsorption of antigen. The plates were blocked with PBS/Tween and rinsed three times; to each well were added 50 μl of test human plasma (diluted 1:100 in PBS/Tween) or purified IgG (50 μg/ml), and the plates were incubated for 2 h at ambient temperature. The wells were washed three times with PBS/Tween, 50 μl of alkaline phosphatase (AP)–conjugated goat anti–human IgG (heavy + light chain; Zymed Laboratories, Inc., San Francisco, CA) diluted 1:1,000 were added to each well, and plates were incubated at ambient temperature for 1 h. After five subsequent washes with PBS/Tween, the color reaction was initiated, and the OD at 405 nM was recorded as described above.

Purification of Ab1. IgG was isolated from OG plasma by ammonium sulfate precipitation and DEAE cellulose chromatography, as described (18), while $\alpha_{IIb}\beta_3$ was purified from washed platelets according to the method of Fitzgerald et al. (16). $\alpha_{IIb}\beta_3$-specific antibodies were affinity-purified by adsorption to $\alpha_{IIb}\beta_3$, coupled to CNBr-activated Sepharose 4B (1.5 mg antigen/ml beads; Pharmacia Fine Chemicals, Uppsala, Sweden). 2 ml of packed beads was incubated overnight at 4°C with 30 mg of OG IgG in 2 ml of 20 mM Tris, pH 7.4. Bound antibody was eluted by addition of 100 mM glycine, pH 3.0, and the eluate was neutralized by addition of 1.5 M Tris, pH 8.8. Fab were prepared by digestion of affinity-purified IgG with mercaptopapain (Sigma Chemical Co., St. Louis, MO) in PBS containing 10 mM cysteine and 2 mM EDTA, as described (14). Intact IgG and Fc fragments were removed by protein A-Sepharose (Pharmacia Fine Chemicals) chromatography. Purity of Fab preparations was confirmed by SDS-PAGE.

Preparation of Rabbit Polyclonal Ab2. Fab fragments of the purified OG IgG specific for $\alpha_{IIb}\beta_3$ were used to immunize two New Zealand White rabbits. For primary immunizations, each rabbit was injected subcutaneously in the back with 50 μg of affinity-purified OG Fab emulsified in CFA. Booster injections (50 μg) were given in incomplete Freund's adjuvant on day 7, 14, 35, and every 2 wk thereafter. Sera were collected from ear arteries on day 1, 21, and every 15 d thereafter. Sera were stored at 4°C before use. IgG was isolated from sera of immunized rabbits, as described above for human IgG, and adsorbed by successive passages over columns containing a 50-fold molar excess of normal human IgG plus IgM linked to Sepharose 4B (Pharmacia Fine Chemicals) a minimum of three times or until all detectable reactivity with isotypic or allotypic determinants was removed.

Specificity of Ab2 for OG IgG. 50 μl of normal human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) or affinity-purified OG IgG in 0.05 M sodium carbonate buffer, pH 9.6, was added to each well of a microtiter plate (Immulon II; Dynatech Laboratories, Inc., Alexandria, VA), and plates were incubated at 4°C overnight. The wells were blotted dry, to each well were added 200 μl of 67 mM $NaHPO_4$, 67 mM Na_2PO_4, 150 mM NaCl, pH 7.4 containing 0.05% Tween 20 (PBS/Tween), and plates were incubated for 1 h at ambient temperature to block remaining binding sites. After three rinses with PBS/Tween, 50 μl of rabbit Ab2 (5 μg/ml in PBS/Tween) was added to each well, and plates were incubated for 1 h. The wells were washed with PBS/Tween three more times, then to each well were added 50 μl of AP-conjugated goat anti–rabbit IgG (heavy + light chain; Zymed, Inc.), diluted 1:1,000 in PBS/Tween, and plates were incubated for 1 h. After an additional five washes, the color reaction was initiated, and the OD at 405 nm for each well was recorded at intervals using an automated microplate reader (EIA reader, model 255; Bio-Rad Laboratories, Richmond, CA).

ELISA Screen of Human IgG for OG Id. 50 μl of a 10 μg/ml solution of murine monoclonal antibody HB43 (anti–human IgG

Fc) in 0.05 M sodium carbonate buffer, pH 9.6, was added to the wells of a microtiter plate (Immulon II; Dynatech Laboratories, Inc.). Plates were then incubated either at ambient temperature for 3 h or at 4°C overnight to permit maximal adsorption of added HB43, with comparable results. The wells were then rinsed six times with PBS/Tween. To block nonreacted surfaces, the wells were then incubated for 60 min at ambient temperature with 250 μl PBS/Tween containing 2% (wt/vol) BSA, and rinsed six times with PBS/Tween. Human plasmas or IgG preparations to be tested were first diluted 1:100 or 1:1,000 in PBS/Tween. 50 μl of each human test sample was added to each well (in triplicate), and plates were incubated for 1 h at ambient temperature. The wells were rinsed six times with PBS/Tween, 50 μl of affinity-purified Ab2 (4 μg/ml) was added to each well, and plates were incubated for 1 h. The wells were then washed with PBS/Tween three times, to each well was added 50 μl of alkaline-phosphatase conjugated goat anti-rabbit IgG (Zymed, Inc.) diluted 1:1,000 in PBS/Tween, and plates were incubated for 1 h. After an additional five washes, the color reaction was initiated, and the OD at 405 nm of each well was recorded in an EIA reader (Bio-Rad Laboratories) at appropriate intervals (nominally, at 30 min).

Binding to Ab2 to Purified Proteins. Human plasma fibronectin and type I collagen were purified as previously described (19, 20). Human plasma vitronectin was purified as described (21) and was a gift from Dr. Brunhilde Felding-Haberman (La Jolla, CA). Human plasma vWf, human fibrinogen (fraction I-2), fragment X, fragment D_{100}, and fragment E were purified as described (22, 23) and generously provided by Dr. Zaverio Ruggeri (La Jolla, CA). To adsorb antigen, 50 μl of carbonate buffer containing the purified protein was added to each well of a microtiter plate, and plates were incubated overnight at 4°C. The concentrations of each protein that enabled maximal adsorption were determined beforehand and were: 2 μg/ml type I collagen; 5 μg/ml fibrinogen; 10 μg/ml fibronectin; 4 μg/ml fragment D_{100}; 3 μg/ml fragment E; 7.5 μg/ml fragment X; 2 μg/ml vitronectin; and 5 μg/ml vWf. The wells were blocked with PBS/Tween, and after three rinses with PBS/Tween, the binding of Ab2 (50 μl vol containing 0–50 μg/ml in PBS/Tween) was measured as described above. In competition assays, 25 μl of PBS/Tween containing the inhibitory protein at 0.2, 1, 2, 5, 10, 25, 50, or 100 μg/ml was added at the same time as Ab2, the mixtures were incubated for 2 h at ambient temperature, and the plates were processed as described above.

To assess the effect of selected fibrinogen-specific, murine monoclonal antibodies on the binding of Ab2 to fibrinogen, wells coated with fibrinogen, as described above, were then incubated with 100 μl of murine antibody IgG in PBS/Tween for 2 h at ambient temperature. The wells were then rinsed three times, Ab2 (10 μg/ml in PBS/Tween) was added, the plates were incubated for an additional 2 h, and the reactions were assessed as described above. Murine IgG was added at a concentration of 1, 2.5, 5, or 25 μg/ml. The murine antibodies developed and characterized by Dr. Z. Ruggeri and co-workers (24, 25) are: anti-RGDS (LJ-134B29; anti-Aα566-580); anti-RGDF (LJ-155B9; anti-Aα87-100); anti-L10 (LJ-Z-69/8; anti-γ400-411); and anti-Bβ (LJ-33.4; specific for an unidentified epitope(s) in the NH_2-terminal region).

Western Blot. The specificity of the antifibrinogen activity of Ab2 was further analyzed by Western blot (18). SDS-PAGE was performed as previously described (18) using a Laemmli buffer system and 10% polyacrylamide resolving slab gels. Purified human fibrinogen (fractions I-2 and I-9), prepared as described (26) and generously provided by Dr. David Amrani (Milwaukee, WI), and fragment D_{100}, were subjected to electrophoresis together with normal human Fab and OG Fab. Reduction was accomplished by addition of 5% 2-mercaptoethanol. After electrophoresis, proteins were transferred to 0.2 μm pore-size nitrocellulose membranes (Bio-Rad Laboratories) in the following manner. Acrylamide gels and membranes were equilibrated in transfer buffer (192 mM glycine, 25 mM Tris, 20% methanol, pH 8.3), and transfer of proteins was accomplished at 30 V for 30 min followed by 60 V for 3 h at 4°C. After transfer, the remaining active sites of the nitrocellulose membrane were blocked by incubating the membrane in PBS/Tween containing 0.05% NaN_3 and 1% nonfat dry milk. The membrane was then washed three times for 10 min each in PBS/Tween. The same buffer containing 1% nonfat dry milk was used to dilute the primary antibodies or the purified conjugated antibody. The membrane strips were incubated with the primary antibody, i.e., rabbit Ab2 or polyclonal rabbit anti–human fibrinogen (Dako Corp., Carpinteria, CA), overnight (16–20 h) at room temperature with constant, gentle, orbital agitation. Then, strips were washed three times for 10 min and incubated with a 1:1,000 dilution of alkaline-phosphatase conjugated goat antibodies against rabbit IgG (heavy + light chain; Zymed, Inc.). After 2–3 h and three additional washes, the binding of this antibody was revealed by incubating the strips in freshly prepared substrate solution consisting of 66 μl of a stock solution of nitroblue tetrazolium (50 mg/ml in 70% dimethylformamide) and 33 μl of a stock solution of bromo-4-chloro-3-indolylphosphate (50 mg/ml in 100% dimethyl-formamide) in 10 ml of 100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5. To stop color development, the membranes were rinsed in distilled water for 10 min and allowed to dry.

Peptides. Peptides were synthesized using F-moc chemistry and an automated peptide synthesizer (MilliGen/Biosearch, Burlington, MA) then purified by reverse-phase HPLC, as previously described (27).

Results

Comparison of Human IgG that Bind $\alpha_{IIb}\beta_3$. Both OG and ES IgG bind weakly, if at all, to $\alpha_v\beta_3$ (Fig. 1), unlike AF IgG and each of the alloantibodies tested, namely GAS and KRO. Since OG IgG is known to bind to free β_3 in Western blot assays (4), two explanations for these findings are possible. Either the OG epitope(s) on β_3 is masked in the $\alpha_v\beta_3$ complex or there exist both β_3-reactive and $\alpha_{IIb}\beta_3$-restricted IgG antibodies. Because the anti-β_3 eluted from Western blot membranes binds to $\alpha_{IIb}\beta_3$ on intact platelets, and antibodies eluted from intact platelets or purified $\alpha_{IIb}\beta_3$ bind solely to β_3 in subsequent Western blots (4), the latter explanation is not likely. The lack of binding of LUC IgG to $\alpha_v\beta_3$ is expected since it contains isoantibodies that predominantly bind α_{IIb} (3, 8). Moreover, the difference in binding of AF and OG (or ES) to $\alpha_v\beta_3$ is also consistent with a difference in epitope location on β_3. While AF is now known to bind to epitopes located within β_3 324–422 (9), OG binding is predominantly to epitopes within β_3 7-52 (Honda, S., Y. Honda, and T. J. Kunicki, unpublished observation).

Rabbit Polyclonal Ab2. After adsorption with pooled, normal human IgG plus IgM, Ab2 IgG binds to OG IgG but not to pooled normal human IgG or to any individual normal human IgG, and binding of Ab2 to OG IgG is inhibited by purified $\alpha_{IIb}\beta_3$ in a dose-dependent manner (Fig. 2). Thus, Ab2 contains predominantly those antiidiotypic

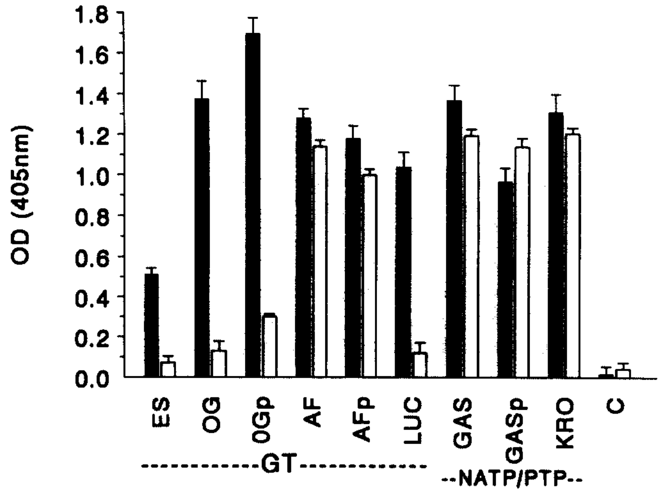

Figure 1. Binding of human IgG to β_3 integrins. Purified human $\alpha_{IIb}\beta_3$ (■) or $\alpha_v\beta_3$ (□) was adsorbed onto wells of a microtiter tray at identical concentrations (2 μg/ml). Plasmas (suffix "p") or IgG (all others) from a group of donors known to contain $\alpha_{IIb}\beta_3$-reactive antibodies were allowed to incubate with antigen, then bound human IgG was detected with rabbit anti–human IgG. Of the donors tested, IgG from ES, LUC, and the propositus OG binds exclusively or much more strongly to $\alpha_{IIb}\beta_3$, while IgG from AF, GAS, and KRO binds equally well to both $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$. In each case, the mean ± SD is depicted (for $\alpha_{IIb}\beta_3$, n = 4; for $\alpha_v\beta_3$, n = 2). As a negative control, the lack of binding of IgG from normal subjects (C) is conspicuous (for $\alpha_{IIb}\beta_3$, n = 10; for $\alpha_v\beta_3$, n = 2).

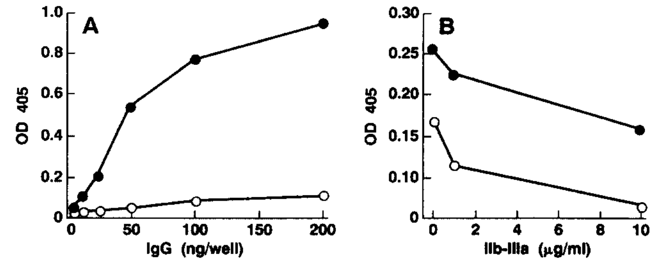

Figure 2. Reactivity of rabbit polyclonal Ab2. (*A*) Binding of Ab2 to OG IgG (*solid circles*) or a pool of normal human IgG (*open circles*) in an ELISA. OG IgG or normal IgG were adsorbed onto wells of microtiter trays. Bound Ab2 was detected by AP-conjugated goat anti–rabbit IgG. The amount of IgG as antigen is indicated on the abscissa. (*B*) Competitive ELISA wherein the ability of purified $\alpha_{IIb}\beta_3$ to inhibit the binding of Ab2 to solid-phase Ab1 is measured. OG IgG was used at either 25 ng/well (*open circles*) or 50 ng/well (*solid circles*). Purified $\alpha_{IIb}\beta_3$ was added to the well at the same time as Ab2. Bound rabbit IgG was detected with AP-conjugated goat anti–rabbit IgG. The concentration of $\alpha_{IIb}\beta_3$ used is indicated on the abscissa.

antibodies that are classified Ab2β, i.e., those that mimic antigen epitopes and block the binding of Ab1 to antigen.

To determine the extent to which the OG idiotype is used by $\alpha_{IIb}\beta_3$-specific antibodies from other individuals, we tested the binding of Ab2 to IgG in sera, plasmas, and IgG preparations from nonimmunized volunteers and persons who have been previously determined to have developed antibodies against $\alpha_{IIb}\beta_3$ as a result of auto-, allo-, or isoimmunization (Fig. 3 *A*). From this preliminary screening, it is evident that Ab2 binds to a restricted subset of human antibodies reactive with $\alpha_{IIb}\beta_3$, i.e., only those antibodies generated by certain

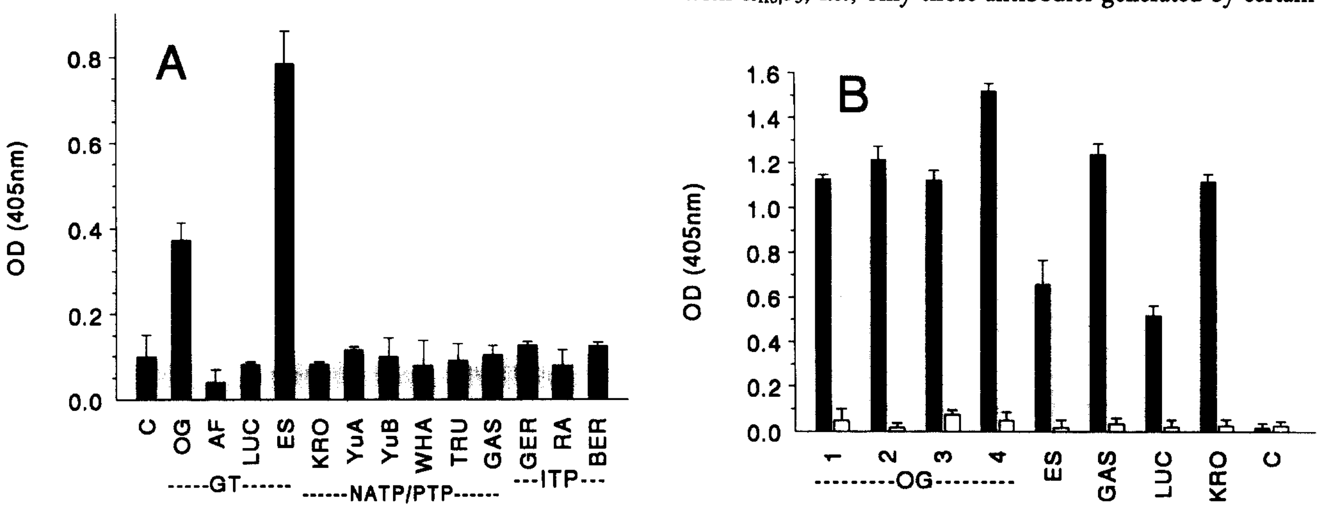

Figure 3. (*A*) Binding of Ab2 to human IgG. Plasma IgG from donors indicated on the abscissa was specifically captured by adsorption to murine mAb HB43 (anti–human IgG Fc)-coated microtiter trays. These donors included: 13 nonimmunized volunteers (C); four patients with Glanzmann thrombasthenia (GT) who have documented $\alpha_{IIb}\beta_3$-reactive isoantibodies (OG, AF, LUC, and ES); six individuals known to have developed $\alpha_{IIb}\beta_3$-reactive alloantibodies as a result of neonatal alloimmunization of posttransfusion purpura (NATP/PTP) (KRO, YuA, YuB, WHA, TRU, GAS); and three ITP patients documented to have $\alpha_{IIb}\beta_3$-reactive autoantibodies (GER, RA, and BER). The binding of Ab2 to captured human IgG is expressed as optical density recorded at 405 nm (ordinate). All values represent mean (bar) ± SD (for controls, n = 13; all others, n = 4). The binding of Ab2 to IgG from each donor was tested on at least two occasions, with equivalent results. (*B*) Lack of binding of Ab2 to antigen-captured Ab1. Microtiter trays were coated with $\alpha_{IIb}\beta_3$. The binding of human IgG to solid-phase $\alpha_{IIb}\beta_3$ was detected by the subsequent binding of rabbit anti–human IgG (■). In parallel wells, bound human IgG was detected by rabbit anti-OGId (Ab2) (□). Human antibody sources are as described in (*A*). OG samples are: (1 and 2) two preparations of OG IgG; (3) OG plasma; and (4) affinity-purified OG IgG (adsorbed by and eluted from $\alpha_{IIb}\beta_3$). Mean ± SD is depicted (for control, n = 4; for all others, n = 2).

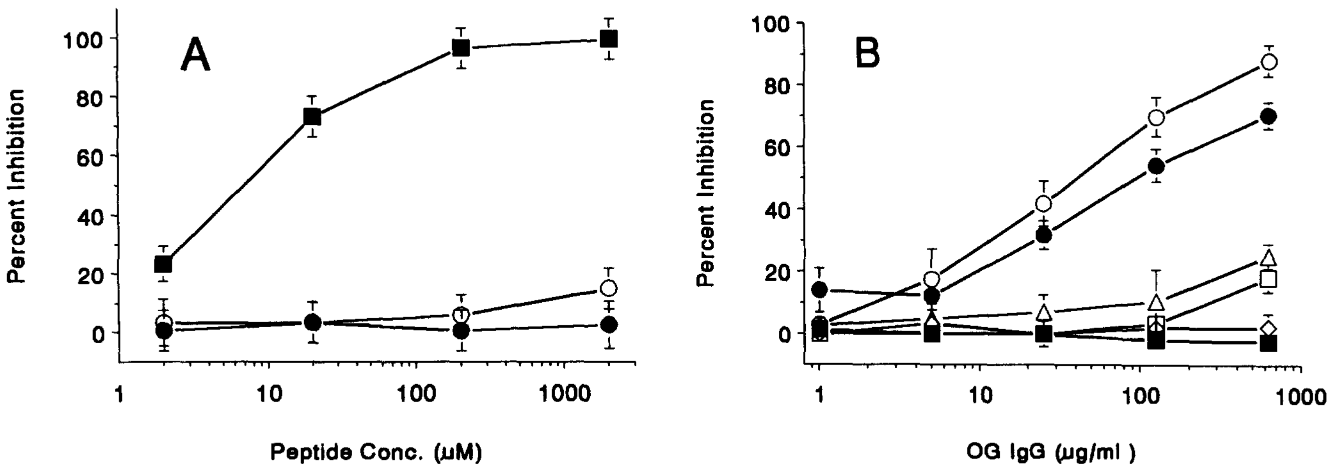

Figure 4. (*A*) Effect of peptides on OG IgG binding to $\alpha_{IIb}\beta_3$. The effect on OG IgG binding of increasing concentrations (abscissa) of the peptides RGDW (●) or L10 (○) was measured. The percent inhibition of binding of OG IgG (ordinate) in the presence of increasing peptide (μM) (abscissa) is plotted. As a positive control, the inhibition of binding of murine mAb OPG2 by RGDW was also tested (■). Values represent mean ± SD (n = 4). (*B*) Inhibition by OG IgG of the binding of selected murine mAb to $\alpha_{IIb}\beta_3$. The binding of murine mAbs OPG2 (○), 7E3 (●), anti-LIBS2 (■), AP2(△), SZ21 (◇), and AP5 (□) in the presence and absence of OG IgG was determined. The percent inhibition of binding of each mAb (ordinate) in the presence of increasing OG IgG (μg/ml) (abscissa) is plotted. Values represent mean ± SD (n = 4).

individuals with the hereditary disorder GT. The presence of the OG idiotype (OG and ES positive; AF, LUC, and remaining donors negative) correlates well with the observed binding properties of IgG from these donors (Fig. 1). Thus, the OG idiotype-positive individuals, OG and ES, are the sole subjects whose IgG antibody binds to β_3 but also distinguishes $\alpha_{IIb}\beta_3$ from $\alpha_v\beta_3$.

When patient IgG is permitted to bind to $\alpha_{IIb}\beta_3$-coated microtiter plates (Fig. 3 *B*), significant levels of $\alpha_{IIb}\beta_3$-specific antibody are bound, as detected with rabbit anti-human IgG (*solid bars*). However, bound OG idiotype-positive IgG (OG or ES IgG) cannot be detected by the rabbit antiidiotype Ab2, presumably because Ab2β antiidiotypes, which are the predominant component of this antiidiotype reagent, cannot access the specific paratopes once they are in contact with antigen. However, OG IgG eluted from $\alpha_{IIb}\beta_3$-coated microtiter plates, is strongly bound by Ab2 in subsequent ELISA (4).

Peptide and MAb Competition. The binding of OG IgG (Ab1) to $\alpha_{IIb}\beta_3$ is not inhibited by either the peptide RGDW or the γ-chain decapeptide L10 (Fig. 4 *A*), even when peptide concentrations as high as 2 mM were used. As a control, specific inhibition of mAb OPG2 by RGDW was observed ($ID_{50} \approx 6$ μM). Of six $\alpha_{IIb}\beta_3$-specific mAb tested, OG IgG

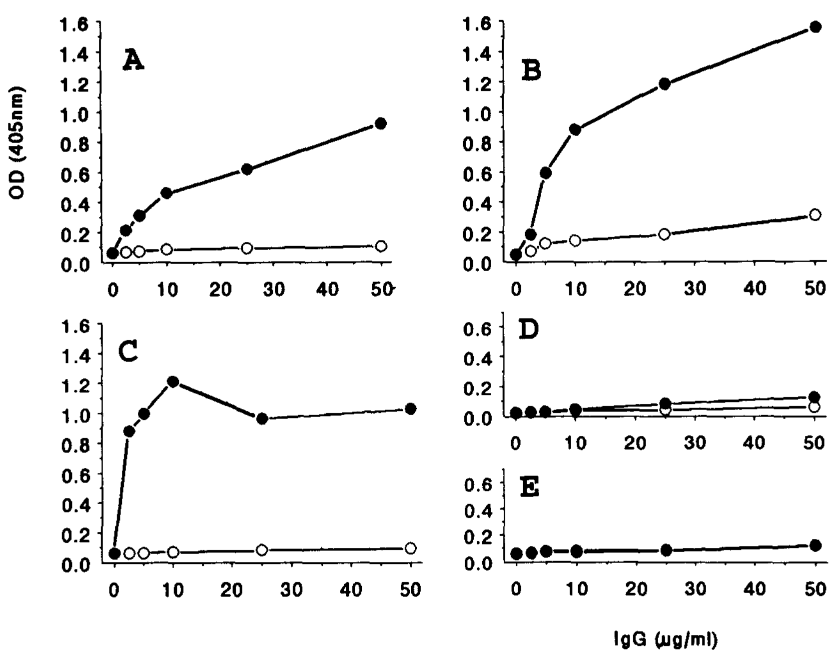

Figure 5. Binding of Ab2 to selected human adhesive proteins. Depicted is the binding of Ab2 (●) or IgG from nonimmunized rabbits (○) to purified human (*A*) fibrinogen; (*B*) von Willebrand factor; (*C*) vitronectin; (*D*) fibronectin; or (*E*) type I collagen. The concentration of Ab2 or nonimmune IgG (μg/ml) added is indicated on the abscissa. In *E*, the two lines are superimposable. Values depicted are the mean of duplicate determinations for a single representative experiment.

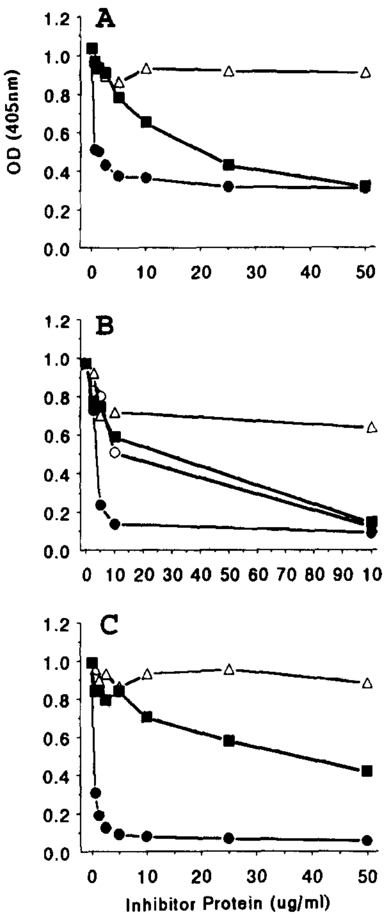

Figure 6. Competitive binding of adhesive proteins. The binding of Ab2 to (*A*) fibrinogen; (*B*) von Willebrand factor; or (*C*) vitronectin was measured in the presence of increasing amounts of vitronectin (●), fibrinogen (■), von Willebrand factor (○), or type I collagen (△). The concentration of inhibitor protein (μg/ml) is indicated on the abscissa. Values represent the mean of duplicate determinations in a single representative experiment.

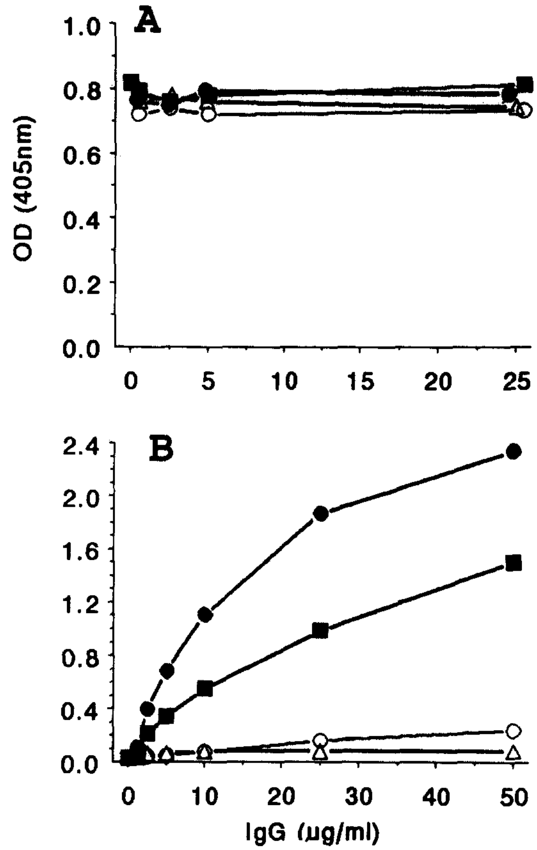

Figure 7. (*A*) Effect of murine monoclonal anti-RGDS (●), anti-RGDF (■), anti-L10 (△), or anti-Bβ chain (○) on the binding of Ab2 to solid-phase intact fibrinogen. The concentration of inhibitor monoclonal antibody (μg/ml) is indicated on the abscissa. (*B*) Comparative binding of Ab2 to intact fibrinogen (●), fragment X (■), fragment D (△), and fragment E (○). The concentration of Ab2 added (μg/ml) is indicated on the abscissa.

strongly inhibited the binding of only 7E3 or OPG2 (Fig. 4 *B*), two IgG mAb known to bind to distinct sites within the fibrinogen recognition pocket of $\alpha_{IIb}\beta_3$. 7E3 and OPG2 are both complex-dependent mAb. As controls, no inhibition of the binding of the complex-specific mAb AP2 (epitope unknown), AP5 (anti-$\beta_3$1-5), SZ21 (anti-$\beta_3$27-32), or LIBS2 (anti-$\beta_3$602-690) was observed. Although not tested here, ES IgG has also been shown to significantly inhibit the binding of 7E3 to $\alpha_{IIb}\beta_3$ (2).

As shown in Fig. 5, Ab2 also binds to fibrinogen, vWf and vitronectin but not to fibronectin, collagen type I, or the control protein, albumin. The binding of Ab2 to any one of these three adhesive proteins is completely inhibited by either of the remaining two (Fig. 6), suggesting that Ab2 recognizes cross-reactive or identical epitopes on these three proteins.

We previously determined that the binding of Ab1 to $\alpha_{IIb}\beta_3$ could not be inhibited by RGD-containing peptides, such as RGDW, or the carboxyl-terminal decapeptide of the fibrinogen gamma A chain (L10) (Fig. 4). Likewise, the binding of Ab2 to fibrinogen is not inhibited by any of three murine monoclonal antibodies specific for the three putative integrin binding sites on this molecule, namely RGDS and RGDF on the Aα chain or L10 (Fig. 7 *A*). These findings are supported by a comparison of the binding of Ab2 to intact fibrinogen and its major plasmin-derived fragments. Thus, in comparison to intact fibrinogen, Ab2 binds less strongly to fragment X and not at all to fragment D or fragment E (Fig. 7 *B*). Finally, by Western blot assay, Ab2 binds preferentially to a protein band whose electrophoretic mobility is consistent with that of the Bβ chain (Fig. 8).

Since the differential binding of Ab2 to fibrinogen fragments and the results of immunoblot assays suggest that Bβ15-42 might participate in epitopes recognized by Ab2, we tested the ability of three overlapping Bβ peptides, Bβ15-28, Bβ24-33, and Bβ32-42, to inhibit binding of Ab2 to either fibrinogen or vitronectin. None of the three peptides inhibits Ab2 binding even at concentrations as high as 2 mM (data not shown).

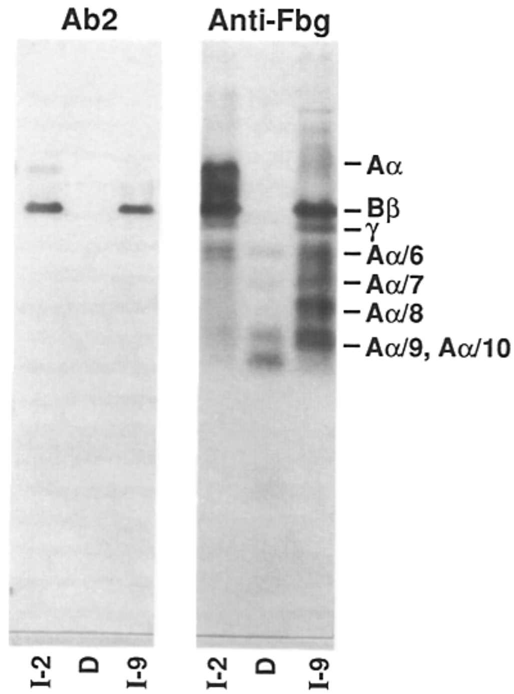

Figure 8. Binding of Ab2 to OG Fab and individual fibrinogen chains as determined by Western blot assay. Proteins were reduced and separated on polyacrylamide slab gels and transferred to nitrocellulose membranes. The following proteins (left to right) were tested: 10 μg purified human fibrinogen fraction I-2; 10 μg human fibrinogen fragment D_{100} (D); and 10 μg human fibrinogen fraction I-9. The left panel depicts a membrane incubated with Ab2. The right panel depicts a membrane incubated with rabbit polyclonal anti–human fibrinogen. To the right are indicated the fibrinogen bands that represent intact Aα, Bβ, and γ chains, as well as the major plasmin-generated fragments of the Aα chain, designated Aα/6, Aα/7, Aα/8, Aα/9, and Aα/10.

Discussion

Since the initial description of the idiotype network, there has been a great deal of interest in the potential role of this network in the regulation of the immune response and in the exploitation of antiidiotypes for the development of vaccines or inhibitors of the immune response, because certain antiidiotypes bear the "internal images" of the primary antigen (28). Yet another interesting and practical application of antiidiotypes stems from the capacity of antiidiotypes to mimic functional protein recognition site(s) of the primary antigen. Anti-Id antibodies are classified into four major categories, Ab2α, -β, -γ, and -ϵ, but only Ab2β, which bind to the paratope (28) of Ab1 and carry the internal image of the antigen, or Ab2γ, which recognize antigen combining-site–associated idiotopes, are able to interfere with the interaction of Ab1 with antigen. In this study, we conclude that Ab2 contains predominantly Ab2β and/or Ab2γ, because purified $\alpha_{IIb}\beta_3$ inhibits the binding of Ab2 to OG IgG, and Ab2 is precluded from binding to Ab1 when Ab1 is bound to $\alpha_{IIb}\beta_3$. This conclusion is supported by the finding that Ab2 binds strongly to an epitope(s) common to three physiologic ligands of $\alpha_{IIb}\beta_3$, i.e., fibrinogen, vWf, and Vn.

Since OG isoantibody binds to β_3 and inhibits ADP- or thrombin-induced platelet aggregation, it is likely that it binds close to or at recognition site(s) on β_3 for fibrinogen. There are two loci on β_3 that have been implicated as fibrinogen contact sites. The first, flanking Asp119, is thought to engage the RGDS sequence of the fibrinogen Aα chain (29, 30). The second, encompassing amino acids 209–222, is thought to interact with a different site on fibrinogen (31). Our results indicate that the OG isoantibody (Ab1) does not bind to either of these loci. First, neither RGD-containing peptides nor our murine monoclonal antibody OPG2 (32), which contains an RYD sequence and binds to the first recognition site on β_3, inhibit the binding of OG isoantibody. Second, our murine monoclonal IgM antibody AP6 (33), which binds to the sequence β_3 209–222, also fails to inhibit the binding of OG isoantibody. Third, by comparing the binding of OG isoantibody to recombinant human β_3, *Xenopus* β_3, and several chimeras of human β_3:*Xenopus*β_3, we have found that the epitope recognized by OG isoantibody is determined by sequences within residues 7–53 of β_3 (S. Honda, Y. Honda, and T. J. Kunicki, unpublished observation).

The fact that the putative adhesive sequences of fibrinogen, i.e., RGDS (Aα 572–575) or L10 (decapeptide γ402–411) are not involved is supported by ELISA results wherein Ab2 binds to fibrinogen fragment X which does not contain the α COOH-terminal RGDS site, and Ab2 binding to human fibrinogen is not inhibited by murine monoclonal antibodies specific for either Aα 566-580 (encompassing RGDS), Aα 87-100 (encompassing RGDF), or the γ COOH-terminal decapeptide. Thus, the epitopes within fibrinogen recognized by Ab2 are distinct from the RGD sequences. We find common epitopes in Vn and vWf. By ELISA, the binding of Ab2 to Vn or vWf is stronger than that observed with fibrinogen, and the epitopes on Vn and vWf are similar if not identical to those fibrinogen, since any one of these proteins is able to completely inhibit the binding of Ab2 to the other two. Other RGD-containing ligands, namely, collagen type I and fibronectin, are not recognized by Ab2, consistent with the conclusion that Ab2 binds to RGD-independent sequences.

Without further localization of the cross-reactive regions on the fibrinogen Bβ chain, Vn, or vWf, it is not possible to precisely identify the epitopes for Ab2 solely by sequence homologies even if a few homologous short acid sequences are present. We will use a number of approaches to further localize the epitopes in question, including screening of random peptide libraries to find sequences that bind with high affinity to Ab2 and testing of additional proteolytic fragments of the fibrinogen Bβ chain or well-defined fibrinogen variants. Nonetheless, our results, in light of the fact that OG IgG inhibits platelet aggregation and fibrinogen binding and Ab2 mimics the primary antigen, support the hypothesis that the Bβ chain is involved in the fibrinogen binding to platelet $\alpha_{IIb}\beta_3$ in a manner that has not yet been characterized.

Ab2 reacts solely with OG isoantibodies (Ab1) and $\alpha_{IIb}\beta_3$-specific isoantibodies from a second GT patient ES. In the limited comparison of four GT patient isoantibodies conducted in this study, ES IgG and OG IgG have the most similar specificities, with respect to $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$. On the other hand, the two idiotype-negative IgG isoantibodies, from patients AF and LUC, exhibit specificities different from one another and from ES or OG IgG. Thus, it is not surprising that the antiidiotype reagent can distinguish ES and OG IgG from the remaining two isoantibodies. In addition, Ab2 never binds to IgG from nonimmunized volunteers or to other $\alpha_{IIb}\beta_3$-specific human IgG antibodies that were generated through different immune mechanisms, e.g., alloimmunization or autoimmunity.

The finding that Ab2 binds to ES IgG indicates that these two unrelated GT patients have independently generated antibodies of similar specificity which share a common idiotype. It has not been logistically feasible to measure direct competition between OG IgG and ES IgG since that would require substantial quantities of affinity-purified, labeled IgG, and these human antibodies are a limited resource. Additional evidence that ES and OG share the OG idiotype derives from the observation of Coller et al. (2) that ES IgG blocks the binding to $\alpha_{IIb}\beta_3$ of the mAb 7E3. In this study, we further establish a close relationship between ES IgG and OG IgG by showing that the latter also inhibits binding of 7E3 to $\alpha_{IIb}\beta_3$. Moreover, we show that OG IgG also blocks the binding of our mAb OPG2. 7E2 and OPG2 are effective inhibitors of fibrinogen binding to human platelets, and 7E3 is the first mAb of its kind to be tested in humans in vivo, as an inhibitor of thrombus formation (34, 35). The epitope(s) recognized by 7E3, OPG2, ES IgG, and OG IgG are obviously of biological and clinical relevance, and are at least spatially related.

Since Ab2 binds to the Bβ chain of fibrinogen, our study raises the possibility that a site(s), likely located within Bβ1-42, makes contact with a recognition site on β_3, one that is defined by the OG epitope(s). Although there has not yet been any report of an interaction of the fibrinogen Bβ chain with $\alpha_{IIb}\beta_3$ or any other integrin, Hamaguchi et al. (36) have demonstrated that platelet spreading on fibrin is mediated by a site within Bβ15-42. The receptor on endothelial cells that recognizes this site is believed to be a 130-kD surface protein not related to the integrins (37). We found that three overlapping peptides representing sequences within Bβ15-42 had no effect on the binding of Ab2 to fibrinogen or vitronectin. However, this negative result does not conclusively rule out that the epitope is present in this region of the Bβ chain, since the true epitope may be more complex than a simple linear sequence. Further studies are clearly warranted.

The OG idiotype is the first idiotype to be described that is associated with human IgG inhibitors of $\alpha_{IIb}\beta_3$ function. Since the antibodies bearing this idiotype bind so closely to the ligand recognition pocket of $\alpha_{IIb}\beta_3$, it is not unexpected that antiidiotypic antibodies should bind to ligands that interact with this recognition pocket. However, it is truly serendipitous and significant that each of the molecular interactions involved in this idiotype network, i.e., binding of OG idiotype to $\alpha_{IIb}\beta_3$, OG idiotype to antiidiotype, and antiidiotype to protein ligands, is mediated by a protein recognition motif that is independent of any ligand:$\alpha_{IIb}\beta_3$ interaction yet defined. Our findings represent an excellent example of the manner in which the characterization of the molecular basis of an immune response and the underlying idiotype network can substantially improve our understanding of biologically relevant protein:protein recognition mechanisms.

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References

1. Kunicki, T.J., and P.J. Newman. 1992. The molecular immunology of human platelet proteins. *Blood.* 80:1386.
2. Coller, B.S., E.I.B. Peershke, U. Seligsohn, L.E. Scuddler, A.T. Nurden, and J.P. Rosa. 1986. Studies on the binding of an alloimmune and two murine monoclonal antibodies to the platelet glycoprotein IIb-IIIa complex receptor. *J. Lab. Clin. Med.* 107:384.
3. Rosa, J.P., N. Kieffer, D. Didry, D. Pidard, T.J. Kunicki, and

A.T. Nurden. 1984. The human platelet membrane glycoprotein complex GP IIb-IIIa expresses antigenic sites not exposed on the dissociated glycoproteins. *Blood.* 64:1246.
4. Kunicki, T.J., K. Furihata, B. Bull, and D. Nugent. 1987. The immunogenicity of platelet membrane glycoproteins. *Transfus. Med. Rev.* 1:21.
5. Jallu, V., M. Pico, J. Chevaleyre, G. Vezon, T.J. Kunicki, and A.T. Nurden. 1992. Characterization of an antibody to the integrin $\beta 3$ subunit (GPIIIa) from a patient with neonatal thrombocytopenia and an inherited deficiency of GPIIb-IIIa complexes in platelets (Glanzmann's thrombasthenia). *Human Hyb. Antib.* 3:93.
5a. Ishida, F., Y. Gruel, E. Brojer, D.J. Nugent, and T.J. Kunicki. Repertoire cloning of a human IgG inhibitor of $\alpha_{IIb}\beta_3$ function. The OG idiotype. *Mol. Immunol.* In press.
6. George, J.N., J.P. Caen, and A.T. Nurden. 1990. Glanzmann's thrombasthenia: the spectrum of clinical disease. *Blood.* 75:1383.
7. White, G.C., E.F. Workman, and R.L. Lunblad. 1978. Thrombin binding to thrombasthenic platelets. *J. Lab. Clin. Med.* 91:76.
8. Degos, L., A. Dautigny, J.C. Brouet, M. Colombani, and N. Ardailou. 1975. A molecular defect in thrombasthenic platelets. *J. Clin. Invest.* 56:236.
9. Jallu, V., M. Diaz-Ricard, A. Ordinas, M. Pico, and A.T. Nurden. 1994. Two human antibodies with different epitopes on integrin β_3 of platelets and endothelial cells. *Eur. J. Biochem.* 222:743.
10. Kunicki, T.J., and R.H. Aster. 1979. Isolation and immunologic characterization of the human platelet alloantigen, PlA1. *Mol. Immunol.* 16:353.
11. Newman, P.J., R.S. Derbes, and R.H. Aster. 1989. The human platelet alloantigens, PLA1 and PLA2, are associated with a leucine33/proline33 amino acid polymorphism in membrane glycoprotein IIIa, and are distinguishable by DNA typing. *J. Clin. Invest.* 83:1778.
12. Furihata, K., D.J. Nugent, A. Bissonette, R.H. Aster, and T.J. Kunicki. 1987. On the association of the platelet-specific alloantigen, Pen^a, with glycoprotein IIIa. Evidence for heterogeneity of glycoprotein IIIa. *J. Clin. Invest.* 80:1624.
13. Wang, R., K. Furihata, J.G. McFarland, K. Friedman, R.H. Aster, and P.J. Newman. 1992. An amino acid polymorphism within the RGD binding domain of platelet membrane glycoprotein IIIa is responsible for the formation of the Pen^a/Pen^b alloantigen system. *J. Clin. Invest.* 90:2038.
14. Kekomaki, R., B. Dawson, J. McFarland, and T.J. Kunicki. 1991. Localization of human platelet autoantigens to the cysteine-rich region of glycoprotein IIIa. *J. Clin. Invest.* 88:847.
15. Fujisawa, K., P. Tani, T.E. O'Toole, M.H. Ginsberg, and R. McMillan. 1992. Different specificities of platelet-associated and plasma autoantibodies to platelet GPIIb-IIIa in patients with chronic immune thrombocytopenic purpura. *Blood.* 79:1441.
16. Fitzgerald, L.A., B. Leung, and D.R. Phillips. 1985. A method of purifying the platelet membrane GPIIb-IIIa complex. *Anal. Biochem.* 151:169.
17. Smith, J.W., D.J. Vestal, S.V. Irwin, T.A. Burke, and D.A. Cheresh. 1990. Purification and functional characterization of integrin alpha V beta 5. An adhesion receptor for vitronectin. *J. Biol. Chem.* 265:11008.
18. Tomiyama, Y., R. Kekomäki, J. McFarland, and T.J. Kunicki. 1992. Antivinculin antibodies in sera of patients with immune thrombocytopenia and in sera of normal subjects. *Blood.* 79:161.
19. Miller, E.J., and S. Gay. 1982. Collagen: an overview. *Methods Enzymol.* 82:3.
20. Vuento, M., and A. Vaheri. 1979. Purification of fibronectin from human plasma by affinity chromatography under nondenaturing conditions. *Biochem. J.* 183:331.
21. Yatohgo, T., M. Izumi, H. Kashiwagi, and M. Hayashi. 1988. Novel purification of vitronectin from human plasma by heparin affinity chromatography. *Cell Struct. Funct.* 13:281.
22. Fujimura, Y., K. Titani, L.Z. Holland, S.R. Russell, J.R. Roberts, J.H. Elder, Z.M. Ruggeri, and T.S. Zimmerman. 1986. von Willebrand factor. A reduced and alkylated 52/48 kDa fragment beginning at amino acid resideu 449 contains the domain interacting with platelet glycoprotein Ib. *J. Biol. Chem.* 261:381.
23. Felding-Habermann, B., Z.M. Ruggeri, and D.A. Cheresh. 1992. Distinct biological consequences of integrin $\alpha_v\beta_3$-mediated melanoma cell adhesion to fibrinogen and its plasmic fragments. *J. Biol. Chem.* 267:5070.
24. Cheresh, D.A., J.W. Smith, H.M. Cooper, and V. Quaranta. 1989. A novel vitronectin receptor integrin ($\alpha_v\beta_x$) is responsible for distinct adhesive properties of carcinoma cells. *Cell.* 57:59.
25. Savage, B., and Z.M. Ruggeri. 1991. Selective recognition of adhesive sites in surface-bound fibrinogen by GP IIb-IIIa on nonactivated plateelts. *J. Biol. Chem.* 266:11227.
26. Kirschbaum, N.E., M.W. Mosesson, and D.L. Amrani. 1992. Characterization of the gamma chain platelet binding site on fibrinogen fragment D. *Blood.* 79:2643.
27. Kunicki, T.J., E.F. Plow, R. Kekomaki, and D.J. Nugent. 1991. Human monoclonal autoantibody 2E7 is specific for a peptide sequence of platelet glycoprotein IIb. Localization of the epitope to IIb231-238 with an immunodominant Trp235. *J. Autoimmun.* 4:415.
28. Jerne, N.K., J. Roland, and P.A. Cazenave. 1982. Recurrent idiotypes and internal images. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:243.
29. D'Souza, S.E., M.H. Ginsberg, T.A. Burke, S. C.T. Lam, and E.F. Plow. 1988. Localization of an Arg-Gly-Asp recognition site within an integrin adhesion receptor. *Science (Wash. DC).* 242:91.
30. Loftus, J.C., T.E. O'Toole, E.F. Plow, A. Glass, A.L. Frelinger III, and M.H. Ginsberg. 1990. A β_3 integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. *Science (Wash. DC).* 249:915.
31. Charo, I.F., L. Nannizzi, D.R. Phillips, M.A. Hsu, and R.M. Scarborough. 1991. Inhibition of fibrinogen binding to GPIIb-IIIa by a GP IIIa peptide. *J. Biol. Chem.* 266:1414.
32. Tomiyama, Y., E. Brojer, Z.M. Ruggeri, S.J. Shattil, J. Smiltneck, J. Gorski, A. Kumar, T. Kieber-Emmons and T.J. Kunicki. 1992. A molecular model of RGD ligands: antibody D gene segments that direct specificity for the integrin $\alpha IIb\beta 3$. *J. Biol. Chem.* 267:18085.
33. Kouns, W.C., B. Steiner, T.J. Kunicki, S. Moog, J. Jutzi, L.K. Jennings, J. Cazenave, and F. Lanza. 1994. Reformation of the fibrinogen binding site on platelets isolated from a patient with the Strasbourg I variant of Glanzmann's thrombasthenia. *Blood.* 84:1108.
34. Coller, B.S., L.E. Scudder, H.J. Berger, and J.D. Iuliucci. 1988. Inhibition of human platelet function in vivo with a monoclonal antibody: with observations on the newly dead as experimental subjects. *Ann. Intern. Med.* 109:635.
35. Coller, B.S. 1992. Inhibitors of the platelet glycoprotein IIb/IIIa receptor as conjunctive therapy for coronary artery thrombolysis. *Coron. Artery Dis.* 3:1016.
36. Hamaguchi, M., L.A. Bunce, L.A. Sporn, and C.W. Francis. 1993. Spreading of platelets on fibrin is mediated by the amino terminus of the β chain including peptide β15-42. *Blood.* 81:2348.
37. Erban, J.K., and D.D. Wagner. 1992. A 130-kDa protein on endothelial cells bind to amino acids 15-42 of the $B\beta$ chain of fibrinogen. *J. Biol. Chem.* 267:2451.