Low platelet counts alone do not cause bleeding in an experimental model of immune thrombocytopenic purpura in mice

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Background and Objectives. The physiopathogenesis of hemorrhagic phenomena in patients with autoimmune thrombocytopenic purpura is associated with low platelet levels. In the present work the relation between thrombocytopenia and bleeding was examined. The possible participation of endothelial cells in bleeding was also investigated.

Design and Methods. Immune thrombocytopenia and bleeding were studied in mice injected with anti-mouse and anti-human platelet polyclonal rabbit IgG. Platelet levels were measured at different times and bleeding signs were systematically recorded. ANOVA tests were used to compare platelet levels. Binding of anti-platelet antibodies to vascular endothelial cells was analyzed by immunohistochemistry.

Results. Three different doses of anti-platelet IgG caused the same low platelet levels, but bleeding occurred only with high doses of anti-platelet IgG irrespective of the platelet levels. No inflammation around blood vessels was observed in paraffin-embedded tissue sections of organs. Immunohistochemistry demonstrated anti-platelet antibodies bound to vascular endothelium.

Interpretation and Conclusions. We showed lack of correlation between platelet counts and bleeding in a murine model. The binding of anti-platelet IgG to endothelial cells of small vessels is an indication that antibody-mediated endothelial activation, in addition to thrombocytopenia, could be participating in bleeding.

Key words: immune thrombocytopenic purpura, hemorrhagic disease, murine model, anti-platelet antibodies, endothelial cells.

Haematologica 2003; 88:679-687
http://www.haematologica.org/2003_06/679.htm

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Autoimmune thrombocytopenic purpura (AITP) is a hemorrhagic disorder widely thought to be caused by excessive destruction of anti-platelet autoantibody-coated platelets by macrophages of the reticuloendothelial system, occurring mainly in the spleen and in the liver.[1-4]

The platelet membrane glycoproteins gpIIb-IIIa ($\alpha_{IIb}\beta_3$, classical fibrinogen receptor) and gpIb-V-IX (classical von Willebrand factor receptor)[5-9] are the main targets of autoantibodies in patients with AITP (16.7-83.3% and 13.3-83%, respectively).[10] Kosugi *et al.*[11] demonstrated that 29% of AITP patients also have specific autoantibodies to $\alpha V\beta_3$ (classical vitronectin receptor). There are also reports that other membrane proteins have epitopes recognized by anti-platelet autoantibodies, for example gpIa-IIa (classical collagen receptor) and CD62.[12,13] Our previous studies, using a phage display library, as well as those of other groups, have shown that the sera of patients with AITP contain a complex mixture of anti-platelet autoantibodies with a wide range of specificities for a number of different epitopes, some of them corresponding to autoantigens.[14-16] There is, therefore, the possibility that these autoantibodies may react with other cells and add to the pathogenesis of AITP.

There is evidence that platelet and endothelial cells have a common origin. Hematopoietic and vascular cells have a common progenitor stem cell in embryonic stages[17] and, in adult life, $CD34^+$ stem cells from bone marrow can form new blood vessels.[18] Platelets bear many membrane proteins also found in endothelial cells: a) gpIIb-IIIa ($\alpha_{IIb}\beta_3$) complex is an integrin receptor found exclusively in megakaryocytes and platelets, but shares the β_3 chain with integrin $\alpha_v\beta_3$ present in megakaryocytes, platelets, endothelial cells and other cells;[19,20] b) gpIb-V-IX, a leucine rich-receptor, is also found in endothelial cells;[21,22] c) E-selectin, P-selectin, CD40 and ICAM-1 are receptors that participate in platelet rolling and are found in both cellular types.[23-25] Moreover, the vascular endothelium is constantly in contact with platelets and actively participates in many hemostatic mechanisms such as platelet rolling, platelet activation and inactivation, thrombus formation and regulation, etc.[26,27] However, the possible participation of endothelial cells in the pathophysiology of AITP has received little attention.

Experimental animal models of AITP have been used to study mechanisms of platelet destruction,[28] treatment schemes,[29-31] the role of immunoglobulin receptors in different reactions[32-35] and to characterize antiplatelet

antibodies interfering with normal platelet function.[36] In the present study we describe the influence of different doses of anti-platelet antibodies on thrombocytopenia and bleeding, a point that has not been extensively explored.[37,38] BALB/c mice received rabbit immune polyclonal anti-human and anti-mouse platelet IgG and the kinetics of thrombocytopenia and hemorrhagic signs were recorded. Furthermore, binding of anti-platelet antibodies to vascular endothelial cells was analyzed by immunohistochemistry.

Design and Methods

Mice and platelet counts

Eight-week old BALB/c male mice were used. Mice were originally purchased from Jackson Laboratories (Bar, Harbor Maine, USA) in 1982, and have been maintained in controlled conditions through b × s mating at our animal facilities for more than 20 generations. Fifty microliters of whole blood were obtained from the retro-orbital plexus by puncture, under ether anesthesia, and collected in 1 mL polypropylene tubes (Eppendorf; Marsh Biochemical Products, Rochester, NY, USA) containing 0.5 mL of phosphate-buffered saline (PBS) with 2% ethylenediaminetetra-acetic acid disodium (EDTA). The platelets were counted in an automatic cell counter (Coulter Electronics, Hialeah, FL, USA).

Preparation of human platelets to use as immunogen

Three outdated O Rh$^+$ platelet concentrates, kindly provided by the Banco Central de Sangre, Centro Médico Nacional Siglo XXI, México, were centrifuged at 800 g for 10 minutes. The pellet of platelets was washed three times with PBS (pH 7.4) containing 10 mM-EDTA and 0.02%-BSA (bovine serum albumin), and then 0.6×10^9 platelets/mL were frozen at –70°C overnight. Platelets were thawed and washed five times in PBS. Aliquots of 1mL of platelets were frozen at -20°C.[39]

Preparation of mouse platelets to use as immunogen

Blood from forty mice was harvested from the retro-orbital venous plexus by puncture and collected in polypropylene tubes containing 0.1 mL of acid-citrate-dextrose (ACD, 38 mM citric acid, 75 mM trisodium citrate, and 100 mM dextrose). Platelet-rich plasma was obtained by centrifugation at 320 g for 10 minutes.

After a second centrifugation at 800 g for 10 minutes platelets were prepared as described above.

Rabbit anti-mouse and anti-human platelet antiserum

Six New Zealand female rabbits were immunized by the platelet preparations to produce anti-human and anti-mouse platelet polyclonal antibodies. One milliliter (0.6×10^9 platelets) of each platelet preparation was thawed and injected subcutaneously into the rabbits on days 1, 15, 30 and 45. The first dose was injected in a mixture with 1mL of complete Freund's adjuvant, the subsequent doses were injected with 1 mL of incomplete Freund's adjuvant. Animals were bled two weeks after the last injection. IgG anti-platelet antibodies were purified using a Protein A-Sepharose column (Sigma Chemical Co, St Louis, MO, USA). A control antibody was prepared from the sera of 2 rabbits injected only with PBS and adjuvant.

Preparation of human platelet proteins to use as antigen in the ELISA

The platelet pellets of 30 (O Rh$^+$) outdated platelet concentrates, kindly provided by the Banco Central de Sangre, Centro Médico Nacional Siglo XXI, México, were obtained by centrifugation at 320 g for 10 minutes and then pooled and washed 3 times with TBS-EDTA, pH 7.4 (Tris-HCl 20 mM, NaCl 150 mM, EDTA 1 mM). The pellets were then treated with lysis buffer TBS-1% triton, pH 7.4 (Tris-HCl 20 mM, NaCl 150 mM, $CaCl_2$ 1 mM, triton 1%, leupeptin 10^{-5}M, phenylmethylsulphonyl fluoride (PMSF 1 mM) (Boehringer Mannheim GmBh, Germany) for 30 min, centrifuged at 1800 g for 30 min and the supernatant collected and frozen at -70°C.

Enzyme-linked immunosorbent assay (ELISA)

Each well of a 96-well microtiter plate (Maxisorp, Nunc, Roskilde, Denmark) was coated with 100 µL of 100 µg/mL platelet proteins in carbonate buffer (50 mM sodium carbonate, pH 9.8) at 37°C for 60 min and washed five times with PBS-0.3% Tween (pH 7.4), followed by blocking with PBS-1% BSA-0.3% Tween (pH 7.4) at 37°C for 30 min. After five washes plates were incubated with 100 µL of rabbit sera diluted 1:100 in PBS-1% BSA-0.3% Tween (pH 7.4) at 37°C for 60 min. The plates were washed again and incubated at 37°C for 60 min with 100 µL of phosphatase-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St Louis, MO, USA) diluted 1:5000. Then *p*-nitrophenylphosphate in diethanolamine buffer (pH 9.8) was added, and after incubation of the plates at 37°C for 30 min, the absorbance was measured at 405 nm.

Immune thrombocytopenia in Balb/c mice

In the first experiment mice were bled under ether anesthesia as mentioned above and injected intraperitoneally (ip) with a single dose of purified anti-mouse or anti-human platelet IgG or control IgG (0.1, 0.5 and 1.0 mg/mouse in 100 µL sterile PBS). The intraperitoneal route was selected to avoid the possibility of intravascular clogging by instantaneous exposure of platelets to high concentrations of the anti-platelet IgGs. Three mice were used in each

experimental group. Blood samples were drawn every 24 hours and the external bleeding signs (ecchymoses, petechiae, blood in stools) were recorded. In a second experiment mice were bled (as above) and injected with 0.1 and 1.0 mg/mouse of anti-mouse platelet IgG every 24 hours, over 4 days (2 mice/group) and their external bleeding signs (ecchymoses, petechiae, blood in stools) were recorded. In a third experiment mice were bled (as above) and injected with 1.0 mg of purified anti-mouse platelet IgG or purified control IgG or PBS only. Mice were bled for platelet counts and sacrificed 0, 2, 4, 8, or 24 hours after injection (3 mice per each experimental group). The mice was sacrificed by exposure to a CO_2 atmosphere. Signs of external and internal bleeding were recorded and the organs (liver, kidney, small intestine, gut, spleen, lungs, heart, and skin) were collected in PBS-10% formalin for microscopic examination. Three additional mice (1 control, 2 injected with 0.1 mg/mouse or 1 mg/mouse of purified anti-mouse platelet IgG) were killed 4 hrs after injection by cervical dislocation to avoid congestion caused by the CO_2 atmosphere.

Histopathology and immunohistochemistry

Organs collected in PBS-formalin were embedded in paraffin, sectioned (6 mm thickness) and stained with hematoxylin-eosin. Histologic sections were examined by light microscopy. Cryostat sections (5 μm) of fresh-frozen tissue were post-fixed in acetone. Sections were treated with 3% hydrogen peroxide. Blank sections were then incubated with PBS, negative control sections with control IgG and test sections with rabbit anti- mouse platelet IgG (1 mg/100 μL). After incubation for 30 min, the sections were treated with goat anti-rabbit IgG conjugated with horseradish peroxidase (Zymed, San Francisco, CA, USA) diluted 1:40 (30 minutes). After three washes with PBS, the sections were treated for 1 to 2 minutes with freshly prepared 3'3'-diaminobenzidine (Sigma Chemical Co., St Louis, MO, USA). The slides were counterstained with hematoxylin. Sections were mounted on glass slides with cytosel mounting medium (Stephen Scientific, Riverdale, NJ, USA).

Statistical analysis

Intergroup differences of platelet counts were compared with analysis of variance (ANOVA) followed by Tukey's studentized range analysis; a repeated measurements design was used when needed. Statistical analysis was performed using the SAS (SAS Institute Inc., Cary, NC27511, USA, 6.02) statistical software program.

Image processing

Color photographs were digitalized (200 dpi) and processed as a whole image using Adobe Photoshop™ 5.5. The images were filtered isolating the red channel and setting its source components as follows: red +76, green +36, blue -72, constant 20. Afterwards the whole image was monochromized. A high resolution (1200 × 1200 dpi) output device was used for printing.

Results

Specificity of rabbit anti-platelet antiserum

Platelets have activation receptors pertaining to highly conserved protein families; for example, there is a 79% homology between human and mouse gpIIa,[40] and a 70% homology between human and mouse E-selectin,[41] We, therefore, decided to test the capacity of the anti-mouse-platelet IgGs to react with human platelets *in vitro*, and the potential of heterologous antibodies to produce thrombocytopenia and bleeding *in vivo*. Anti-human-platelet and anti-mouse-platelet rabbit sera were tested by ELISA using human platelet proteins as antigen. Although anti-human-platelet rabbit serum showed a higher reactivity (O.D. = 2.470), anti-mouse-platelet rabbit serum also showed high reactivity (O.D. = 1.485); pre-immune serum did not react (O.D. = 0.161).

Immune thrombocytopenia in Balb/c mice

To induce immune thrombocytopenia, anti-human and anti-mouse platelet immunoglobulins were injected intraperitoneally into male mice. Polyclonal antibodies were used since AITP patients are known to have a mixture of autoantibodies with different reactivities.[14] When anti-mouse-platelet IgG were injected, at the three different doses used, thrombocytopenia was detected in all mice 1 day after injection, and all mice recovered normal platelet counts after 3 days (Figure 1A). Thrombocytopenia was also induced when anti-human-platelet IgG was injected (Figure 1B) but only with the higher doses, the low dose (0.1 mg/mouse) having no effect. No statistically significant differences ($p>0.05$) were obtained for platelet counts among mice injected with anti-mouse-platelet IgG (0.1, 0.5 or 1.0 mg/mouse) and mice injected with anti-human-platelet IgG (0.5 or 1.0 mg) on the first day after treatment. In contrast, control IgG did not cause thrombocytopenia but a slight thrombocytosis was observed in all mice: platelet counts increased 29% on the first day, and 52% ($p<0.05$) by the third day after treatment (Figure 1C). Outstandingly, there was no correlation between platelet levels and hemorrhagic signs: bleeding depended on antibody dose used regardless of platelet level. Mice injected with 0.1 mg anti-human-platelet IgG had neither thrombocytopenia nor bleeding signs. Mice injected with 0.1 mg anti-mouse-platelet IgG or 0.5 mg anti-human-platelet IgG had severe thrombocytopenia but did not show any signs of bleeding. In contrast, mice injected with

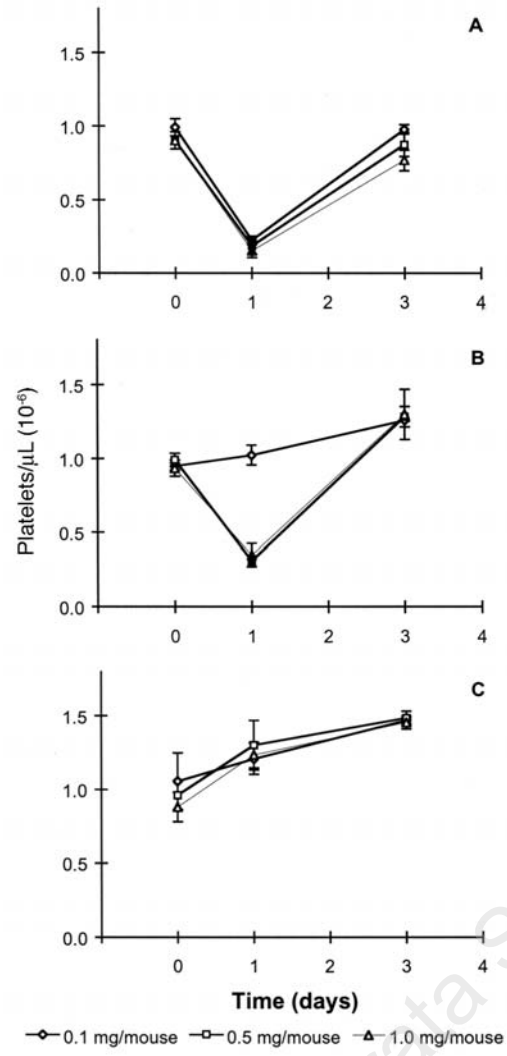

Figure 1. Thrombocytopenia development caused by a single dose of rabbit anti-platelet purified IgG. Mice received 0.1, 0.5 or 1.0 mg/mouse of purified IgG intraperitoneally in 100 mL sterile PBS. Platelet counts were determined at the indicated times, using an automatic cell counter. (A) Anti-mouse platelet IgG. (B) Anti-human platelet IgG. (C) Control IgG. Results are shown as mean platelet count ± standard error for groups of 3 mice each; in all cases when the mean platelet count was low the 3 mice in the corresponding group presented thrombocytopenia. Empty symbols correspond to groups of mice without bleeding signs; filled symbols correspond to groups of mice presenting signs of bleeding.

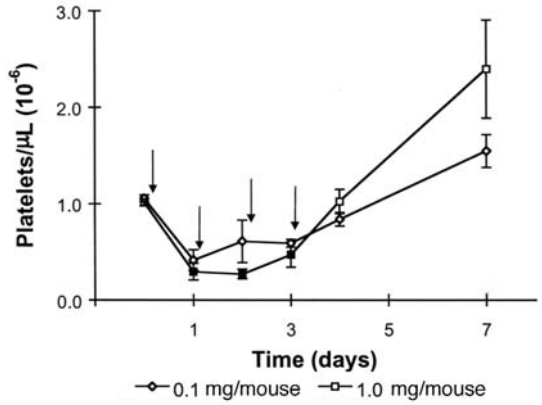

Figure 2. Thrombocytopenia development caused by repeated injections of antibody. Mice received 4 injections of 0.1 or 1.0 mg/mouse intraperitoneally in 100 mL sterile PBS, at the times indicated by vertical arrows. Platelets counts were determined at the indicated times, using an automatic cell counter. Results are shown as mean platelet counts ± standard error for groups of 2 mice each. Empty symbols correspond to groups of mice without bleeding signs; filled symbols correspond to groups of mice presenting signs of bleeding.

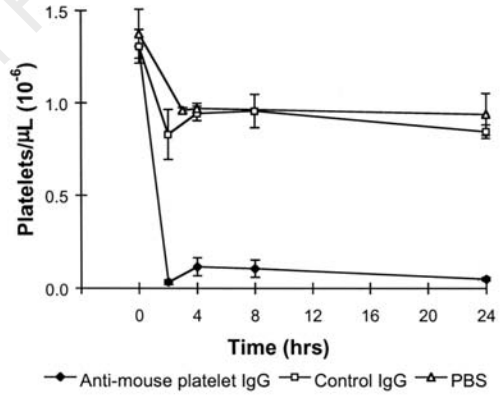

Figure 3. Early development of thrombocytopenia caused by a single dose of rabbit anti-mouse platelet purified IgG. Mice received 1.0 mg/mouse purified IgG intraperitoneally in 100 μL sterile PBS. Negative control mice were injected with 1.0 mg/mouse of control IgG in 100 μL sterile PBS or 100 μL sterile PBS alone. Platelet counts were determined at the indicated times, using an automatic cell counter. Results are shown as mean platelet counts ± standard error for groups of 3 mice each; in all cases when the mean platelet count was low the 3 mice in the corresponding group presented thrombocytopenia. Empty symbols correspond to groups of mice without bleeding signs; filled symbols correspond to groups of mice presenting signs of bleeding.

0.5 mg anti-mouse-platelet IgG or 1.0 mg anti-human-platelet IgG showed thrombocytopenia plus moderate hemorrhagic signs: petechiae in ears and feet. Furthermore, mice injected with 1.0 mg anti-mouse-platelet IgG showed platelet count drops and intestinal bleeding was evident (blood in feces), as were ecchymoses on the head and ears, and hematomas in the retro-orbital plexus (at the site of puncture). On the 3rd day after injection, bleeding stopped in all mice. At all times studied, the prothrombin time[42] and activated partial thromboplastin time[43] were normal in all mice (*data not shown*).

Since injection of anti-platelet IgG caused thrombocytopenia on day 1 and mice were shown to recover by day 3, repeated injections of antibodies were applied every 24 hours for 4 consecutive days

to maintain low platelet counts. Two doses of anti-mouse-platelet antibodies were used (0.1 and 1.0 mg/ mouse). Both doses produced thrombocytopenia. Platelet counts were kept low during the first 3 days, but even though mice received the last injection on the 3rd day, platelet counts recovered to normal levels on day 4 ($p<0.10$) (Figure 2). Mice injected with 0.1 mg/mouse of anti-mouse platelet IgG did not present any external signs of hemorrhage while mice injected with the 1.0 mg/mouse dose presented severe bleeding on the first day: hematomas, petechiae and ecchymoses. On the 2nd day, petechiae and ecchymoses disappeared, hematomas were smaller but external signs of intestinal bleeding appeared. On the 3rd day, only blood in feces was observed, and by the 4th and 7th days no signs of further bleeding were observed. As in the previous experiment, there was no correlation between platelet counts and hemorrhagic signs.

Detailed thrombocytopenia kinetics, external and internal hemorrhagic signs

To examine the relation between thrombocytopenia and bleeding more closely, mice received 1.0 mg/mouse of purified anti-mouse-platelet IgG; platelet counts and external and internal examinations of the mice were performed 0, 2, 4, 8, and 24 hrs after injection. The number of circulating platelets decreased within the first 2 hrs, and remained at an average of 6% of the normal level for 24 hrs after anti-platelet IgG injection (Figure 3). Circulating platelets in mice injected with the same amount of control IgG also decreased but only an average 31.5 %. The same reduction in platelet levels was observed in mice injected with PBS. Mice injected with anti-platelet IgG had similar external signs of bleeding as those described above, as soon as 4 hrs after injection, and these signs were more apparent at 8 and 24 hrs after IgG injection (Figure 4A). External signs of intestinal bleeding were observed in one out of 3 mice examined 4 hrs after IgG injection, and in all six mice examined 8 and 24 hrs after anti-platelet injection. Internal signs of bleeding (Figure 4B) were also evident 4 and 8 hrs after IgG injection: hematomas were observed in several organs (intestine, lungs, heart, liver, and kidney) of some mice. Internal hemorrhagic signs persisted 24 hrs after anti-platelet IgG injection (Table 1).

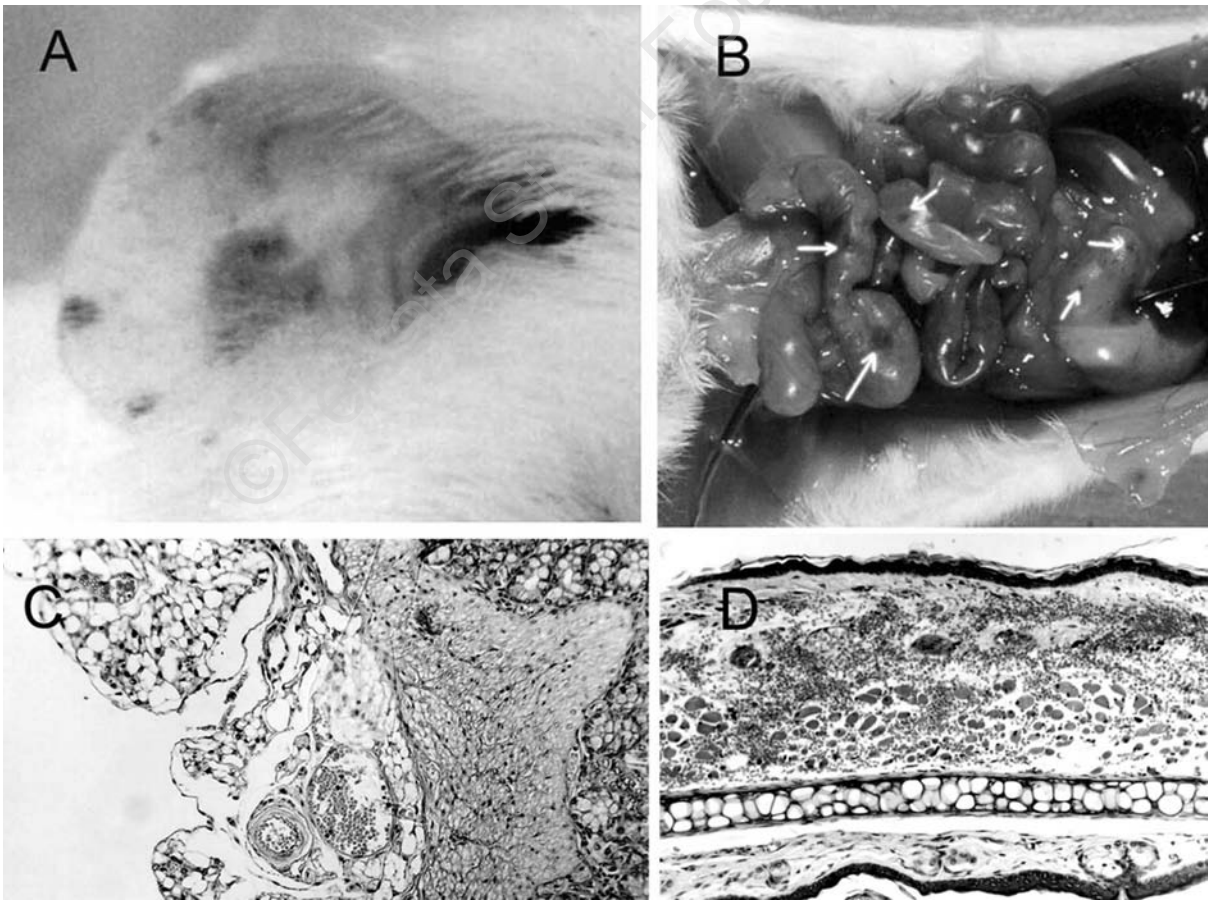

Figure 4. External and internal bleeding signs. Mice received a single dose of 1 mg/mouse of anti-mouse platelet IgG and were examined 4 hrs after IgG injection. A) Ear with external petechiae and ecchymoses. B) Ecchymoses in stomach, gut and small intestine. C) Histologic section of intestine, 4 hrs after anti-mouse-platelet IgG injection. Note vascular congestion without extravasation (40×). D) Histologic section of an ear, 4 hrs after anti-mouse-platelet IgG injection. Note congestion and extravasation (10×).

Table 1. Early kinetics of bleeding in the mice.

	External bleeding				Internal bleeding								
	Head	*Legs*	*Ears*	*Tail*	*Liver*	*Kidney*	*Gut*	*Small intestine*	*Heart*	*Lung*	*Fat tissue (sc)*	*Abdominal tissue (sc)*	*Stomach*
4 hrs													
Petechiae		1*	2										
Ecchymoses			1										
Hematoma		1			2	1	1	1			1		
8 hrs													
Petechiae		1	1	1								1	1
Ecchymoses		1					1						
Hematoma		1					3		3	1	1	1	1
24 hrs													
Petechiae	1	2	2								1	1	2
Ecchymoses							2					1	1
Hematoma		2					2				1		

**Number of mice out of 3 in each experimental group presenting the corresponding bleeding sign in the referred site.*

Histopathology

Microscopic evaluation of paraffin-embedded tissue sections of organs revealed incipient congestion 2 hrs after treatment, and moderate to severe congestion at later times. Skin, kidney, liver, gut, small intestine, lungs and stomach presented hemorrhagic foci 4, 8 and 24 hours after IgG injection (Figures 4C, D). No inflammation around blood vessels was observed at any time in any section studied. Sections from mice injected with control IgG did not show anomalies.

Evidence of anti-platelet IgG binding to endothelial cells

The hypothesis that the binding of anti-platelet antibodies to endothelial cells is involved in bleeding was tested by immunohistochemistry because: 1) there was no correlation between platelet counts and hemorrhage, 2) animals treated with higher doses of anti-platelet antibodies showed more bleeding manifestations, and 3) no perivascular inflammatory response was observed. Besides, it is well known that endothelial cells bear glycoprotein molecules on their surface membrane similar to some glycoproteins on platelet membranes; thus, anti-platelet IgG could contain antibodies that bind to endothelium. Cryostat sections of fresh frozen liver incubated with anti-mouse-platelet IgG showed that anti-platelet IgG did in fact bind only to endothelial cells, while hepatic cells did not react with this IgG. Control IgG did not bind to endothelial cells or to hepatic cells (Figure 5).

Discussion

Our results demonstrate immunologic cross-reactivity between anti-mouse-platelet IgG and human platelets *in vitro*. Moreover anti-human-platelet IgG caused thrombocytopenia and moderate bleeding in mice *in vivo*, this possibly as a result of the known similarities between mouse and human gpIIb-IIIa,[40] gpIb-V-IX, and between other proteins such as CD40, P-selectin and E-selectin.[41] Both *in vitro* and *in vivo* the amount of anti-platelet IgG (Figure 1) required to react with the homologous platelet antigens was less than the quantity required to react with the heterologous platelet antigens, as expected for non-identical proteins and/or because of the presence of non-shared proteins in human and mouse platelets.

This experimental murine model also reproduced the external hemorrhagic signs observed in AITP patients. Internal bleeding was detected in many organs and external signs of intestinal bleeding occurred in mice injected with the highest dose of homologous anti-platelet IgG. A delay was observed between thrombocytopenia, which occurred 2 hrs after anti-mouse platelet IgG injection, and external or internal bleeding, which took place only 4 hrs after anti-platelet IgG injection.

Interestingly, external signs of hemorrhage appeared only in thrombocytopenic mice injected with the medium or high doses of anti-platelet IgG, but not in similarly thrombocytopenic mice injected with a low dose. This lack of correlation shows that low platelet counts by themselves do not explain bleeding, and suggests an additional mechanism involving high doses of anti-platelet IgG. Given the possibility that antibody excess could form soluble immune complexes, as suggested by Nieswandt *et al.*,[37] we microscopically examined histologic sections but did not find any inflammatory reactions in or around the blood vessels at 4, 8 or 24 hrs after antibody injection.

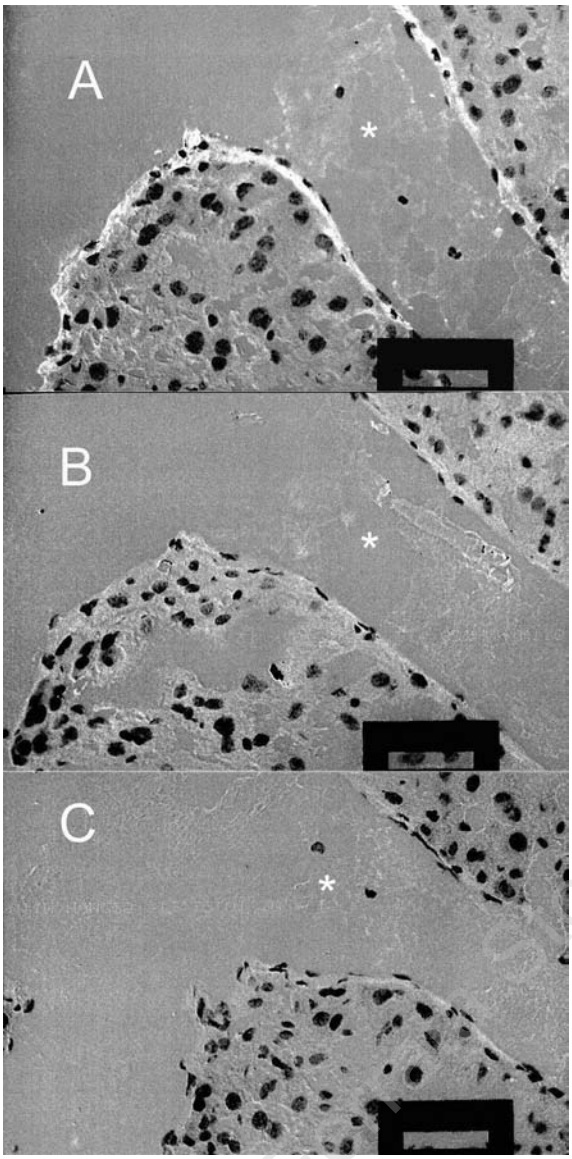

Figure 5. Immunohistochemistry showing anti-platelet antibodies bound to endothelial cells in fresh-frozen liver sections using as the second antibody, anti-rabbit IgG conjugated with horseradish peroxidase, developed with diaminobinzidine. A) Anti-mouse platelet IgG. B) Control IgG. C) PBS. Asterisk indicates vessel lumen.

Traditionally, AITP has been classified as an organ-specific autoimmune disease, and thrombocytopenia has been considered sufficient to explain bleeding.[1,2] However, we found that the anti-platelet IgG employed also bind to endothelial cells, as expected because platelets and endothelium share many proteins.[19-25] Nakajima *et al.*[44] also demonstrated that an anti-gpIIb-IIIa monoclonal antibody specifically binds to human umbilical vein endothelial cells with a high dissociation constant (K_d 40.2 nM). On the other hand, Rodríguez-Calvillo *et al.*[45] obtained a monoclonal antibody against a single endothelial protein of 35 kDa which produced thrombocytopenia and bleeding in mice. Excess anti-platelet antibodies bound to endothelial cells could activate the cytoskeleton, disrupting endothelial junctions and allowing extravasation, and could also interfere with the anticoagulant-procoagulant hemostatic equilibrium.[46,47] Further investigation is required to elucidate the precise participation of these mechanisms in AITP. Our hypothesis is in accordance with other previous suggestions and information. Koenig *et al.*[48] found that one of three patients with immune thrombocytopenic purpura had autoantibodies reacting with a cryptic endothelial antigen. In systemic lupus erythematosus, anti-endothelial autoantibodies have been described as possible mediators of antibody-dependent endothelial cell damage.[49-51] Endothelial activation and damage by anti-platelet antibodies reacting with protein targets of endothelial cells has also been described by Hashemi *et al.*[52] and Glynne *et al.*[53]

AITP is often a chronic disease[1-3] because of a continuous production of autoantibody, but in our model all mice receiving a single anti-platelet injection recovered normal platelet counts and no external hemorrhagic signs were observed three days after treatment. Nieswandt *et al.*[36] produced thrombocytopenia that lasted 3-4 days, using a single dose of monoclonal antibodies; when this single dose was divided to simulate a continuous process, the thrombocytopenia lasted the same time. In our study, in an attempt to mimic the chronic disease, mice were injected every 24 hours for 4 consecutive days. Although thrombocytopenia was extended for 2 days more, unexpectedly, one day after the last boost, platelet counts returned to normal and every sign of hemorrhage disappeared (Figure 2). The possible explanations for this remission of thrombocytopenia and bleeding could be: a) increased platelet destruction is accompanied by a compensatory increase in platelet production by megakaryocytes, as observed in AITP patients;[54] b) xenoantibodies bound to platelets are being removed from blood circulation;[28] c) anti-platelet IgG clearance interrupts activation of endothelial cells. On the other hand, the total IgG fraction used in the experiment contains, in addition to the anti-platelet antibodies responsible for the thrombocytopenia, non-specific IgG that is known to prevent platelet phagocytosis by macrophages, thus inducing remission in AITP patients.[2,34,35] In fact, we observed thrombocytosis when control IgG was used, and also following the thrombocytopenic episode 7 days after the mice had received 1 or 4 doses of anti-mouse-platelet IgG. This set of observations is in agreement with the clinical success of infusion of high-dose intravenous immune globulin G (IVIgG).[2]

References

1. George JN, el-Harake MA, Raskob GE. Chronic idiopathic thrombocytopenic purpura. N Engl J Med 1994;331:1207-11.
2. George JN, Woolf SH, Raskob GE, Wasser JS, Aledort LM, Ballem PJ, et al. Idiopathic thrombocytopenic purpura: a practice guideline developed by explicit methods for the American Society of Hematology. Blood 1996;88:3-40.
3. Frederiksen H, Schmidt K. The incidence of idiopathic purpura in adults increase with age. Blood 1999;94:909-13.
4. Alves-Rosa F, Stanganelli C, Cabrera J, van Rooijen N, Palermo MS, Isturiz MA. Treatment with liposome-encapsulated clodronate as a new strategic approach in the management of immune thrombocytopenic in a mouse model. Blood 2000; 96:2834-40.
5. Hou M, Stockelberg D, Kutti J, Wadenvick H. Antibodies against platelet GPIb/IX, GPIIb/IIIa, and other platelet antigens in chronic idiopathic thrombocytopenic purpura. Eur J Haematol 1995;55:307-14.
6. Kosugi S, Tomiyama Y, Shiraga M, Kashiwagi H, Mizutani H, Kanakura Y, et al. Platelet-associated anti glycoprotein GP IIb-IIIa autoantibodies in chronic immune thrombocytopenic purpura mainly recognize cation-dependent conformations: comparison with the epitopes of serum antibodies. Thromb Haemost 1996;75:339-45.
7. Kiefel V, Freitag E, Kroll H, Santoso S, Mueller-Eckhardt C. Platelet autoantibodies (IgG, IgM, IgA) against glycoproteins IIb/IIIa and Ib/IX in patients with thrombocytopenia. Ann Hematol 1996;72:280-5.
8. Hou M, Stockelberg D, Kutti J, Wadenvik H. Immunoglobulins targeting both gpIIb/IIIa and gpIb/IX in chronic idiopathic thrombocytopenic purpura (ITP): evidence for at least two different IgG antibodies. Br J Haematol 1997; 98:64-7.
9. Hurlimann-Forster M, Steiner B, von Felten A. Quantitation of platelet-specific autoantibodies in platelet eluates of ITP patients measured by novel ELISA using the purified glycoprotein complexes gpIIb/IIIa and gpIb/IX as antigens. Br J Haematol 1997;98:328-35.
10. Wang ZY, Shen ZX. Megakaryocytes and platelets in immune thrombocytopenic purpura. Bailliére's Clin Hematol 1997;10: 89-107.
11. Kosugi S, Tomiyama Y, Honda S, Kashiwagi H, Shiraga M, Tadokoro S, et al. Anti-αVβ3 antibodies in chronic immune thrombocytopenic purpura. Thromb Haemost 2001;85:36-41.
12. Pfueller SL, David R, Firkin BG, Bilston RA, Cortizo WF, Raines G. Platelet aggregating IgG antibody to platelet surface glycoproteins associated with thrombosis and thrombocytopenia. Br J Haematol 1990;74:336-41.
13. Bierling P, Bettaieb A, Fromont P, Favrin M, Duedari N. Anti-GMP140 (CD62) autoantibody in a patient with autoimmune thrombocytopenic purpura. Br J Haematol 1994;87:631-3.
14. Gevorkian G, Manoutcharian K, Almagro JC, Govezensky T, Dominguez V. Identification of autoimmune thrombocytopenic purpura-related epitopes using phage-display peptide library. Clin Immunol Immunopathol 1998;86:305-9.
15. Gevorkian G, Manoutcharian K, Govezensky T, Cano JA, Domínguez V, Santamaría H, et al. Identification of mimotopes of platelet autoantigens associated with autoimmune thrombocytopenic purpura. J Autoimmunity 2000;15:33-40.
16. Fischer P, Jendreyko N, Hoffmann M, Lerch H, Uttenreuther-Fischer MM, Chen PP, et al. Platelet-reactive IgG antibodies cloned by phage display and panning with IVIG from three patients with autoimmune thrombocytopenia. Br J Haematol 1999;105:626-40.
17. Kallianpur A, Jordan J, Brandt S. The SCL/TAL-1 gene is expressed in progenitors of both the hematopoietic and vascular systems during embryogenesis. Blood 1994;83:1200-8.
18. Bhattacharya V, McSweeney PA, Shi Q, Bruno B, Ishida A, Nash R, et al. Enhanced endotheliazation and microvessel formation in polyester grafts seeded with CD34+ bone marrow cells. Blood 2000;95:581-5.
19. Okada Y, Copeland BR, Hamann GF, Koziol JA, Cheresh DA, del Zoppo GJ. Integrin αVβ3 is expressed in selected microvessels after cerebral ischemia. Am J Pathol 1996; 149:37-44.
20. Byzova TV, Rabbani R, D'Souza S, Plow EF. Role of αVβ3 in vascular biology. Thromb Haemost 1998;80:726-34.
21. Rajagopalan V, Essex DW, Shapiro SS, Konkle BA. Tumor necrosis factor-α modulation of glycoprotein Ibα expression in human endothelial and erythroleukemia cells. Blood 1992; 80:153-61.
22. Beacham DA, Lian J, Wu G, Konkle BA, Ludlow LB, Shapiro SS. Arterial shear stress stimulates surface expression of the endothelial glycoprotein Ib complex. J Cell Biochem 1999; 73:508-21.
23. Bombeli T, Schwartz BR, Harlan JM. Adhesion of activated platelets to endothelial cells: evidence for a gpIIbIIIa-dependent bridging mechanism and novel roles for endothelial intercellular adhesion molecule 1 (ICAM-1), $\alpha_v\beta_3$ integrin, and gpIbα. J Exp Med 1998;187:329-39.
24. Yang J, Furie BC, Furie B. The biology of P-selectin glycoprotein ligand-1: its role as a selectin counterreceptor in leucocyte-endothelial and leucocyte-platelet interaction. Thromb Haemost 1999;81:1-7.
25. Henn V, Steinbach S, Buchner K, Presek P, Kroczek R. The inflammatory action of CD40 ligand (CD154) expressed on activated human platelets is temporally limited by coexpressed CD40. Blood 2001;98:1047-54.
26. Andre P, Denis CV, Ware J, Saffaripour S, Hynes RO, Ruggeri ZM, et al. Platelets adhere to and translocate on von Willebrand factor presented by endothelium in stimulated veins. Blood 2000;96:3322-8.
27. Yeh C, Wang WC, Hsieh TT, Huang TF. Agkistin, a snake venom-derived glycoprotein Ib antagonist, disrupts von-Willebrand factor-endothelial cell interaction and inhibits angiogenesis. J Biol Chem 2000;275:18615-8.
28. Ishihara T, Akizuki S, Yokota T, Takahashi M, Uchino F, Matsumoto N. Foamy cells associated with platelet phagocytosis. Am J Pathol 1984;114:104-11.
29. Mizutani H, Furubayashi T, Imai Y, Kashiwagi H, Honda S, Take H, et al. Mechanisms of corticosteroid action in immune thrombocytopenic purpura (ITP) experimental studies using ITP-prone mice, (NZW x BXSB) F1. Blood 1992;79:942-7.
30. Mizutani H, Furubayashi T, Kashiwagi H, Honda S, Take H, Kurata Y, et al. Effects of splenectomy on immune thrombocytopenic purpura in (NZW x BXSB) F1 mice: analyses of platelet kinetics and anti-platelet antibody production. Thromb Haemost 1992;67:563-6.
31. Nemoto K, Mae T, Saiga K, Matsuura E, Koike T. Autoimmune-prone (NZW x BXSB) F1 mice escape severe thrombocytopenia after treatment with deoxispergualin, an immunosuppressant. Br J Haematol 1995;91:691-6.
32. McKenzie SE, Taylor SM, Malladi P, Yuhan H, Cassel DL, Chien P, et al. The role of the human Fc receptor Fc gamma RIIa in the immune clearance of platelets: a transgenic mouse model. J Immunol 1999;162:4311-8.
33. Wallace PK, Keler T, Coleman K, Fisher J, Vitale L, Graziano RF, et al. Humanized mAb H22 binds the human high affinity Fc receptor for IgG (FcγRI), blocks phagocytosis, and modulates receptor expression. J Leuk Biol 1997;62:469-79.
34. Samuelsson A, Towers TL, Ravetch JV. Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor. Science 2001;291:484-6.
35. Lin S, Kinet J. Giving inhibitory receptors a boost. Science 2001;291:445-6.
36. Nieswandt B, Bergmeier W, Rackebrandt K, Gessner JE, Zirngibl H. Identification of critical antigen-specific mechanisms in the development of immune thrombocytopenic purpura in mice. Blood 2000;96:2520-7.
37. Nieswandt B, Echtenacher B, Wachs FP, Schröder J, Gessner JE, Schmidt RE, et al. Acute systemic reaction and lung alterations induced by antiplatelet integrin gpIIb/IIIa antibody in mice. Blood 1999;94:684-93.
38. Hansen RJ, Balthasar JP. Pharmacokinetics, pharmacodynamics, and platelet binding of an anti-glycoprotein IIb/IIIa monoclonal antibody (7E3) in the rat: a quantitative rat model of immune thrombocytopenic purpura. J Pharmacol Exp Ther 2001;298:165-71.
39. Woods VL Jr, Oh EH, Mason D, McMillan R. Autoantibodies against the platelet glycoprotein IIb/IIIa complex in patients with chronic ITP. Blood 1984;63:368-75.
40. Cieutat AM, Rosa JP, Letourneur F, Poncz M, Rifat S. A comparative analysis of cDNA-derived sequences for rat and

mouse b3 integrins (gpIIIa) with their human counterpart. Biochem Biophys Res Comm 1993;193:771-8.
41. Becker-Andre M, Hooft van Huijsduijnen R, Losberger C, Whelan J, Delamarter JF. Murine endothelial leukocyte-adhesion molecule 1 is a close structural and functional homologue of the human protein. Eur J Biochem 1992;206: 401-11.
42. Miletich JP. Prothrombin time. In: Beutler E, Litchman MA, Coller BS, Kipps TJ, Editors. Williams Hematology. New York: McGraw-Hill; 1995. p. L82-4.
43. Miletich JP. Activated partial thromboplastin time. In: Beutler E, Litchman MA, Coller BS, Kipps TJ, eds. Williams Hematology. New York: McGraw-Hill; 1995. p. L85-6.
44. Nakajima T, Koyama T, Kakishita E, Nagai K. Inhibitory effects of TTP sera on binding of anti-platelet glycoprotein IIb-IIIa monoclonal antibodies to human vascular endothelial cells. Am J Hematol 1987;25:115-8.
45. Rodríguez-Calvillo M, Gabari I, Duarte M, Mazzolini G, Rifón J, Rocha E, et al. Thrombopenic purpura induced by a monoclonal antibody directed to a 35-kilodalton surface protein (p35) expressed on murine platelets and endothelial cells. Exp Hematol 2001;29:589-95.
46. Hajjar KA, Esmon NL, Marcus AJ, Muller WA. Vascular function in hemostasis. In: Beutler, Litchman MA, Coller BS, Kipps TJ, Seligsohn U, editors. Williams Hematology. New York: McGraw-Hill; 2001. p. 1451-69.
47. Colman RW, Clowes AW, George JN, Hirsh J, Marder VJ. Overview of hemostasis. In: Colman RW, Hirsh J, Marder VJ, Clowes AW, George JN, editors. Hemostasis and Thrombosis: Basic Principles and Clinical Practice. Philadelphia: Lippincott Williams & Wilkins; 2001. p. 3-16.
48. Koenig DW, Barley-Maloney L, Daniel TO. A western blot assay detects autoantibodies to cryptic endothelial antigens in thrombotic microangiopathies. J Clin Immunol 1993;13:204-11.
49. Meroni PL, Khamashta MA, Youinou P, Shoenfeld Y. Mosaic of antiendothelial antibodies. Review of the first international workshop on antiendothelial antibodies: clinical and pathological significance. Lupus 1995;4:95-9.
50. Li JS, Liu MF, Lei HY. Characterization of anti-endothelial cell antibodies in the patients with systemic lupus erythematosus: a potential marker for disease activity. Clin Immunol Immunopathol 1996;79:211-6.
51. Moscato S, Pratesi F, Bongiorni F, Scavuzzo MC, Chimenti D, Bombardieri S, et al. Endothelial cell binding by systemic lupus antibodies: functional properties and relationship with anti-DNA activity. J Autoimmun 2002;18:231
52. Hashemi S, Drouin J, Trudel E, Aye MT, Couture R, Page D, et al. Characterization of novel platelet and endothelial cell target antigens in a family with genetic susceptibility to autoimmunity. Am J Hematol 1991;38:293-303.
53. Glynne P, Salama A, Chaudhry A, Swirsky D, Lightstone L. Quinine-induced immune thrombocytopenic purpura followed by hemolytic uremic syndrome. Am J Kidney Dis 1999;33:133-7.
54. Harker LA, Finch CA. Thrombokinetics in man. J Clin Invest 1969;48:963-74.

Pre-publication Report & Outcomes of Peer Review

Contributions

VD, TG, GG and CL contributed equally to this work and were primarily responsible for its conception and design, analysis and interpretation of data.

We thank Raúl Ambriz from Banco Central de Sangre, Centro Médico Nacional, SXXI, IMSS for kindly providing platelet concentrates. We appreciate the specialized assistance of Gerardo Arrellín and Eduardo López in the histopathology studies, Raúl Nieto in performing the platelet counts, Ismael Rodríguez for the immunohistochemical stains, and Gabriel Garduño for image processing.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Carlo L. Balduini, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Professor Balduini and the Editors. Manuscript received June 13, 2002; accepted April 18, 2003.

In the following paragraphs, Professor Balduini summarizes the peer-review process and its outcomes.

What is already known on this topic

It is sometimes observed that bleeding tendency in autoimmune thrombocytopenic purpura is more severe than expected on the basis of platelet count, but the reason for this phenomenon is unknown.

What this study adds

The authors of this paper used an animal model to demonstrate that antibodies against glycoproteins of platelet membrane also recognized endothelial cells. Moreover, they observed that different doses of antibodies induced the same degree of thrombocytopenia but different severity of bleeding. The results of this study suggest that an antibody-induced endothelial damage could be involved in the pathogenesis of bleeding in immune thrombocytopenia and open a new area of investigation in this field.