Serum platelet-reactive and glycoprotein (GP) Iib-GPIIa--reactive IgG and F(ab')$_2$ was examined in 39 patients with classic autoimmune thrombocytopenic purpura (ATP), two patients with anti-PLA1 antibody and 25 control subjects in an enzyme-linked immunosorbent assay. IgG was purified by diethyl aminoethyl chromatography and centrifuged at 100,000g before testing of the supernatant. Significant IgG binding (threefold to fourfold control IgG binding) was noted with 8 of 17 ATP patients' IgG, 2 anti-PLA1 IgGs, and 2 ATP patients with multiple platelet transfusions. However, F(ab')$_2$ fragments of nine of nine positive ATP IgGs were nonreactive; F(ab')$_2$ from the two anti-PLA1 and two multiply transfused ATP IgGs were as reactive as their intact IgG. Antiplatelet or anti--GPIIb-GPIIa reactivity of ATP IgG could not be adsorbed to fixed platelets or solid-phase GPIIb-GPIIa and eluted with 0.1 mol/L glycine, pH 2.5. However, binding of IgG to GPIIb-GPIIa could not be inhibited with F(ab')$_2$ of ATP IgG or Fc fragments of control subjects. When platelet-- or GPIIb--

DIOPATHIC thrombocytopenic purpura (ITP) is generally considered an autoimmune disorder (autoimmune thrombocytopenic purpura [ATP]) in which antibody is directed against the host's platelets and megakaryocytes. However, older as well as recent studies from several laboratories have questioned the pathophysiologic relevance of detectable IgG on the patient's platelets, because the amount is 10- to 100-fold greater than that required for platelet or red blood cell (RBC) destruction in other immunologic thrombocytolytic or hemolytic disorders, and elevated platelet IgG is often noted in patients with apparent nonimmune thrombocytopenia. Indeed, the argument has been made that because some patients with peripheral platelet destruction of “non-immune” origin have similar elevated platelet-associated IgG levels as ATP patients, the elevated platelet-associated IgG merely reflects the larger size of platelets (megathrombocytes) seen in these disorders. The nonspecific nature of platelet-associated IgG is supported by older observations that platelet subclass IgG of ATP patients has the same relative distribution as serum IgG. Furthermore, platelet IgG correlates with plasma IgG levels; the same has been noted for platelet and plasma albumin.

Further experiments in guinea pigs showed that platelets and/or megakaryocytes are capable of taking up isotopically labeled IgG infused into the animal, via fluid phase endocytosis, and that platelet $\alpha$-granules contain plasma IgG. Indeed, platelet surface IgG in normal subjects was shown to represent less than 1% of total platelet IgG, and the elevated platelet-bound IgG reported by previous investigators really represents total platelet IgG, ie, $\alpha$-granule plus surface IgG. Nevertheless, experiments designed to measure surface IgG with specific monoclonal anti-Fc reagents do not elevate elevated platelet IgG on platelets of ATP patients, albeit at 20-fold lower levels than previously reported.

These observations prompted a reassessment of detectable antiplatelet IgG antibody in the sera of ATP patients. In attempting to affinity purify and characterize the platelet-reactive IgG, we noted that detectable antiplatelet reactivity was not F(ab')$_2$ mediated but rather immune complex mediated and caused by the formation of sulfhydryl-linked aggregates of IgG that form on storage of ATP IgG at $-20^\circ$C. The present report documents these observations.

MATERIALS AND METHODS

Patient population. Sera were obtained from 39 patients with the diagnosis of classic ATP (female: male ratio, 2.6:1), two patients with anti-PLA1 antibody after posttransfusion purpura, and 25 control subjects. Two female ATP patients had a history of numerous platelet and blood transfusions.

Platelet preparation. Platelets were prepared from EDTA anticoagulated blood and washed in human Ringer-10 mmol/L EDTA as described previously.

Sera. Sera were prepared from whole blood, after incubation at 37°C for 1 hour, or from EDTA plasma by adding 1 mol of 0.025 mol/L CaCl$_2$ to 1 vol of plasma for 1 hour at 37°C. The supernatants were separated by centrifugation and stored at $-20^\circ$C or used fresh.

Purified IgG. IgG was purified from sera by diethyl aminoethyl (DEAE) ion exchange chromatography. All preparations were centrifuged at 100,000g for 3 minutes in an Air Driven Ultracentri...
fuge (Airfuge; Beckman Instruments, Irvine, CA) before utilization in the enzyme-linked immunosorbent assays (ELISAs) or gel filtration experiments.

F(ab')2 fragments. These were prepared by pepsin digestion (Sigma, St Louis, MO) and protein A affinity chromatography.\textsuperscript{16,17} F(ab')2 fragments were verified by molecular weight with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).\textsuperscript{16,17}

Fc fragments. Purified IgG was digested with 10% papain (Sigma) in the presence of 0.01 mol/L cysteine, overnight at 37°C, and then passed through a protein A column. Fc was eluted with 0.1 mol/L glycine, pH 2.5, neutralized with 1 mol/L Tris and then applied to an anti-F(ab')2 affinity column (containing 3.5 mg of antibody coupled to cyanogen-bromide activated Sepharose 4B [Sigma]) to remove undigested IgG. Although SDS-PAGE and silver staining had shown absence of F(ab')2 or IgG contamination, a sensitive ELISA with alkaline-phosphatase-conjugated antibody coupled to cyanogen-bromide activated Sepharose 4B (Sigma) in the presence of 0.01 mol/L cysteine, overnight at 37°C, showed its presence (5% to 10% contamination) before affinity purification.

Affinity purification of IgG with fixed platelets or solid-phase glycoprotein (GP) IIb-GPIIIa. Washed platelets, 2 x 10\(^6\), fixed in 1% paraformaldehyde and washed in Ringer-EDTA or GP IIb-GPIIIa (2.5 mg) coupled to 1.5 mL of Affigel-10 beads (BioRad, Richmond, CA), were exposed to 1 mg/mL IgG overnight at 4°C on a rotating shaker (10 rpm). Platelets or beads were then washed in Ringer-EDTA, sedimented, and eluted with 0.1 mol/L glycine pH 2.5 or 4 mol/L MgCl\(_2\).

Platelet GPIIb-GPIIIa. Outdated platelet-rich plasma was obtained from the NYU Medical Center Blood Bank. Platelets were sedimented, washed in Ringer-EDTA (containing the protease inhibitors: 5 \(\mu\)mol/L soybean trypsin inhibitor, 10 \(\mu\)mol/L benzamidine, and 2 \(\mu\)mol/L phenylmethylsulfonyl fluoride [PMSF]), resuspended in 1% ammonium oxalate for 15 minutes at room temperature, sedimented, washed twice in Ringer-EDTA, and then solubilized in 1% Triton X-100, 0.15 mol/L NaCl plus the above protease inhibitors at 4°C for 2 hours. The platelet extract was then centrifuged at 30,000 \(\times g\) for 15 minutes at 4°C, the sediment discarded, and the supernant applied to a concanavalin A (Sigma) column equilibrated with 20 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L CaCl\(_2\), 1% Triton X-100, and 0.05% sodium azide, pH 7.4. The column was washed in the above solution until no protein was detectable in the flow-through solution by 280-nm absorbance. The column was then eluted with 100 mmol/L \(\alpha\)-methyl-D-mannose (Sigma) in the washing buffer.\textsuperscript{18,19} The eluate was then applied to a heparin-sepharose column to remove thrombospondin.\textsuperscript{19} Remaining fibrinogen was removed by affinity chromatography with a specific murine IgG1-k monoclonal antibody (MoAb) against fibrinogen (LK-F, which we have prepared). Four milligrams of MoAb LK-F was coupled to 1 mL of wet cyanogen bromide-activated sepharose-4B. The flow-through material was pure GPIIb-GPIIIa on SDS-PAGE with silver stain as determined by molecular weight, shift in mobility of both bands after reduction with mercaptoethanol, and reactivity of GPIIIa on immunoblot with a specific MoAb against GPIIb, LK-2.\textsuperscript{20}

ELISA. Washed platelets in Ringer-EDTA (1 x 10\(^7\)) or GPIIb-GPIIIa (250 ng) in PBS-Ca\(^{2+}\) (1 mmol/L) were applied to polyvinyl 3911 microtiter plates (Falcon, Oxnard, CA) overnight at 4°C. Plates were washed twice with 1% bovine serum albumin (BSA) in 0.01 mol/L phosphate-buffered saline (PBS)-Ca\(^{2+}\) (2 mmol/L), pH 7.4 and blocked with 1% BSA-PBS for 60 minutes at 37°C on a horizontal shaker (50 rpm). In some experiments, plates were blocked with 3% fat-free milk in PBS-Ca\(^{2+}\), pH 7.4. Fluid contents were removed by decanting and IgG, F(ab')2, or Fc fragments were added at serial doubling dilutions in 1% BSA-PBS starting at 60 \(\mu\)g/mL (3 \(\mu\)g/well) for 1 hour at 37°C on a shaker. The plates were washed three times with 20 mmol/L Tris buffer, 250 mmol/L saline (TBS)-0.05% Tween 20 (Sigma), pH 7.4, and then incubated with a 1:1,000 dilution of goat antihuman IgG (\(\gamma\) chain-specific) or antihuman F(ab')2 coupled to alkaline phosphatase (Sigma) for 1 hour at 37°C on a shaker. Wells were washed three times in TBS-Tween 20, followed by addition of the appropriate substrate.
for 60 minutes at room temperature. The plate was then read in a Dynatech MR60 microtiter plate reader at 405 nmol/L (Dynatech, Alexandria, VA).

RESULTS

Reactivity of patient IgG with platelets and GPIIb-GPIIIa. Significant binding of ATP IgG to GPIIb-GPIIIa (three to fourfold control binding) was noted with 8 of 17 patient IgG preparations. Figure 1 (right panel) shows the mean reactivity of the eight positive patient IgG preparations versus the mean of 10 control IgG preparations with purified GPIIb-GPIIIa. The optical density (OD) of the eight positive-reactive IgG preparations varied from 1.050 to 1.600 at 60 µg/mL of IgG. Note that mean reactivity of control IgG with blank microtiter wells blocked with 1% BSA-PBS was negligible. Similar results were noted for patient IgG on blank wells. The OD of the nine negative-reacting IgG preparations varied from 0.220 to 0.450 at 60 µg/mL. Figure 1 (left panel) shows the reactivity of five positive patient IgG preparations versus 10 control IgG preparations with intact platelets. Note the positive reactivity of patients' IgG with as little as 190 ng/well.

The eight ATP patients with positive-reacting IgG for GPIIb-GPIIIa were compared clinically with the nine ATP patients with nonreactive IgG. No differences were noted for positive- and negative-reactive patients, respectively, between age at onset of disease, 33 ± 6 (SEM) versus 42 ± 6 years, P > .1; platelet count at presentation, 25,000 ± 6,000 versus 21,000 ± 7,000/µL; duration of disease, 14 ± 4 versus 11 ± 4 years; splenectomy 2 of 7 versus 3 of 9 patients; and positive response to corticosteroids 4 of 5 versus 5 of 7 patients.

Absorption and elution of anti-GPIIb-GPIIIa IgG reactivity from fixed platelets or solid-phase GPIIb-GPIIIa. Figure 2A shows that absorption of a patient's IgG with fixed platelets removed reactivity with GPIIb-GPIIIa, whereas the eluate from the platelets contained a modestly higher specific activity than the original IgG. The increase in specific activity is considerably less than that expected for a classic antibody elution purification. Similar results were also

Fig 2. Reactivity of an ATP patient's IgG with GPIIb-GPIIIa after absorption to and elution from platelets or solid-phase GPIIb-GPIIIa. (A) Fixed platelets, 2 x 10^9 or (B) GPIIb-GPIIIa coupled to Affigel-10 beads were incubated with 1 mg/mL of an ATP patient's IgG overnight at 4°C followed by washing and elution with 0.1 mol/L glycine, pH 2.5. Original IgG, absorbed supernatant IgG, and acid-eluted IgG were then reacted with purified GPIIb-GPIIIa in a solid-phase ELISA as in Fig 1. Doubling serial dilutions are given. (□) ATP IgG; (○) control IgG; (▲) ATP eluate; (●) ATP supernatant.
SERUM PLATELET-REACTIVE IgG OF ATP PATIENTS

GPIIb-GPIIIa Target Antigen

![Graph showing reactivity of IgG versus F(ab')2 for patients and controls.](image)

Fig 3. Reactivity of ATP, control, and anti-PLA1 intact IgG versus their corresponding F(ab')2 fragments with purified GPIIb-GPIIIa in a solid-phase ELISA, as in Fig 1. (Left) ATP IgG versus its F(ab')2 fragment; (center) control IgG versus F(ab')2 fragment; (right panel) anti-PLA1 IgG versus F(ab')2 fragment.

Obtained with IgG from four other patients, as well as with platelets as the ELISA antigen (data not shown). Comparable absorption and elution data for anti-GPIIb-GPIIIa of six positive-reactive patients were obtained with solid-phase GPIIb-GPIIIa coupled to Affigel 10, as antigen (Fig 2B).

Reactivity of IgG versus F(ab')2 for patients and controls. Figure 3 shows the lack of reactivity of ATP patients' F(ab')2 with platelet GPIIb-GPIIIa compared with intact IgG (left panel). In contrast, pepsin digestion of IgG from a PLA1 antibody did not remove its reactivity with GPIIb-GPIIIa (right panel). Two additional ATP patients with

Platelet Target Antigen

![Graph showing reactivity of IgG versus F(ab')2 for patients and controls.](image)

Fig 4. Reactivity of ATP, control, and anti-PLA1 intact IgG versus their corresponding F(ab')2 fragments with intact platelets on microtiter plates, as in Fig 1, except for blocking of plates with 5% fat-free milk. ATP 1 and ATP 2 refer to IgG preparations from two different ATP patients.
multiple platelet transfusions showed binding of their F(ab')2 fragments to GPIIb-GPIIIa (data not shown).* Similar results were obtained with intact platelets as ELISA antigen (Fig 4). These data indicated that binding of IgG from nontransfused ATP patients to platelets was not F(ab')2 dependent and suggested that this binding could be immune complex mediated rather than caused by the presence of a specific antibody to GPIIb/GPIIIa on platelets.

Attempts to block binding of patient IgG to GPIIb/GPIIIa with F(ab')2 or Fc fragments. We reasoned that binding of intact IgG to GPIIb-GPIIIa could be caused by a cooperative effect of F(ab')2 and Fc portions of the intact IgG, both needed to provide sufficient avidity of binding. If this were the case, addition of either fragment of the IgG should block the binding. Figure 5 shows lack of inhibition of the binding of patient IgG to GPIIb-GPIIIa under conditions where the ratio of F(ab')2 prepared from the ATP patient's IgG to the patient's IgG was 80:1. Similar results were obtained with an F(ab')2 and intact IgG preparation from a second patient. This could not be attributed to lack of saturation of GPIIb-GPIIIa binding with IgG because experiments with decreasing concentrations of GPIIb-GPIIIa from 250 to 31 ng/well with constant F(ab')2 at 15 µg/well and IgG at 1.5 µg/well also showed no inhibition by F(ab')2 with two different patient IgG preparations (data not shown).

Similar results were obtained with Fc fragments of normal IgG. Lack of inhibition of the binding of two different patients' IgG to GPIIb-GPIIIa was noted under conditions where the ratio of normal human Fc to patient IgG concentration was 52:1. Purified Fc did not bind to GPIIb-GPIIIa at 3 µg/well (data not shown).

High molecular weight ATP patient IgG reacts with GPIIb-GPIIIa. Figure 6A shows the Sephacryl-300 gel filtration profile of an ATP patient's DEAE-IgG that had been spun at 100,000g immediately before gel filtration. A high molecular weight protein region was observed in the void volume. Similar results were obtained with IgG preparations from three additional ATP patients. When individual collection tubes were assayed for reactivity with GPIIb-GPIIIa, activity was found in the void volume fractions, not in the 7S IgG region. Similar anti–GPIIb-GPIIIa reactivity was noted in the void volume of IgG passed over Sephacryl-300 in five additional patients (in two of those patients protein regions in the high molecular weight fraction were not prominent). Figure 6B shows the results obtained with a control IgG preparation. No reactivity with GPIIb-GPIIIa was found in any of the fractions, as was the case with six additional control IgG preparations. Similar results were obtained with intact platelets as ELISA antigen (data not shown).

Characterization of the high molecular weight IgG fraction by immunoblotting. We further examined the content of the void volume fraction of ATP IgG obtained by Sephacryl-300 gel filtration. Figure 7 shows an immunoblot of reduced void volume fraction (lane B) and 7S IgG (lane A) of an ATP patient with platelet-reactive IgG, using γ-chain–specific goat antihuman IgG. No reactivity was noted with anti-IgM, µ-chain–specific antibody. Reduction of the fractions with 0.6 mol/L mercaptoethanol for 3 minutes with boiling showed heavy-chain IgG at ~55 Kd for the respective fractions in lanes D and C. Attempts at obtaining intact IgG via more gentle reduction were unsuccessful. Similar results were obtained with two other ATP patients. Thus, platelet-reactive IgG of the void volume gel filtration fraction is composed of IgG aggregates held together by sulphhydryl bonds. No preferential distribution of IgG subclass was noted in four different platelet-reacting void volume fractions, using type-specific antibody in an ELISA (data not shown).

Because platelets or GPIIb-GPIIIa bind fibrinogen, we considered the possibility that the high molecular weight fraction might represent antifibrinogen-fibrinogen complexes or polymerized cryofibrinogen-fibrinogen-fibrin complexes. Attempts at demonstrating antifibrinogen antibody

*Reactivity with GPIIb-GPIIIa was unexpected because specific alloantibodies are uncommon after platelet transfusions. Reactivity could have been against GPIIb-GPIIIa polymorphisms described for GPIIb and GPIIIa.
using purified fibrinogen in a solid phase ELISA were unsuccessful with five of five reactive void volume fractions. Similar negative results were obtained for the detection of fibrinogen in 6 reactive void volume fractions when tested in immunoblots with an MoAb (LK-F) with specificity for fibrinogen (data not shown).

**Effect of storage at −20°C on anti–GPllb-GPllla reactivity.**

The sera used for the above experiments had all been stored at −20°C for prolonged periods of time (6 months to 5 years) after blood withdrawal, before the experiments were performed. It was considered of interest to test sera from freshly withdrawn blood as well. Surprisingly, such sera or IgG preparations lacked binding activity for GPllb-GPllla. However, after storage for 3 or 6 months, significant binding activity developed. Figure 8 shows the reactivity of 5 of 19 ATP patients' anti–GPllb-GPllla IgG from freshly prepared IgG (tested within 1 to 2 days of blood withdrawal), and IgG stored at −20°C for 3 months and 6 months. This is compared with control IgG stored at −20°C for 3 and 4 months. Note the lack of patient anti–GPllb-GPllla reactivity with fresh samples compared with fresh control samples. Note the increasing patient anti–GPllb-GPllla reactivity with storage at −20°C for 3 and 6 months. No increase in reactivity was noted with 16 of 16 control IgG preparations. Nonreactivity of freshly prepared IgG was similarly noted in an additional 19 of 20 ATP patient sera. A similar increase in platelet-binding IgG reactivity of ATP patients was noted on storage at −20°C (data not shown).

**DISCUSSION**

These data indicate the absence of classic F(ab')2-mediated antiplatelet reactivity in the stored sera and/or IgG of nine nontransfused ATP patients whose DEAE-purified IgG bound to platelets and/or GPllb-GPllla,
detectable when tested at 150 ng/well. However, positive F(ab')\textsubscript{2} reactivity was noted with two anti-PLA\textsubscript{1} antibodies as well as with the IgG of two ATP patients with a history of multiple blood and platelet transfusions. The reactive IgG from ATP patients behaved like classic antibody in that they could be adsorbed and eluted from fixed platelets or solid-phase GPIIb-GPIIIa. However, the reactive IgG did not fractionate in the 7S region on Sephacryl gel filtration, whereas it did fractionate in the void volume region, suggesting the presence of a high molecular weight IgG aggregate. This was confirmed by immunoblotting with anti-IgG (\gamma-chain specific) antibody which showed greater than 250-Kd high molecular weight bands that were reduced to a 55-Kd band with mercaptoethanol, typical of IgG heavy chain.

These findings were surprising because we expected to find F(ab')\textsubscript{2} antiplatelet IgG reacting with platelets. This supposition was based on clinical observations on the transfer of a humoral factor across the placenta leading to thrombocytopenic purpura in neonates of ATP women, the classic experiments of Harrington et al\textsuperscript{4} showing a thrombocytopenic humoral factor in the plasmas of 62% of ATP patients, and the demonstration that this humoral factor was in the globulin fraction of a patient's plasma. There is a paucity of data on the binding of F(ab')\textsubscript{2} fragments of antiplatelet IgG from ATP patients to platelets. A review of 24 publications on antiplatelet IgG in ATP patients\textsuperscript{1} (plus 12 additional references since 1985) shows only one that reports F(ab')\textsubscript{2} binding to platelets of five of seven reactive IgG samples.\textsuperscript{21} However, no mention was made of the purity of the F(ab')\textsubscript{2} fragments, and a qualitative immunofluorescence test was used. Of interest is a report of \textsuperscript{125}I-IgG production by spleens of three ATP patients in which F(ab')\textsubscript{2} fragments of platelet-reactive \textsuperscript{125}I-IgG bound to platelets at 89%, 63%, and 55% of intact IgG.\textsuperscript{22} Because equal cpm of \textsuperscript{125}I-IgG versus \textsuperscript{125}I-F(ab')\textsubscript{2} was added to

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Fig 7. Immunoblot of an ATP patient's IgG obtained from various Sephacryl 300 gel filtration fractions. Gel-filtered void volume (lane B) and 7S IgG (lane A) fractions were electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose, and then immunoblotted with goat antihuman IgG (\gamma-chain specific). Lanes D and C refer to their respective mercaptoethanol-reduced samples.

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Fig 8. Effect of storage of ATP IgG at -20°C on anti-GPIIb-GPIIIa reactivity. (Left) Freshly prepared ATP IgG (1 to 2 days after blood withdrawal) was compared with identical aliquots stored at -20°C for 0, 3, and 6 months for their ability to react with purified GPIIb-GPIIIa. (Right) Similar studies were performed with control IgG aliquots stored for 0, 3, and 4 months at -20°C.
platelets, it is likely that $^{125}$I-F(ab')$_2$ binding represented 37% to 59% of $^{125}$I-IgG. Thus, some F(ab')$_2$ binding was shown.

An alternative mechanism for IgG binding to platelets is via immune complexes that could bind to the platelet Fc receptor. Three laboratories have reported the presence of elevated immune complexes in ATP patients.23-25 Our initial observation suggested that platelet-reactive IgG could indeed be immune-complex mediated because purified antiplatelet IgG from nontransfused patients did not fractionate on gel filtration in the 7S region, but rather in the void volume high molecular weight fraction. However, these IgG immune complexes did not contain IgM, were not blocked from binding to GPIIb-GPIIIa by isolated Fc fragments, and did not dissociate into the 150,000 molecular weight of IgG on SDS-PAGE. Reduction with mercapto-ethanol converted the complex to its 55,000 molecular weight heavy chain on immunoblot with anti-IgG (γ-chain specific), indicating that the IgG-IgG complex was held together by sulfhydryl bonds. The mechanism of binding of ATP IgG aggregates to GPIIb-GPIIIa is of interest because binding was neither blocked by patient F(ab')$_2$ nor normal Fc fragments. Thus, ATP IgG aggregates bind to GPIIb-GPIIIa by an undefined mechanism.

Freshly prepared IgG from ATP patients did not contain these complexes; neither was it observed in stored control samples.† Thus, stored ATP sera or IgG (at −20°C) was unique in that it developed oxidized sulfhydryl-linked aggregates on storage. We considered the possibility that the development of sulfhydryl-oxidized IgG aggregates on storage of ATP IgG could be caused by a unique IgG subclass composition of the aggregate. For example, IgG3 has a propensity to undergo aggregation, presumably because the hinge region is rich in sulfhydryl groups.27 However, no preferential distribution of IgG subclass was noted in the void volume platelet-reactive immune com-

†George26 has reported the presence of platelet-reactive IgG in control plasma stored at −20°C. It is of interest that 80% of this reactivity could be removed after centrifugation at 12,000g for 10 minutes at 4°C.26

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Serum platelet-reactive IgG of autoimmune thrombocytopenic purpura patients is not F(ab')2 mediated and a function of storage

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