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IVIg inhibits reticuloendothelial system function and ameliorates murine passive-immune thrombocytopenia independent of anti-idiotype reactivity

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Summary. Although the mechanism of action of intravenous immunoglobulin (IVIg) in treating antibody-dependent thrombocytopenia remains unclear, most studies have suggested that IVIg blocks the function of Fc receptors in the reticuloendothelial system (RES) and/or the protective effect may be due to the presence of variable region-reactive (anti-idiotype) antibodies within IVIg. We evaluated the effect of IVIg on platelet counts in a murine model of passively induced immune thrombocytopenia (PIT). Although IVIg was unable to neutralize the binding of two platelet-specific monoclonal antibodies to their target antigens either *in vivo* or *in vitro,* it was able to prevent PIT as well as ameliorate pre-established PIT mediated by these antibodies. IVIg adsorbed against the antibody used to induce thrombocytopenia or endogenous murine immunoglobulin also protected against PIT, indicating that antibodies with anti-idiotype activity present in IVIg are not necessary for its effective treatment of PIT. IVIg significantly blocked the ability of the RES to clear antibody-sensitized red blood cells. $F(ab')_2$ fragments of IVIg, which are unable to block the RES but retain the idiotypic regions, were ineffective at protecting mice from PIT. Our data suggest that IVIg exerts its rapid effect by inhibiting RES function and that anti–idiotype interactions are not required.

Keywords: thrombocytopenia, IVIg, platelet, anti-idiotype, mouse.

Intravenous immunoglobulin (IVIg) is prepared from the pooled plasma of a large number of individuals and contains antibodies specific for many antigens, including other antibodies, i.e. anti-idiotypes (Dietrich & Kazatchkine, 1990; Dwyer, 1992; Glotz *et al*, 1993; Ronda *et al*, 1994; Amital *et al*, 1996; Schussler *et al*, 1998; Fischer *et al*, 1999; Macias *et al*, 1999). It is currently used to treat several autoimmune diseases (Godeau *et al*, 1993; Amital *et al*, 1996; Fazekas *et al*, 1997; Stangel *et al*, 1998) and to ameliorate both allogeneic (Reed *et al*, 1987; Glotz *et al*, 1993) and xenogeneic (Gautreau *et al*, 1995; Schussler *et al*, 1998) graft rejection. IVIg is also used to treat antibody-dependent thrombocytopenia resulting from autoimmune and non-autoimmune diseases such as idiopathic thrombocytopenic purpura (Imbach *et al*, 1981), systemic lupus erythematosus (DeVita *et al*, 1996; Lesprit *et al*, 1996), heparin-induced thrombocytopenia (Frame *et al*, 1989; Winder *et al*, 1998; Warkentin & Kelton, 1994), post-transfusion purpura (Nugent, 1992) and some patients with human immunodeficiency virus-associated thrombocytopenia (Bussel & Haimi, 1988; Jahnke *et al*, 1994). Amelioration of disease activity has been attributed to the presence of functional anti-idiotype antibodies in IVIg (Dietrich & Kazatchkine, 1990; Dwyer, 1992; Glotz *et al*, 1993; Ronda *et al*, 1994; Amital *et al*, 1996; Schussler *et al*, 1998; Macias *et al*, 1999). These anti-idiotype antibodies may bind autoantibodies, neutralize their function and form IgG multimers, as well as form immune complexes with endogenous immunoglobulin. These complexes may, individually or in concert, block the function of phagocytic cells within the reticuloendothelial system (RES) (reviewed in Dwyer, 1992; Hurez *et al*, 1993).

The contribution of anti-idiotypes in IVIg to the reversal of thrombocytopenia has been difficult to resolve with *in vivo* studies. Although anti-idiotype-dependent neutralization of platelet-reactive autoantibody has been demonstrated *in vitro* (Berchtold *et al*, 1989; Rossi *et al*, 1989) and an *in vivo* decrease in platelet-associated autoantibody after IVIg therapy has been shown (Bussel *et al*, 1983; Winiarski *et al*, 1983; Bussel, 1989; Boughton *et al*, 1994), Barbano

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et al (1989) found IVIg to be ineffective at autoantibody neutralization in idiopathic thrombocytopenic purpura (ITP). To investigate the action of IVIg, we have used a model of passive-immune thrombocytopenia (PIT) in mice.

Murine models of thrombocytopenia can be produced through the use of platelet-specific polyclonal or monoclonal antibodies (Alves-Rosa *et al*, 2000; Nieswandt *et al*, 2000; Samuelsson *et al*, 2001). Using the PIT model, we found that IVIg was unable to block the binding of two platelet-specific antibodies to their target antigen either *in vitro* or *in vivo*. IVIg was, however, able to prevent the onset of thrombocytopenia and ameliorate pre-existing PIT. IVIg adsorbed against the anti-platelet antibody used to induce PIT or against murine immunoglobulin was as effective at preventing PIT as standard IVIg. These results suggest that IVIg exerts its rapid effect by inhibiting the RES and that anti–idiotype interactions are not required.

MATERIALS AND METHODS

Reagents

The monoclonal antibody specific for glycoprotein (GP)IIb (clone MWReg30; rat IgG_1, κ), the unconjugated or fluorescein isothiocynate (FITC)-conjugated antibody against GPIIIa (clone 2C9.G2; hamster IgG, group 1, κ), and the affinity-purified anti-Ly-76 monoclonal antibody (clone TER-119; rat $IgG1_2b$, κ) were purchased from BD PharMingen (Mississauga, ON, Canada). Goat anti-rat IgG-FITC, and affinity-purified unconjugated and alkaline phosphatase conjugated goat anti-human IgG (H+L) were from Cedarlane Laboratories (Hornby, ON, Canada). The IVIg used (Gamimune) was from Bayer, Inc. (Elkhart, IN, USA). Human serum albumin was purchased from Miles Laboratories Canada (Etobicoke, Canada). Ovalbumin and maltose was purchased from Sigma-Aldrich (Oakville, ON, Canada). CNBr-activated Sepharose 4B, Protein G Sepharose, QAE Sephadex and Sephadex G-150 were purchased from Amersham Pharmacia Biotech (Baie d'Urfé, PQ, Canada). Outbred CD1 mouse serum was obtained from Cedarlane (Hornby, Canada). FITC and pepsin were purchased from Sigma-Aldrich.

Mice. CD1 and CB.17

Severe combined immunodeficient (SCID) virgin female mice (6–8 weeks of age) were purchased from Charles River Laboratories (Montreal, PQ, Canada) and housed in the St. Michael's Hospital Research Vivarium. SCID mice were housed under gnotobiotic conditions.

Induction of passive-immune thrombocytopenia (PIT)

PIT was induced by intraperitoneal injection of monoclonal anti-platelet antibody (2 μg rat anti-mouse GPIIb, or 10 μg hamster anti-mouse GPIIIa) in 200 μl phosphate-buffered saline (PBS) pH 7·2; 24 h later, 100 μl of whole blood was collected via the tail vein into microvette tubes (Sarstedt, Montreal, PQ, Canada) pre-loaded with 10 μl of 1% EDTA in PBS, pH 7·2, and platelets enumerated by flow cytometry. Using this method, all strains of mice tested became transiently thrombocytopenic by 24 h after infusion of anti-platelet antibody; platelet numbers returned to normal by d 3 after infusion. The mean platelet counts in unmanipulated SCID mice and outbred CD1 mice were $916 \pm 51{\cdot}9 \times 10^9$/l and $1567 \pm 97{\cdot}8 \times 10^9$/l respectively. For IVIg pretreatment, mice were first injected intraperitoneally with the indicated amounts of IVIg, control protein or buffer 24 h before induction of PIT. Blood samples were taken 24 h after induction of PIT. Initial experiments demonstrated that the protective effect of IVIg was equally successful whether the intraperitoneal or intravenous route (three injections of 333 μl over an 8 h period) was used (data not shown); we have used the intraperitoneal route because of the large volume (1 ml per mouse) of IVIg injected. Control mice were pretreated with an equivalent amount of human albumin in 10% maltose–PBS buffer or buffer alone. Red blood cell (RBC) counts were unaffected in untreated PIT mice ($8{\cdot}24 \pm 0{\cdot}37 \times 10^{12}$/l) or IVIg-treated PIT mice ($8{\cdot}42 \pm 0{\cdot}43 \times 10^{12}$/l) compared with unmanipulated mice ($8{\cdot}74 \pm 0{\cdot}23 \times 10^{12}$/l).

Platelet enumeration

Mouse blood (5μl) was diluted into 100 μl of 1% EDTA–PBS. The blood was then further diluted in PBS to a final dilution of 1:12 000. The samples were acquired for 2 min on a flow-rate-calibrated FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA), using forward scatter (FSC) versus side scatter (SSC) to gate platelets. Reference samples were incubated with FITC-conjugated anti-mouse platelet antibody to ensure that the proper platelet gate was set. RBC were used as an internal reference standard in all samples; any sample varying by $>10\%$ of the mean RBC count in untreated mice was discarded.

Anti-idiotype depletion of IVIg

Mouse. Outbred CD1 mouse serum was passed over a Protein G column to isolate IgG. Bound IgG was eluted from the column with 0·1 mol/l glycine–HCl, pH 2·7 and immediately adjusted to pH 7·2 by the addition of 2 mol/l Tris base, pH 8·0. The IgG was then dialysed in 10 mmol/l PBS, pH 7·2. The resulting purified immunoglobulin was coupled to CNBr-activated Sepharose 4B according to the manufacturer's directions (Amersham Pharmacia Biotech). IVIg was depleted of mouse IgG-reactive components by three rounds of incubation with the IgG-coupled Sepharose using a batch method. There was no detectable total protein loss in the IVIg preparation after this manipulation.

Rat. Purified rat IgG and anti-GPIIb were separately coupled to CNBr-activated Sepharose 4B, as above. IVIg was depleted of rat IgG-reactive components by three rounds of incubation with rat IgG-Sepharose followed by a further three incubations with anti-GPIIb-Sepharose using a batch method to remove any possible anti-GPIIb-reactive 'anti-idiotypic-like' antibodies.

Analysis of anti-idiotype-depleted IVIg

The effectiveness of anti-idiotype depletion was assessed using enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated with 1 μg/ml (50 μl/well) of the purified $F(ab')_2$ fragments of CD1 IgG or rat anti-GPIIb IgG, in

50 mmol/l carbonate–bicarbonate buffer, pH 9·6 overnight at 4°C. Plates were then washed with 0·05% Tween-20 in PBS and then blocked (200 μl/well) for 2 h with 0·2% Tween-20 in PBS. IVIg or the two anti-idiotype-depleted IVIg batches were serially diluted in PBS and added (50 μl/well) to the plates for 2 h at room temperature. Plates were then washed and 2 μg/ml (50 μl/well) alkaline phosphatase-conjugated goat anti-human IgG (H+L) added for 1·5 h at 20°C. Plates were washed again with Tween–PBS and 100 μl of substrate solution (5 mmol/l *p*-nitrophenyl phosphate) was added. Absorbance was read at 405 nm.

Analysis of platelet-associated IgG

Anti-GPIIb antibody (2 μg) in 50 μl of PBS, pH 7·2 was incubated with serial dilutions of IVIg, human serum albumin or ovalbumin in an equal volume of PBS at 20°C for 1 h. The mixtures were added to 2×10^5 CD1 mouse platelets in a total volume of 150 μl of PBS at 20°C for 1 h. Platelets were washed twice in PBS and stained with 1 μg/ml affinity-purified goat anti-rat IgG-FITC at 20°C for 1 h. Cells were then washed, resuspended in PBS and acquired on a FACScan flow cytometer.

Preparation of F(ab′)2

Human. IVIg was precipitated in 50% saturated ammonium sulphate, dialysed into 10 mmol/l Tris, 150 mmol/l NaCl, pH 8·3 and passed over a QAE Sephadex column. The IVIg was dialysed into 0·2 mol/l acetate buffer, pH 4·5 and incubated at a concentration of 10 mg/ml with 0·1 mg/ml pepsin for 20 h at 37°C. Digestion was stopped by the addition of 2 mol/l Tris base. The digested IVIg fractions were then separated by passage over a $90 \times 1{\cdot}5$ cm Sephadex G-150 column. The $F(ab')_2$ fractions of IVIg were further enriched by passage over Protein-G-Sepharose to remove any remaining Fc fragments or whole IgG and was then dialysed into 10 mmol/l PBS, pH 7·2, concentrated to 16·5 mg/ml and filter-sterilized before injection. Purity was determined to be > 96% by high performance liquid chromatography (HPLC) analysis.

Murine. Purified IgG (2 mg/ml) in 0·2 mol/l acetate buffer (pH 4·5) was incubated with 0·1 mg/ml pepsin for 20 h at 37°C. Digestion was stopped by addition of 2 mol/l Tris base, pH 8·0. Fc fragments and undigested IgG were removed by Protein-A-sepharose affinity chromatography. $F(ab')_2$ purity was >96% using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE).

RES blockade

Packed RBC (500 μl) from SCID mice were added to 500 μl of 80 μg/ml FITC in PBS and incubated at 20°C for 0·5 h with gentle rocking. The cells were then washed with PBS to remove free FITC. To opsonize the RBC, they were incubated for another 0·5 h with 5 μg/ml monoclonal anti-mouse Ly-76 antibody. Cells were then washed with PBS and resuspended in PBS before intravenous injection into SCID mice that were either untreated or pretreated with IVIg. Injection of 200 μl of labelled cells (100 μl packed RBC + 100 μl of PBS; representing 1/10 the blood volume of a mouse) resulted in ≈ 10% of the RBCs in the circulation being FITC-labelled. Blood samples taken at the times indicated were analysed using flow cytometry.

Statistical analysis

Data are expressed as mean ± SEM. The Mann–Whitney *U*-test (non-parametric) was used to determine the significance of observed differences between the various groups of mice; the significance level was set at $P < 0{\cdot}05$.

RESULTS

IVIg does not inhibit the binding of monoclonal anti-GPIIb to platelets

The rat anti-GPIIb antibody used to induce PIT was incubated with titrations of IVIg or control proteins (human albumin or ovalbumin) for 1 h at room temperature. The ability of the rat anti-GPIIb antibody to bind to platelets was not neutralized by previous exposure to IVIg or other proteins *in vitro* (Fig 1A). Mice were pretreated with 2 g/kg of IVIg (a standard human dose for treating ITP) or

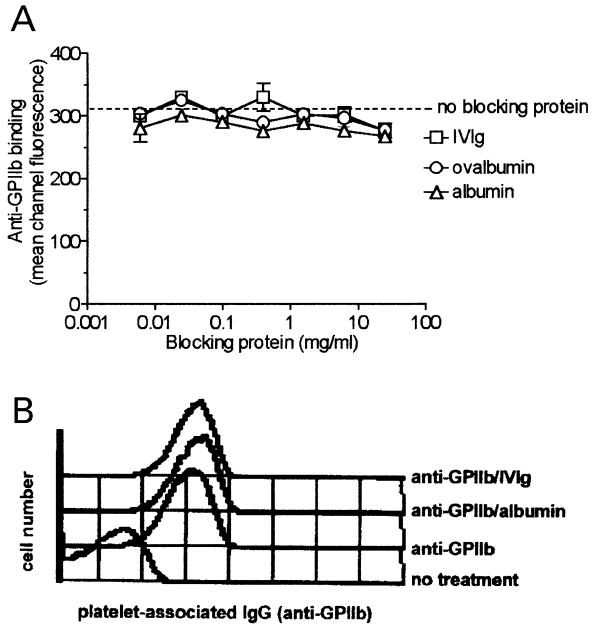

Fig 1. (A) IVIg does not inhibit platelet-specific antibody binding *in vitro*. Anti-GPIIb was pre-incubated with serial dilutions of IVIg, human albumin or ovalbumin for 1 h before incubation with CD1 mouse platelets, followed by anti-rat IgG-FITC. The *y*-axis represents anti-platelet antibody binding measured using flow cytometry, in arbitrary mean log fluorescence units. The *x*-axis represents the concentration of blocking protein used. The dotted line denotes the binding of platelet-specific antibody in the absence of blocking protein. Data are the mean ± SEM from three separate experiments. (B) IVIg does not affect the ability of the anti-GPIIb antibody to bind platelets *in vivo*. Mice were either untreated, or pretreated with albumin or IVIg 1 d before administration of anti-platelet antibody. Platelets were analysed for platelet-associated antibody (rat anti-mouse GPIIb) using flow cytometry. The *x*-axis represents antibody binding to platelets displayed as mean channel fluorescence in a log scale. Each histogram is of one representative mouse, $n = 5$ mice per group.

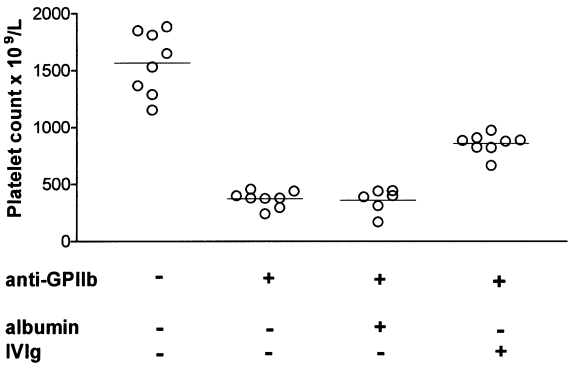

Fig 2. IVIg protects CD1 mice from PIT. The groups are as in Fig 1B. Each data point represents an individual mouse. Platelet counts were taken 1 d after PIT induction, and analysed using flow cytometry as described in *Materials and methods*. $P < 0{\cdot}001$ for column 4 versus column 2 and column 3.

human albumin, 1 d before injection of platelet-specific antibody. Platelet enumeration 1 d after injection of the anti-platelet antibody revealed that neither IVIg nor albumin prevented the antibody binding to platelets *in vivo* compared with platelets taken from mice receiving no pretreatment (Fig 1B).

IVIg protects mice from passive-immune thrombocytopenia (PIT)

Compared with unmanipulated control mice, CD1 mice treated with IVIg before induction of PIT were protected from thrombocytopenia (Fig 2, column 4). Control mice receiving no IVIg pretreatment (Fig 2, column 2), or human serum albumin (Fig 2, column 3), displayed significant thrombocytopenia. Mice injected with a different platelet-specific antibody (hamster anti-GPIIIa, which was also not neutralized by IVIg) were likewise found to undergo amelioration of thrombocytopenia by pretreatment with IVIg (data not shown). In the absence of any measurable ability of IVIg to inhibit anti-platelet antibody binding (Fig 1), IVIg retained its ability to protect against thrombocytopenia.

IVIg protects against PIT in the absence of anti-idiotypic–like interactions

To address whether anti-idiotypic antibodies in IVIg were required for the rapid amelioration of PIT, we first analysed IVIg for murine or rat IgG-reactive antibodies. It was found that there was significant reactivity of antibodies in human IVIg with the $F(ab')_2$ fragments of mouse IgG (Fig 3A) and the rat anti-GPIIb antibody used to induce PIT (Fig 3C). However, when these murine IgG or rat anti-GPIIb reactive antibodies were depleted from the IVIg (Fig 3A and C), the

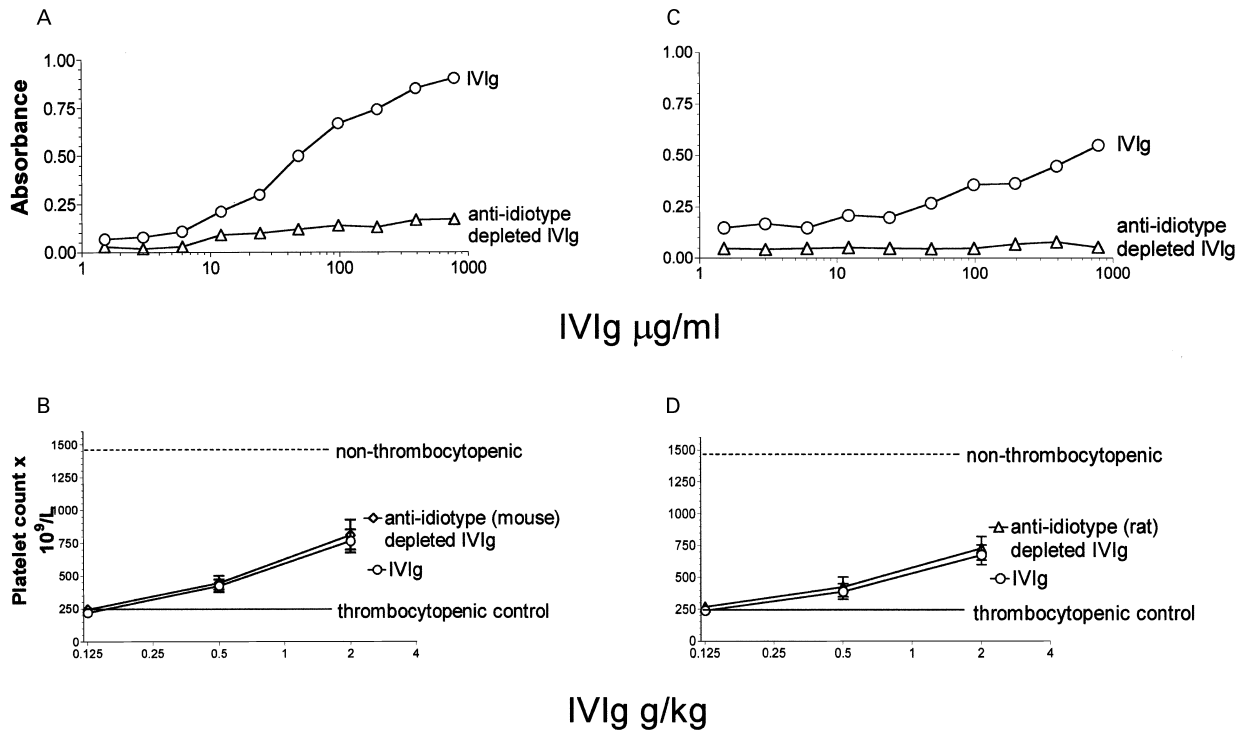

Fig 3. (A) Depletion of mouse IgG-reactive anti-idiotypic antibodies in IVIg. IVIg was depleted of anti-idiotypic antibodies and analysed for reactivity with $F(ab')_2$ fragments of murine IgG from CD1 mice by ELISA. The *x*-axis represents concentration of applied samples, the *y*-axis is absorbance measured at 405 nm. (B) IVIg depleted of murine IgG-reactive anti-idiotypic antibodies protects against PIT. CD1 mice were either pretreated with IVIg or anti-idiotype-depleted IVIg 24 h before the induction of PIT. The *x*-axis denotes the amount of IVIg used to treat the mice; the *y*-axis denotes platelet count. Unmanipulated mice (- - -), PIT mice (———), n = five mice for each point. (C.) Depletion of anti-idiotypic antibodies to rat anti-GPIIb in IVIg. IVIg was depleted of rat IgG-reactive antibodies followed by depletion of anti-GPIIb-reactive antibodies and analysed by ELISA against the anti-GPIIb antibody. (D) IVIg depleted of rat anti-GPIIb-reactive antibodies prevents PIT. Mice were treated as described in Fig 3B.

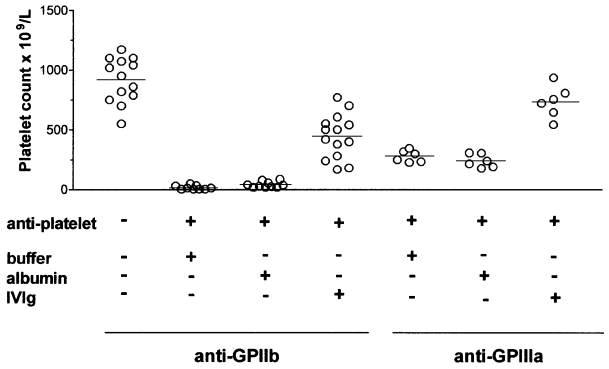

Fig 4. IVIg protects against thrombocytopenia in PIT mice devoid of endogenous immunoglobulin. SCID mice were treated as in Fig 2, using the anti-platelet antibody described below the *x*-axis. $P < 0{\cdot}0001$ for column 4 versus columns 2 or 3 and $P < 0{\cdot}01$ for column 7 versus columns 5 or 6.

depleted IVIg still protected PIT mice against thrombocytopenia in a similar fashion to non-depleted IVIg at all doses tested (Fig 3B and D). To ensure that IVIg was not reacting with any endogenous immunoglobulin, we used the PIT model using SCID mice, which lack endogenous immunoglobulin. IVIg was as successful at protecting PIT-SCID mice against thrombocytopenia induced by either anti-GPIIb (Fig 4, column 4) or anti-GPIIIa (Fig 4, column 7) antibody as it was at protecting outbred CD1 mice (Fig 2, column 4).

IVIg reverses pre-established PIT in SCID mice

To determine whether IVIg could ameliorate pre-existing thrombocytopenia in the PIT model, SCID mice were injected daily with anti-GPIIb antibody to induce stable thrombocytopenia. On d 3 platelet counts were assessed (Fig 5, columns 1 and 3) and mice were randomly assigned to receive either no therapeutic treatment (Fig 5, column 2) or to receive IVIg (Fig 5, column 4). On d 4, compared with the non-treated group, platelet counts revealed that IVIg treatment significantly reversed the thrombocytopenia.

IVIg blocks the murine RES

To assess whether IVIg exerted its protective effect by inhibiting RES function, opsonized red cell clearance studies

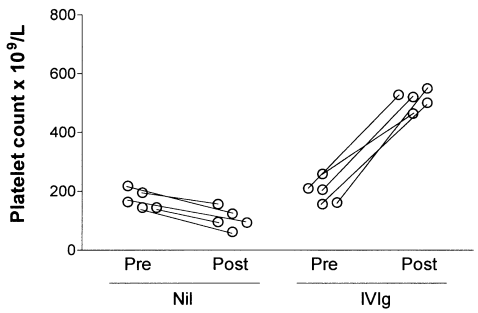

Fig 5. IVIg reverses thrombocytopenia in PIT-SCID mice. SCID mice were injected with 0·5 μg anti-GPIIb antibody daily for 4 d to induce PIT. On d 3, half the PIT-SCID mice were treated with 2 g/kg IVIg. 'Pre' represents d 3 bleed before IVIg treatment; 'Post' is d 4 bleed. $P < 0{\cdot}01$ for column 4 versus column 2.

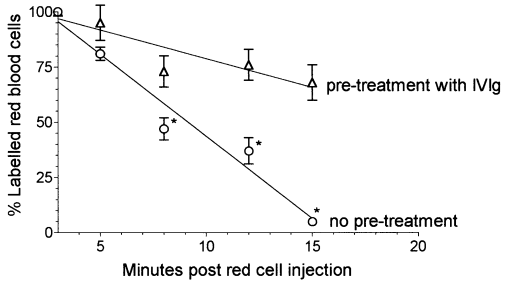

Fig 6. IVIg blocks the murine RES. SCID mice were either untreated or pretreated with 2 g/kg IVIg and then injected intravenously 24 h later with autologous FITC-labelled, monoclonal antibody-sensitized RBC. Whole blood samples were taken at the indicated times and analysed by flow cytometry for the percentage of labelled RBC; the percentage of labelled RBC at 3 min was considered to be 100%. The slope of the line was −6·3 for the control group and −2·1 for the IVIg treated group. *$P < 0{\cdot}01$ (n = five mice per data point). In independent experiments, the clearance rate of unsensitized, FITC-labelled RBC 24 h after injection was negligible (not shown).

were undertaken in IVIg-treated mice. SCID mice were untreated or pretreated with IVIg before intravenous injection with FITC-labelled, antibody-opsonized RBC (Fig 6). Blood samples were analysed for the percentage of FITC-labelled RBC remaining at the indicated times. The half-life of the FITC-labelled RBC in unmanipulated mice was 9 min, whereas the half-life of the labelled RBC in IVIg-treated mice was 22 min (as extrapolated from the curve). Thus, IVIg significantly blocked the ability of the RES to clear opsonized RBC. In contrast to intact IVIg, pretreatment of mice with an equimolar concentration of the $F(ab')_2$ fragment of IVIg did not protect from PIT (Fig 7). Without the ability to block the RES, IVIg was unable to protect mice from PIT.

DISCUSSION

Although the precise mechanism of action of IVIg in the treatment of autoimmune thrombocytopenia is not completely understood, several theories have been proposed; the

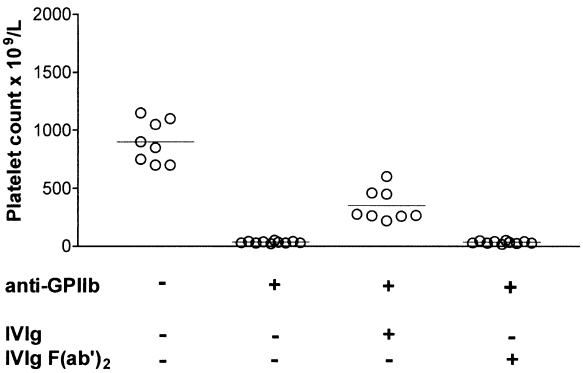

Fig 7. $F(ab')_2$ IVIg does not protect PIT mice from thrombocytopenia. PIT was induced in SCID mice which were either untreated, or pretreated with 1 g/kg whole IVIg or an equimolar quantity of $F(ab')_2$ fragment of IVIg (0·67 g/kg), as described in Fig 2.

two most prevalent include (i) Fc receptor-dependent RES blockade, and/or (ii) anti-idiotypic interactions provided by allogeneic IVIg (Rossi *et al*, 1989; Dwyer, 1992; Hurez *et al*, 1993). These murine studies were performed to simulate the immune thrombocytopenia observed in patients with antibody-dependent forms of thrombocytopenia, such as ITP, and to study the role of IVIg in the amelioration of the thrombocytopenia. The relevance of the PIT model to the understanding of the potential method of action of IVIg in immune forms of thrombocytopenia stems from the knowledge that human IVIg has been successfully used to treat murine and rat models of immune/autoimmune diseases (Saoudi *et al*, 1993; Nicoletti *et al*, 1998; Samuelsson *et al*, 2001), and IVIg has also been demonstrated to contain antibodies with anti-idiotypic activity which inhibit xenogeneic graft rejection and neutralize allo-specific antibodies (Gautreau *et al*, 1995; Schussler *et al*, 1998). Furthermore, $F(ab')_2$ fragments of human IVIg (which possess the idiotypes) have been shown to inhibit rat microglial cell phagocytic function (Stangel *et al*, 2000) and several investigators have in fact described and characterized several functional murine anti-human idiotype antibodies (Mehta & Badakere, 1996; Macias *et al*, 1999; McElveen *et al*, 2000), demonstrating the presence of cross-species variable-region reactive antibodies. A cross-species network-like connectivity has been proposed (Macias *et al*, 1999), and this may be caused by the high degree of conservation (92% amino acid sequence homology for β3) between the murine and human GPIIb–IIIa antigens (Cieutat *et al*, 1993). This high degree of protein homology has useful consequences to aid in the understanding of human immune thrombocytopenia in that platelet-specific antibodies isolated from individuals with HIV-thrombocytopenia can induce thrombocytopenia in mice (Nardi & Karpatkin, 2000).

We sought to determine if human IVIg-induced amelioration of PIT was associated with inhibition of RES function and/or whether the benefit was due to a required contribution of antibodies in the IVIg with anti-idiotype activity. We found that neither the anti-GPIIb or the anti-GPIIIa anti-platelet antibodies were neutralized by exposure to IVIg *in vitro* or *in vivo*, suggesting that IVIg does not contain neutralizing anti-idiotype antibodies reactive with these anti-platelet antibodies. To remove antibodies from IVIg with any possible 'non-neutralizing' anti-idiotypic-like effects in this model system, we depleted IVIg of murine or rat IgG-reactive components and observed that the resultant preparations retained full *in vivo* therapeutic activity.

IVIg reacts with endogenous murine immunoglobulin, and this could potentially contribute to the action of IVIg via formation of antibody–antibody dimers, formation of rheumatoid-factor-like conjugates, or formation of immunoglobulin aggregates which may affect complement–complement receptor-dependent effects; a SCID mouse model of PIT was therefore used. SCID mice are essentially devoid of endogenous immunoglobulin and therefore no reactions between human IVIg and any class of endogenous Ig are possible. IVIg was highly successful at protecting PIT-SCID mice against thrombocytopenia; although pretreatment with a higher dose of IVIg may have resulted in a more dramatic prevention of thrombocytopenia, this was not tested because of the large volume of IVIg required for injection. We conclude that no anti-idiotype-like interactions are required for the observed clinical effect of IVIg in the model used.

It was observed that SCID mice developed a more profound thrombocytopenia than normal mice induced by the monoclonal anti-platelet antibodies. We have not confirmed why this occurs. However, SCID mice are essentially free of B cells, T cells, serum immunoglobulins and are maintained under pathogen-free conditions; thus the RES is not actively functioning. The RES of SCID mice may therefore be more capable of removing opsonized platelets than normal mice.

In a recent report by Samuelsson *et al* (2001), it was found that the rapid protective effect of IVIg in murine ITP (using a passive-immune thrombocytopenic model) was Fc-dependent and complement independent, and that IVIg 'paralysed' phagocytic cells dependent upon the presence of the inhibitory FcγRIIb receptor on monocytes. Extrapolating the results from the Samuelsson *et al* (2001) study coupled with our work reported here, a role for anti-idiotypic interactions being necessary in IVIg-dependent rapid reversal of immune thrombocytopenia is not supported.

It should be noted that although immune thrombocytopenia developing from such diseases as ITP, systemic lupus erythematosus (SLE) and HIV–ITP, among others, are polyclonal antibody-mediated diseases; our work, along with that of others (Nieswandt *et al*, 2000; Samuelsson *et al*, 2001) simulates immune thrombocytopenia(s) through the use of monoclonal anti-platelet antibodies and may therefore not always reflect all of the attributes of these diseases.

To determine whether IVIg interacts with and inhibits FcR/RES function in this PIT mouse model, we performed antibody-sensitized RBC clearance experiments. Fehr *et al* (1982) examined FcR blockade in the action of IVIg in treating ITP; they showed that IVIg was able to significantly inhibit the clearance of anti-D-sensitized RBC in ITP patients. In the current studies, SCID mice pretreated with IVIg also had an impaired ability to clear antibody-sensitized autologous RBC. We conclude that administration of human IVIg to PIT mice caused inhibition of RES function. Although we cannot determine which particular FcR is affected by IVIg in the work presented here, i.e. FcγRI, II or III, work by Samuelsson *et al* (2001) and our group (data not shown) demonstrates that although FcγRII knockout mice develop antibody-induced thrombocytopenia, IVIg is ineffective at disease prevention. We have also demonstrated that IVIg can protect against passive immune thrombocytopenia in non-obese diabetic (NOD)/SCID mice (basal platelet count 660 ± 86·5; PIT 340 ± 34·2; PIT+IVIg 764 ± 63·3), which have a unique high-affinity macrophage FcγRI (Gavin *et al*, 1998) but nevertheless have difficulty in internalizing immune complexes compared with the FcγRI from BALB/c mice (Gavin *et al*, 1996). In addition, a study examining the effect of an anti-FcγRI antibody on a patient with refractory ITP resulted in downmodulation of monocyte FcγRI without any appreciable

change in the platelet count (Ericson *et al*, 1996). Taken together, these results do not support the notion that IVIg may mediate its inhibition of the RES by an FcγRI-dependent mechanism.

To further assess the relevance of RES blockade and/or anti-idiotype-dependent effects of IVIg action in our model, we tested the ability of $F(ab')_2$ fragments of IVIg to prevent PIT. $F(ab')_2$ fragments possess the anti-idiotypic regions of IVIg, but are unable to block the RES because of the lack of the Fc region. We found that, in contrast to intact IVIg, pretreatment of mice with IVIg $F(ab')_2$ did not protect them from PIT. This provides further evidence that in the PIT model human IVIg provides rapid amelioration of thrombocytopenia by a non-anti-idiotype-dependent mechanism; rather, IVIg inhibits the function of the RES.

We observed that IVIg can inhibit immune thrombocytopenia in SCID mice as efficiently as in normal mice. Thus, because SCID mice do not possess B cells, T cells, or B/T cell-dependent cytokines, we can rule out (*a priori*) a role for B and T cells in the effects seen here.

In short, although some or perhaps all of the long-term protective effects of IVIg may rely on functional anti-idiotypic antibodies, such as regulation of antibody production or other forms of immunomodulation, this study provides evidence that the acute protective effect of IVIg can occur independent of anti-idiotypic interactions.

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REFERENCES

Alves-Rosa, F., Stanganell, C., Cabrera, J., van Rooijen, N., Palermo, M.S. & Isturiz, M.A. (2000) Treatment with liposome-encapsulated clodronate as a new strategic approach in the management of immune thrombocytopenic purpura in a mouse model. *Blood*, **96**, 2834–2840.

Amital, H., Swissa, M., Bar-Dayan, Y., Buskila, D. & Shoenfeld, Y. (1996) New therapeutic avenues in autoimmunity. *Research in Immunology*, **147**, 361–376.

Barbano, G., Saleh, M.N., Mori, P.G., LoBuglio, A.F. & Shaw, D.R. (1989) Effect of intravenous gammaglobulin on circulating and platelet-bound antibody in immune thrombocytopenia. *Blood*, **73**, 662–665.

Berchtold, P., Dale, G.L., Tani, P. & McMillan, R. (1989) Inhibition of autoantibody binding to platelet glycoprotein IIb/IIIa by anti-idiotypic antibodies in intravenous gammaglobulin. *Blood*, **74**, 2414–2417.

Boughton, B.J., Cooke, R.M., Smith, N.A. & Simpson, A.W. (1994) Autoimmune thrombocytopenia: anti-glycoprotein IIb/IIIa auto antibodies are reduced after human anti-D immunoglobulin treatment. *Autoimmunity*, **18**, 141–144.

Bussel, J.B. (1989) Modulation of Fc receptor clearance and antiplatelet antibodies as a consequence of intravenous globulin infusion in patients with immune thrombocytopenic purpura. *Journal of Allergy and Clinical Immunology*, **84**, 566–577.

Bussel, J.B. & Haimi, J.S. (1988) Isolated thrombocytopenia in patients infected with HIV: treatment with intravenous immunoglobulin. *American Journal of Hematology*, **28**, 79–84.

Bussel, J.B., Kimberly, R.P., Inman, R.D., Schulman, I., Cunningham-Rundles, C., Cheung, N., Smithwick, E.M., O'Malley, J.Barandun, S. & Hilgartner, M.W. (1983) Intravenous gammaglobulin treatment of chronic idiopathic thrombocytopenic purpura. *Blood*, **62**, 480–486.

Cieutat, A.M., Rosa, J.P., Letourneur, F., Ponez, M. & Rifat, S. (1993) A comparative analysis of cDNA-derived sequences for rat and mouse β3 integrins (GPIIIA) with their human counterpart. *Biochemical and Biophysical Research Communications*, **193**, 771–778.

DeVita, S., Ferraccioli, G.F., DiPoi, E., Bartoli, E. & Bombardieri, S. (1996) High dose intravenous immunoglobulin therapy for rheumatic diseases: Clinical relevance and personal experience. *Clinical and Experimental Rheumatology*, **14**, 85–92.

Dietrich, G. & Kazatchkine, M.D. (1990) Normal immunoglobulin G (IgG) for therapeutic use (intravenous Ig) contain antiidiotypic specificities against an immunodominant, disease-associated, cross-reactive idiotype of human anti-thyroglobulin autoantibodies. *Journal of Clinical Investigation*, **85**, 620–625.

Dwyer, J.M. (1992) Manipulating the immune system with immune globulin. *New England Journal of Medicine*, **326**, 107–116.

Ericson, S.G., Coleman, K.D., Wardell, K., Baker, S., Fanger, M.W., Guyre, P.M. & Ely, P. (1996) Monoclonal antibody 197 (anti-FcγRI) infusion in a patient with immune thrombocytopenia purpura (ITP) results in down-modulation of FcγRI on circulating monocytes. *British Journal of Haematology*, **92**, 718–724.

Fazekas, F., Deisenhammer, F., Strasser-Fuchs, S., Nahler, G. & Mamoli, B. (1997) Randomised placebo-controlled trial of monthly intravenous immunoglobulin therapy in relapsing-remitting multiple sclerosis. Austrian Immunoglobulin in Multiple Sclerosis Study Group. *Lancet*, **349**, 589–593.

Fehr, J., Hofmann, V. & Kappeler, U. (1982) Transient reversal of thrombocytopenia in idiopathic thrombocytopenic purpura by high-dose intravenous immunoglobulin. *New England Journal of Medicine*, **306**, 1254–1258.

Fischer, P., Jendreyko, N., Hoffman, M., Lerch, H., Uttenreuther-Fischer, M.M., Chen, P.P. & Gaedicke, G. (1999) Platelet-reactive IgG antibodies cloned by phage display and panning with IVIG from three patients with autoimmune thrombocytopenia. *British Journal of Haematology*, **105**, 626–640.

Frame, J.N., Mulvey, K.P., Phares, J.C. & Anderson, M.J. (1989) Correction of severe heparin-associated thrombocytopenia with intravenous immunoglobulin. *Annals of Internal Medicine*, **111**, 946–947.

Gautreau, C., Kojima, T., Woimant, G., Cardoso, J., Devillier, P. & Houssin, D. (1995) Use of intravenous immunoglobulin to delay xenogeneic hyperacute rejection. An in vivo and in vitro evaluation. *Transplantation*, **60**, 903–907.

Gavin, A.L., Hamilton, J.A. & Hogarth, P.M. (1996) Extracellular mutations of non-obese diabetic mouse FcγRI modify surface expression and ligand binding. *Journal of Biological Chemistry*, **264**, 17091–17099.

Gavin, A.L., Tan, P.S. & Hogarth, P.M. (1998) Gain-of-function mutations in FcγRI of NOD mice: implications for the evolution of the Ig superfamily. *EMBO Journal*, **17**, 3850–3857.

Glotz, D., Haymann, J.P. & Sansonetti, N. (1993) Suppression of HLA-specific alloantibodies by high-dose intravenous immunoglobulins (IVIg). *Transplantation*, **56**, 335–337.

Godeau, B., Lesage, S., Divine, M., Wirquin, V., Farcet, J.P. & Bierling, P. (1993) Treatment of adult chronic autoimmune thrombocytopenic purpura with repeated high-dose intravenous immunoglobulin. *Blood*, **82**, 1415–1421.

Hurez, V., Kaveri, S.V. & Kazatchkine, M.D. (1993) Normal polyspecific immunoglobulin G (IVIg) in the treatment of autoimmune diseases. *Journal of Autoimmunity*, **6**, 675–681.

Imbach, P., Barandun, S., dApuzzo, V., Baumgartner, C., Hirt, A., Morell, A., Rossi, E., Schöni, M., Vest, M. & Wagner, H.P. (1981) High-dose intravenous immunoglobulin for idiopathic thrombocytopenic purpura in childhood. *Lancet*, **1**, 1228–1231.

Jahnke, L., Applebaum, S., Sherman, L.A., Greenberger, P.A. & Green, D. (1994) An evaluation of intravenous immunoglobulin in the treatment of human immunodeficiency virus-associated thrombocytopenia. *Transfusion*, **34**, 759–764.

Lesprit, P., Mouloud, F., Bierling, P., Schaeffer, A., Cesaro, P., Brun Buisson, C. & Godeau, B. (1996) Prolonged remission of SLE-associated polyradiculoneuropathy after a single course of intravenous immunoglobulin. *Scandinavian Journal of Rheumatology*, **25**, 177–179.

Macias, A., Arce, S., Leon, J., Mustelier, G., Bombino, G., Domarco, A., Perez, R. & Lage, A. (1999) Novel cross-reactive anti-idiotype antibodies with properties close to human intravenous immunoglobulin (IVIg). *Hybridoma*, **18**, 263–272.

McElveen, J.E., Furtado, P.B., Smith, S.J., Clark, M.R., Spendlove, I., Sewell, H.F. & Shakib, F. (2000) Characterisation of a mouse monoclonal anti-idiotype reactive with a V region sequence commonly used by human immunoglobulins. *Journal of Clinical Pathology: Molecular Pathology*, **53**, 77–82.

Mehta, Y.S. & Badakere, S.S. (1996) In-vitro inhibition of antiplatelet autoantibodies by intravenous immunoglobulins and Rh immunoglobulins. *Journal of Postgraduate Medicine*, **42**, 46–49.

Nardi, M. & Karpatkin, S. (2000) Antiidiotype antibody against platelet anti-GPIIIa contributes to the regulation of thrombocytopenia in HIV-1-ITP patients. *Journal of Experimental Medicine*, **191**, 2093–2100.

Nicoletti, A., Kaveri, S., Caligiuri, G., Bariéty, J. & Hansson, G.K. (1998) Intravenous treatment reduces atherosclerosis in apo E knockout mice. *Journal of Clinical Investigation*, **102**, 910–918.

Nieswandt, B., Bergmeier, W., Rackebrandt, K., Gessner, J.E. & Zirngibl, H. (2000) Identification of critical antigen-specific mechanisms in the development of immune thrombocytopenic purpura in mice. *Blood*, **96**, 2520–2527.

Nugent, D.J. (1992) Alloimmunization to platelet antigens. *Seminars in Hematology*, **29**, 83–88.

Reed, E., Hardy, M., Benvenisty, A., Lattes, C., Brensilver, J., McCabe, R., Reemstra, K., King, D.W. & Suciu-Foca, N. (1987) Effect of antiidiotypic antibodies to HLA on graft survival in renal-allograft recipients. *New England Journal of Medicine*, **316**, 1450–1455.

Ronda, N., Haury, M., Nobrega, A., Coutinho, A. & Kazatchkine, M.D. (1994) Selectivity of recognition of Variable (V) regions of autoantibodies by intravenous immunoglobulin (IVIg). *Clinical Immunology and Immunopathology*, **70**, 124–128.

Rossi, F., Dietrich, G. & Kazatchkine, M.D. (1989) Antiidiotypic suppression of autoantibodies with normal polyspecific immunoglobulins. *Research in Immunology*, **140**, 19–31.

Saoudi, A., Hurez, V., de Kozak, Y., Kuhn, J., Kaveri, S.V., Kazatchkine, M.D., Druet, P. & Bellon, B. (1993) Human immunoglobulin presss for intravenous use prevent experimental autoimmune uveoretinitis. *International Immunology*, **5**, 1559–1567.

Samuelsson, A., Towers, T.L., Ravetch, J.V. (2001) Anti-inflammatory activity of, IVIG mediated through the inhibitory Fc receptor. *Science*, **291**, 484–486.

Schussler, O., Genevaz, D., Latremouille, C., Goussev, N., Kaveri, S. & Glotz, D. (1998) Intravenous immunoglobulins for therapeutic use contain antiidiotypes against xenophile antibodies and prolong discordant graft survival. *Clinical Immunology and Immunopathology*, **86**, 183–191.

Stangel, M., Hartung, H.-P., Marx, P. & Gold, R. (1998) Intravenous immunoglobulin treatment of neurological autoimmune diseases. *Journal of the Neurological Sciences*, **153**, 203–214.

Stangel, M., Joly, E., Scolding, N.J. & Compston, D.A. (2000) Normal polyclonal immunoglobulins ('IVIg') inhibit microglial phagocytosis in vitro. *Journal of Neuroimmunology*, **106**, 137–144.

Warkentin, T.E. & Kelton, J.G. (1994) Interaction of heparin with platelets, including heparin-induced thrombocytopenia. In: *Low-Molecular-Weight Heparins in Prophylaxis and Therapy of Thromboembolic Diseases.* (ed. by H. Bounameaux,) pp. 75–127. Marcel-Dekker, NY.

Winder, A., Shoenfeld, Y., Hochman, R., Keren, G., Levy, Y. & Eldor, A. (1998) High-dose intravenous gamma-globulins for heparin-induced thrombocytopenia: a prompt response. *Journal of Clinical Immunology*, **18**, 330–334.

Winiarski, J., Kreuger, A., Ejderhamn, J. & Holm, G. (1983) High dose intravenous IgG reduces platelet associated immunoglobulins and complement in idiopathic thrombocytopenic purpura. *Scandinavian Journal of Haematology*, **31**, 342–348.