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. (Fab')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.

Mice were pretreated 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 μg 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 (8·24 ± 0·37 × 1012/l) or IVIg-treated PIT mice (8·42 ± 0·43 × 1012/l) compared with unmanipulated mice (8·74 ± 0·23 × 1012/l).

Platelet enumeration
Mouse blood (5 μl) 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 FACSscan 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-idiotypie-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 × 10⁵ 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 × 1·5 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 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 < 0·05.

**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

![Image](302x254 to 524x487)

**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.
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\(^\prime\))\(_2\) 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
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 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
two most prevalent include (i) Fc receptor-dependent RES blockade, and/or (ii) anti-idiotypic interactions provided by allogeneic IV Ig (Rossi et al. 1989; Dwyer, 1992; Hurez et al. 1993). These murine studies were performed to simulate the immune thrombocytopenia observed in patients with anti-body-dependent forms of thrombocytopenia, such as ITP, and to study the role of IV Ig in the amelioration of the thrombocytopenia. The relevance of the PT model to the understanding of the potential method of action of IV Ig in immune forms of thrombocytopenia stems from the knowledge that human IV Ig 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 IV Ig has also been demonstrated to contain antibodies with anti-idiotypic activity which inhibit xenogeneic graft rejection and neutralize allo-specific antibodies (Gautreau et al. 2001), and IV Ig 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, (Fab')_2 fragments of human IV Ig (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 (Cloutat 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 IV Ig-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 IV Ig with anti-idiotypic activity. We found that neither the anti-GPIIb nor the anti-GPIIIa anti-platelet antibodies were neutralized by exposure to IV Ig in vitro or in vivo, suggesting that IV Ig does not contain neutralizing anti-idiotypic antibodies reactive with these anti-platelet antibodies. To remove antibodies from IV Ig with any possible `non-neutralizing’ anti-idiotypic-like effects in this model system, we depleted IV Ig of murine or rat IgG-reactive components and observed that the resultant preparations retained full in vivo therapeutic activity.

IV Ig reacts with endogenous murine immunoglobulin, and this could potentially contribute to the action of IV Ig 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 PT was therefore used. SCID mice are essentially devoid of endogenous immunoglobulin and therefore no reactions between human IV Ig and any class of endogenous Ig are possible. IV Ig was highly successful at protecting PT-SCID mice against thrombocytopenia; although pretreatment with a higher dose of IV Ig may have resulted in a more dramatic prevention of thrombocytopenia, this was not tested because of the large volume of IV Ig required for injection. We conclude that no anti-idiotypic-like interactions are required for the observed clinical effect of IV Ig 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 IV Ig in murine ITP (using a passive-immune thrombocytopenic model) was Fc-dependent and complement independent, and that IV Ig ‘paralysed’ phagocytic cells dependent upon the presence of the inhibitory FcyRIIb 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 IV Ig-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 IV Ig interacts with and inhibits FcR/RES function in this PT mouse model, we performed antibody-sensitized RBC clearance experiments. Fehr et al. (1982) examined FcR blockade in the action of IV Ig in treating ITP; they showed that IV Ig was able to significantly inhibit the clearance of anti-ß-sensitized RBC in ITP patients. In the current studies, SCID mice pretreated with IV Ig also had an impaired ability to clear antibody-sensitized autologous RBC. We conclude that administration of human IV Ig to PIT mice caused inhibition of RES function. Although we cannot determine which particular FcR is affected by IV Ig 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, IV Ig is ineffective at disease prevention. We have also demonstrated that IV Ig can protect against passive immune thrombocytopenia in non-obese diabetic (NOD)/SCID mice (basal platelet count 660 ± 86·5: PIT 340 ± 34·2; PIT + IV Ig 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 FcγRII 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 FcγRII 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-idiotypic-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-idiotypic-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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