Autoantibodies purified from therapeutic preparations of intravenous immunoglobulins (IVIg) induce the formation of autoimmune complexes in normal human serum: a role in the *in vivo* mechanisms of action of IVIg?

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Abstract

Although intravenous immunoglobulins (IVIg) are widely used in the treatment of many autoimmune and inflammatory diseases, the mechanisms of action are still unclear in most cases. We have recently reported the presence of soluble autoimmune complexes (auto-IC) in human serum after the addition of a dose of IVIg similar to the one used in therapy. Here, we report the isolation and characterization of the responsible auto-IgG present in IVIg. The auto-IgG were purified by affinity chromatography on serum proteins immobilized on Sepharose. The purified auto-IgG constituted ~3% of the IgG present in IVIg and recognized a wide variety of structures in ELISA as well as many serum proteins on western blots. Auto-IC were formed in human serum following the addition of an amount of purified auto-IgG sufficient to over-saturate the auto-IgG inhibitory mechanisms known to be present in normal serum. These results indicate that most of the IgG present in IVIg are not involved in the formation of the soluble auto-IC, raising the possibility of preparing from IVIg a novel product which could be used for the treatment of the autoimmune diseases in which IC are thought to play an important role.

Introduction

Intravenous immunoglobulins (IVIg) are widely used in the supportive therapy of immunodeficient patients and in the treatment of a wide variety of chronic autoimmune and inflammatory diseases such as immune thrombocytopenia purpura (ITP) and systemic lupus erythematosus (SLE) (1,2). The mechanisms of action of IVIg in most of these diseases are still unclear and are attracting much interest due to increasing IVIg utilization and the possibility of IVIg shortages caused by the limitations in the available volume of human source plasma (3). The proposed mechanisms of action of IVIg in these diseases fall into two general categories: the inhibition of phagocytosis, and the immunomodulatory effects (1,2). The inhibition of phagocytosis has been observed in diseases such as ITP in which platelets opsonized by the pathogenic autoantibodies are no longer phagocytosed shortly after the infusion of IVIg (4). Several mechanisms of inhibition of phagocytosis by IVIg have been suggested and include the direct competitive blockade of the Fcγ receptors (FcγRs) by IgG complexes present in IVIg (5,6). It has been shown recently that IVIg could also inhibit phagocytosis through the inhibitory FcγRIIB (7). Further work by the same group revealed a role for IVIg-sensitive colony stimulating factor (CSF)-1-dependent macrophages in the up-regulation of FcγRIIB on effector macrophages in a murine model of arthritis (8). In the FcγR blockade mechanism, the infusing IVIg has to contain or induce the formation of IgG complexes, which can

interact with the FcγRs. There has been previous work on the characterization of IgG complexes present in IVIg. These studies have shown that, although IVIg was composed mainly of monomeric IgG (>95%), it also contained a small but significant proportion of IgG complexes, which could be involved in the *in vivo* inhibition of phagocytosis (9). These IgG complexes could be the result of idiotype (Id)–anti-Id interactions in the IVIg, caused by the blending of plasma donations from thousands of individuals (10,11). These complexes could alternatively be formed during the industrial fractionation process due to aggregation of IgG molecules (9). It is likely that the therapeutic IVIg component in autoimmune diseases represents only a small proportion of the injected material. This hypothesis is consistent with the very large doses of IVIg (e.g. 1–2 g/kg), which are injected for the short-term treatment of autoimmune diseases (12). Characterization of the active IVIg components is important since it could permit preparation of substitutes or further fractionation of the scarce IVIg preparations into different products for use in the treatment of diseases with different etiologies (e.g. immuno-deficiencies and ITP).

It is now well recognized that the immune system of healthy individuals constantly produces IgM and IgG antibodies that can react with self-structures. These autoantibodies are part of the natural antibody (NA) class, which constitutes a significant part of the serum antibodies (13,14). NA are often polyreactive and can react with various self and non-self structures. They are thought to represent the first line of defense against infectious agents not previously encountered. Binding of NA can result in phagocytosis of the infectious agent and lead to a protective immune response producing high affinity and monospecific IgG antibodies. For reasons that remain unclear, the mechanisms controlling the reactivity or production of autoimmune antibodies may get dysregulated, which can result in the development of various autoimmune and inflammatory diseases (13). Under normal circumstances, the reactivity and production of serum IgG autoantibodies are tightly regulated in order to avoid formation of immune complexes (IC), which could result in inflammation. It has been shown that the activity of autoreactive IgG was constantly inhibited by Id–anti-Id interactions with antibodies of the IgM isotype (15–18). This mechanism was revealed in experiments in which the autoreactivity of serum IgG was shown to significantly increase following purification due to the removal of the inhibitory IgM present in serum. IVIg preparations contain mostly IgG (>98%) with only trace amounts of IgM, IgA and other plasma proteins. Since IVIg are derived from a large pool of plasma donations, it is expected that these preparations contain the wide spectrum of antibodies present in the serum, including the natural and autoreactive antibodies (14).

The presence in human serum of anti-Id IgM able to inhibit the reactivity of autoimmune IgG raised the possibility that this phenomenon may be involved in the mechanism of action of IVIg in some diseases. The large therapeutic doses of IVIg (3) used in some diseases are known to result in a rapid 2–3-fold increase in plasma IgG levels. However, the injected IVIg only contain trace amounts of IgM, indicating that the anti-Id IgM–auto-IgG equilibrium in the patient plasma is expected to be significantly shifted toward autoreactivity following the rapid

infusion of IVIg. It is unclear if the amount of anti-Id IgM present in normal plasma is large enough to inhibit the auto-IgG present in the large doses of injected IVIg. We recently reported that human serum could inhibit the autoreactivity of added IVIg up to a dose corresponding to the addition of twice the endogenous amount of serum IgG. In addition we could detect the presence of soluble autoimmune complexes (auto-IC) in blends of human serum and IVIg (19). These results indicated that injection of large amounts of IVIg could oversaturate the normal mechanisms of control of autoreactive IgG present in human plasma (16–18) and result in formation of auto-IC. In the present study, we further characterized the autoantibodies present in IVIg that are involved in the formation of soluble auto-IC. Purification of the auto-IgG by affinity chromatography showed that they represent only a small proportion of IVIg and retain the auto-IC forming ability of IVIg after addition to normal human serum.

Methods

Purification of polyspecific autoantibodies from IVIg

Serum was depleted of IgG by passage over a column of protein G–Sepharose (Life Technologies, Burlington, Canada). The IgG-depleted serum was dialyzed against 137 mM NaCl in 10 mM phosphate buffer, pH 7.4 (PBS) and the proteins were coupled to CNBr-activated Sepharose (Amersham-Pharmacia, Baie d'Urfé, Canada) as described by the supplier. IVIg (Gamimmune N 10%, Bayer Corporation, Toronto, Canada) were incubated overnight at room temperature with the serum proteins–Sepharose. After washing with PBS, bound IgG were eluted with 100 mM glycine–HCl, pH 2.5. The protein-rich fractions were dialyzed against 40 mM glycine pH 4.5 and concentrated by centrifugation in Centricon filter units (Millipore, Nepean, Canada). Quantification of total protein was done with the Bradford assay (Bio-Rad, Mississauga, Canada) and the IgG content was determined by ELISA as previously described (20).

Antigen-specific ELISA

Microplate wells were coated with human ferritin or thyroglobulin (Calbiochem, La Jolla, CA), bovine casein (BDH Laboratories, Toronto, Canada) or double-strand DNA (dsDNA; Sigma, Oakville, Canada) diluted at 10 μg/ml in 100 mM carbonate buffer, pH 9.7. After overnight incubation at 4°C, wells were blocked with 5% bovine serum albumin (BSA) in 0.05% Tween 20–PBS for 1 h at 37°C. After washing with 0.85% NaCl, samples diluted in 1% BSA–0.05% Tween 20–PBS were distributed into triplicate wells and the plate incubated for 1 h at 37°C. After washing, bound antibodies were detected with peroxidase-labeled goat anti-human IgG (Fc specific; Jackson ImmunoResearch Laboratories, West Grove, PA) and revealed with the *o*-phenyldiamine (OPD) substrate (Abbott Laboratories; Abbott Park, IL). Optical densities were read at 490 nm with a reference wavelength of 630 nm. For the inhibition assays, a 2 h pre-incubation of samples with the tested inhibitor was performed before the ELISA.

Immunoblotting

IgG-depleted serum (10 µg of protein per strip) was subjected to SDS–PAGE (10% polyacrylamide) and proteins were transferred on PDVF membranes (Millipore). Membranes were blocked with 5% BSA in 100 mM NaCl, 10 mM Tris–HCl, pH 7.4 (TBS) for 60 min at room temperature. After washing with TBS, the membranes were incubated for 60 min with the different antibody preparations diluted in 5% BSA–TBS. Following washing, the PVDF membranes were incubated with HPR-conjugated mouse anti-human IgG (Fc specific; Southern Biotech, Birmingham, AL) for 60 min and finally revealed with ECL (Amersham-Pharmacia).

Isolation of IC

IC were isolated by precipitation with 2.5% PEG as previously described (21,22). Briefly, purified auto-IgG preparations (150 µg/ml) were mixed to a solution of 2-fold dilution of complete serum in PBS for a final volume of 500 µl. An equal volume of 5% PEG 6000 (Sigma) was added to diluted samples and incubated overnight at 4°C. Precipitates were then isolated by centrifugation (1500 *g*; 20 min, 4°C), washed twice with 2.5% PEG 6000, and dissolved by incubation for 30 minutes at 37°C in PBS containing 0.05% Tween 20, 10 mM EDTA and 0.01% thimerosal. The IgG content and reactivity of the PEG supernatants and precipitates were tested as above.

Results

Comparative polyreactivity of IVIg and human serum

It has been previously shown that purified IgG are more polyreactive than corresponding amounts of normal human serum (16–18). To determine if a similar difference was observed with IVIg prepared by large-scale industrial fractionation of human plasma, IVIg and human serum were tested by ELISA for polyreactivity with various molecules. The results showed the higher polyreactivity of IVIg (data not shown) and also revealed significant differences in the relative reactivity of IVIg and human serum with serum proteins compared to other molecules. Representative results are shown in Fig. 1 for four target molecules. The IgG dose response curves (0.5 µg/ml to 10 mg/ml) indicated that IVIg was 1000-fold and 250-fold more reactive than human serum for ferritin and thyroglobulin respectively. In contrast, this difference was only 60-fold for casein and 10-fold for DNA. This difference between human and other antigens indicated that the mechanisms controlling the polyreactivity of serum antibodies are much more effective against autoreactive antibodies than against polyreactive antibodies recognizing antigens not normally present in human plasma (e.g. casein and DNA). In the following experiments, we used purified human ferritin as a model for human plasma autoantigens.

Purification of autoantibodies reacting with human serum proteins

In preliminary experiments, we observed that the small proportion of the IVIg reacting with bovine casein (Fig. 1) could be purified from IVIg using affinity chromatography on columns of casein–Sepharose (data not shown). For more direct relevance, the proteins present in human IgG-depleted
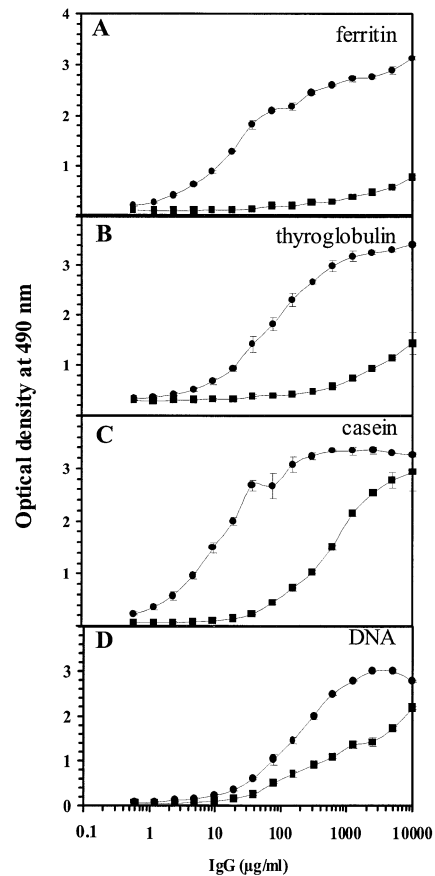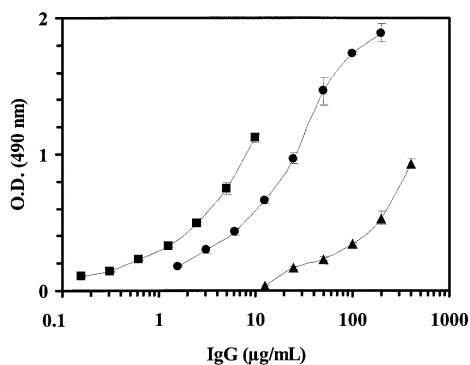

Fig. 1. Reactivity of IVIg and human serum for human ferritin (A), human thyroglobulin (B), bovine casein (C) and dsDNA (D). Reactivity of IgG content from IVIg (closed circles) and human serum (closed squares) was measured by antigen-specific ELISA. The SD of mean O.D. are indicated by bars.

serum were cross-linked in bulk to CNBr-activated Sepharose. The column was then used to purify the serum protein-reactive autoantibodies present in IVIg. Although the procedure should result in the purification of all the autoantibodies reacting with serum proteins present in IVIg, we used the ferritin ELISA to monitor the purification. Representative results are shown in Fig. 2. Affinity chromatography resulted in a very significant depletion of ferritin autoantibodies as shown by the shift of the flow-through curve to the right. Conversely, ferritin autoantibodies were enriched in the eluate fraction as shown by the shift to the left. The observation that the curves had nearly linear dose–response relationships at low O.D. values (<0.4) allowed the calculation of the purification factor and yield. Representative results of a purification experiment are listed in Table 1 and showed that the chromatography depleted ~92% of the anti-ferritin activity of starting IVIg. The glycine–HCl eluate contained <3% of the starting IgG but 78% of the IVIg anti-ferritin activity. The calculated purification factor (27×) is only indicative since it is likely higher due to the fact that the IgG present in the eluate are expected to react with several plasma proteins and not only with ferritin. Thus, the autoantibodies reacting with normal serum proteins can be

significantly enriched by affinity chromatography of the IVIg preparation.

Inhibition of purified autoantibodies reactivity by human serum

We recently reported that human serum can inhibit the ferritin reactivity of IVIg (19). To determine if purified autoantibodies were similarly inhibited, we determined the ability of a fixed volume of human serum containing 25 µg/ml of IgG to inhibit the reactivity of increasing concentrations (0.1 to 25 µg/ml) of purified autoantibodies. The observation that the serum exhibited a very low anti-ferritin reactivity at 25 µg/ml of IgG (Fig. 1) permitted to focus on the reactivity of the added purified autoantibodies. Results are shown in Fig. 3. In panel (A), the ELISA results indicated that the addition of a fixed amount of serum to increasing doses of purified autoantibodies resulted in a significant reduction of the anti-ferritin reactivity at all doses tested. The shapes of the curves suggested that the inhibition was more important at lower doses of purified autoantibodies. Indeed, the results, when expressed as a percentage of inhibition by serum (Fig. 3B), showed that the inhibition was high (>85%) up to a dose of ~3 µg/ml of purified autoantibodies. The inhibition then gradually decreased to reach 20% at the maximal dose of purified autoantibodies tested (25 µg/ml). These results confirmed the previously reported ability of human serum to inhibit the anti-ferritin reactivity of IVIg (19). The inhibition curves obtained with both antibody preparations were very similar in shape. However the amounts of added IgG that can be inhibited by the same volume of serum were significantly different. Indeed a volume of serum containing 25 µg/ml of endogenous IgG could inhibit up to 50 µg/ml of added IVIg (19) but only 3 µg/ml of purified auto-IgG (Fig. 3). It is noteworthy that this differential ratio (16-fold) is similar to the observed auto-IgG purification factor (27-fold, Table 1), indicating that the removal of non-autoreactive IgG which represent the majority of IVIg (97%, Table 1), did not alter the equilibrium existing in serum between auto-IgG and inhibitory IgM.

Diversity of serum proteins recognized by autoantibodies

We have used human ferritin as a model auto-Ag for the ELISA detection of auto-IgG. However IVIg and purified auto-IgG are expected to bind to several serum protein antigens. To evaluate their diversity, we performed western blot experiments with serum proteins. Before electrophoresis, the serum was first depleted of IgG by chromatography on protein–Sepharose to avoid the detection of anti-Id IgG. The serum protein blots were probed with the various fractions from IVIg affinity chromatography, and the binding of antibodies was
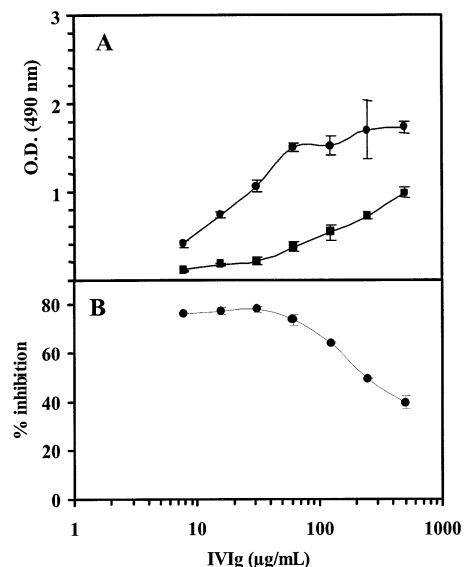
Fig. 3. Autoreactivity of purified auto-IgG for human ferritin in presence of normal serum. (A) ELISA reactivity of auto-IgG in absence (closed circles) and in presence of serum (25 µg/ml of IgG; closed squares). (B) Inhibition of ferritin reactivity of auto-IgG in presence of human serum. The SD of mean O.D. are indicated by bars.

Fig. 2. ELISA reactivity of serum proteins–Sepharose fractions for ferritin. IVIg (closed circles), eluate (closed squares) and flow-through (closed triangles) fractions were serially diluted and tested in the ferritin-specific ELISA. The SD of mean O.D. are indicated by bars.
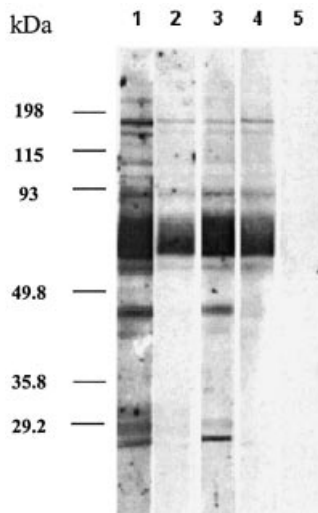
Table 1. Purification of autoreactive IgG

Fractions	Total IgG (mg)	Total reactivity (U)	Specific activity (U/mg)	Purification (X)	Yield (%)
Starting IVIg	14.24	172 606	12 121	–	–
Flow through	11.30	13 831	1224	0.10	8.0
Eluate	0.42	136 197	327 869	27.05	78.9

IVIg were chromatographed on a Sepharose column coupled to human serum proteins. The flow through and glycine pH 2.5 eluate fractions were recovered and analyzed for their IgG content. Quantification of reactivity was estimated using ELISA and human ferritin as coating antigen. One unit of reactivity represents the amount of IgG needed to obtain an O.D. of 0.4.

Fig. 4. Diversity of serum proteins recognized by autoantibodies. IgG-depleted serum proteins were separated by SDS–PAGE and tested in western blot experiments with total IVIg (lane 1; 6.25 µg/ml of IgG), flow-through (lane 2; 6.25 µg/ml of IgG), SP–Sepharose eluate (lane 3; 0.625 µg/ml of IgG) and normal serum (lane 4; 6.25 µg/ml of IgG). Negative control (without IgG; lane 5) shows the absence of reactivity of the anti-human IgG–HRP conjugate. Molecular weights are indicated on the left.

detected with an anti-human IgG conjugate. The results are shown in Fig. 4. The results obtained with IVIg (lane 1) showed the presence of major bands of diverse molecular weights along with a diffuse staining of the blot, suggesting the presence of many minor autoantigens. Experiments with F(ab)2 fragments of IVIg produced similar bands after detection with an anti-F(ab)2-conjugate (data not shown), indicating that the observed bands were not dependent of interactions with the Fc region of IgG. The intense band at 65 kDa observed with all tested antibody preparations corresponds to the molecular weight of albumin and is most likely due to non-specific binding because it was also observed with auto-IgG-depleted IVIg (lane 2). The affinity chromatography was effective in depleting the autoantibodies as seen by the absence of most bands and the clearer background (lane 2). The purified autoantibodies reacted with several proteins with a pattern similar to the starting IVIg (lane 3). Finally, as expected from the ELISA results, normal human serum reacted only weakly with some of the separated proteins (lane 4), yielding a pattern similar to the flow-through fraction of the affinity column. This analysis showed that the autoantibodies present in IVIg and in the purified fraction recognized multiple proteins in human serum.

*Soluble IC in serum in presence of exogenous auto-IgG*

We previously reported the presence in human serum of soluble IC following addition of therapeutic amounts of IVIg. These IC were highly reactive with human ferritin, suggesting a participation of auto-IgG (19). We thus wanted to determine if proportional amounts of purified auto-IgG could similarly induce the formation of auto-IC. The inhibition of purified auto-IgG showed that diluted serum containing 25 µg/ml IgG

could inhibit the ferritin reactivity of 3 µg/ml of purified auto-IgG (Fig. 3). This result indicated that the formation of auto-IC could be optimal at this ratio since the addition of more auto-IgG resulted in increased ferritin reactivity of the blend. The auto-IC present in blends of serum and auto-IgG were isolated by PEG precipitation and analyzed for total and ferritin reactive IgG. In one series of experiments, the IgG were detected with an IgG-specific conjugate. In another, the purified auto-IgG were first labeled with biotin and detected with a streptavidin conjugate allowing the detection of the presence of exogenous auto-IgG in the formed auto-IC. Representative results are shown in Fig. 5. Assay of total IgG (panel A) showed that the IC present in the serum–auto-IgG blend contained significantly more IgG than the sum of those present in the two starting fractions (25 µg versus a total of 11 µg for the two fractions). The IC were also tested for ferritin reactivity using a quantitative assay which allowed the calculation of the percentage of reactivity recovered in the IC fraction. The results (panel B) indicated that only a minor portion of the anti-ferritin reactivity were recovered in the IC formed in the starting serum and auto-IgG fractions. The result with the serum–auto-IgG blend was unexpected since the recovery in the IC was ~200%. This result indicated that the ferritin reactivity of the auto-IC is partly inhibited in the presence of serum components since their isolation resulted in a >100% yield. The detection of only the added auto-IgG using the biotin–streptavidin system produced similar results with an increased amount of auto-IgG present in the IC formed in the blend (panel C) and an even higher increase (350%) of ferritin reactivity in the isolated IC (panel D). These results indicated that the auto-IgG added to the serum participated in the formation of the detected auto-IC.

Discussion

This work has permitted a better characterization of the antibodies present in IVIg which are responsible for the formation of auto-IC when therapeutic amounts of IVIg are mixed with normal human serum. The results showed that those antibodies represent a minor fraction of IVIg (<3%) which are characterized by significant autoreactivity with human proteins. Those antibodies could be easily purified (27-fold) by chromatography on serum proteins immobilized on a solid support, and recognized multiple serum proteins on western blots. Finally, the purified auto-IgG could form auto-IC in normal serum as efficiently as IVIg but at much lower doses proportional to the purification factor. Altogether these results support our previous hypothesis (19) that the control of autoimmune IgG may be impaired in the plasma of IVIg-treated patients, and that the resulting auto-IC could be involved in the therapeutic effects of IVIg particularly in the diseases such as ITP, where IC are thought to play an important role in the early mechanisms of IVIg action after infusion. It remains to be seen if the purified auto-IgG will exhibit the same biological activity as IVIg in *in vitro* and *in vivo* assays; this work is underway in our laboratory.

An involvement of auto-IgG in the mechanisms of action of IVIg has not been specifically studied so far. A possible reason could be the seldom-considered quantitative effect of therapeutic injection of IVIg on the plasma IgG levels. Indeed
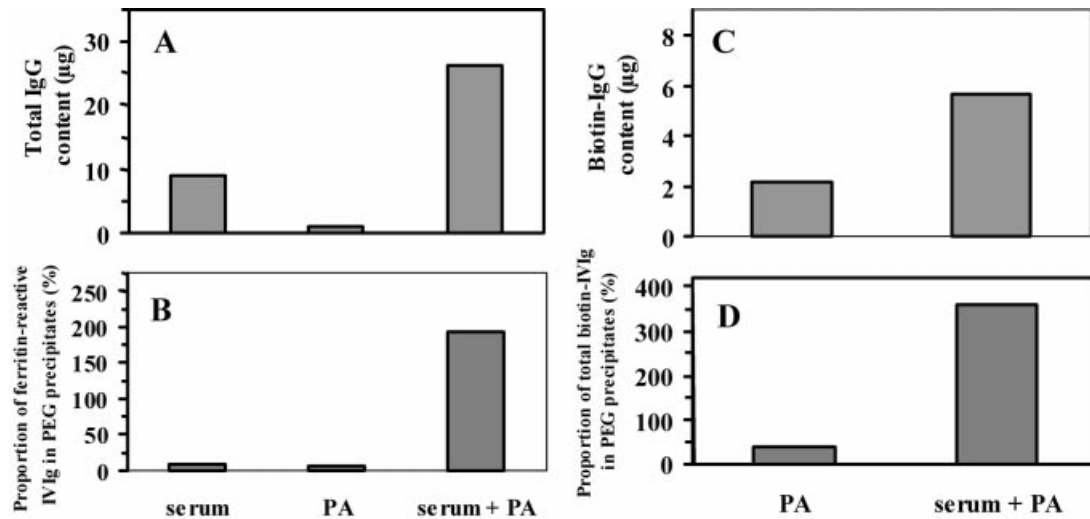
Fig. 5. Analysis by ELISA of the IgG content and ferritin reactivity of PEG-precipitated preparations. (A) Amount of IgG in PEG precipitates. Quantification of IgG was done by ELISA. (B) Proportion of ferritin-reactive IVIg in the PEG precipitates. Ferritin reactivity of serial dilutions of the PEG-precipitated fractions was measured by ELISA. One unit of ferritin reactivity was defined as the volume of each fraction, which produced an O.D. of 0.4. (C) Amount of biotin–IgG in PEG precipitates. Biotin–IgG was quantified by ELISA using streptavidin–HRP conjugate and the starting biotin–IVIg solution as standard. (D) Proportion of ferritin-reactive biotin–IVIg in the PEG precipitates. Ferritin reactivity of serial dilutions of the PEG-precipitated fractions was measured by ELISA and detected using a streptavidin–HRP conjugate. One unit of ferritin reactivity was defined as the volume of each fraction, which produced an O.D. of 0.4. PA: purified auto-IgG.

doses of IVIg now routinely used such as 1–2 g/kg of body weight, can increase plasma IgG levels by a factor of 2–3× (3). Such a sudden increase of plasma IgG can obviously perturb the existing equilibriums. The previously described (15–18) control of auto-IgG by anti-Id IgM could be one of those equilibriums. Our results support this possibility since ferritin-reactive auto-IgG present in IVIg and purified auto-IgG were inhibited by serum up to a threshold similar, in the case of IVIg, to the effect of IVIg infusion on plasma IgG levels [i.e. 50 µg/ml of IVIg added to a volume of serum containing 25 µg/ml of IgG (19)]. Another point to consider is related to the fact that IVIg are injected directly in the plasma and can thus rapidly impact the mechanism of control of auto-IgG. It remains to be seen if the injection of IgG by other routes such as subcutaneous, could affect the therapeutic effects if the auto-IgG can interact with other self-antigens present at injection sites. Characterization of the polyreactivity of purified auto-IgG is under study in our group and should permit a determination of the importance of the intravenous injection.

A potentially important finding in this work is the fact that the auto-IgG purification did not affect the results previously obtained with IVIg (19) except for the doses of IgG necessary to observe the effects. Indeed the saturation curve for serum inhibition with purified auto-IgG was shifted to lower IgG doses compared to the one observed with IVIg [3 µg/ml compared to ~50 µg/ml; Fig. 3 and (19)]. The auto-IC were also formed in human serum in the presence of much less auto-IgG compared to IVIg (150 µg/ml versus 6000 µg/ml). These differences are similar to the observed auto-IgG purification factor (27×, Table 1) and indicate that the vast majority (>97%) of IgG present in IVIg, which are non-reactive with serum proteins, do not appear to participate in the IgM

saturation effect. This result may be considered as un-expected in view of the fact that IVIg are likely to also contain auto-IgG reacting with auto-Ags not present in serum (e.g. tissues or intracellular components) and consequently not present in the purified auto-IgG fraction. Since the serum anti-Id IgM should inhibit all auto-IgG irrespectively of the localization of the auto-Ag, one could have expected that the use of purified serum proteins–specific auto-IgG instead of IVIg in the above experiments could have necessitated the addition of a disproportionately high amount of purified auto-IgG. More work will be necessary to better understand this phenomenon but two explanations can be put forward. The anti-Id IgM interaction with auto-IgG reacting with serum proteins could be highly specific and not influenced by the presence of auto-IgG reacting with other auto-Ags not present in human serum. Another possibility is that the purified auto-IgG isolated by chromatography on immobilized serum proteins are highly polyreactive with human auto-Ags and thus represent the bulk of the auto-IgG present in IVIg. Further characterization of the polyreactivity of the purified auto-IgG with various human tissues will permit a clarification of this point. But the fact that the purified anti-serum proteins auto-IgG reacted with several unrelated structures (data not shown) supports the latter hypothesis.

The chromatography of IVIg on immobilized serum proteins yielded an eluted fraction enriched in autoantibodies, which recognized in western blot experiments a diversity of serum proteins similar to the starting IVIg. The observation that the purified auto-IgG represented only 3% of the starting IVIg may be seen as unexpected in view of previous findings indicating that auto-IgG represented a significant part of serum IgG (13). However it is likely that the purification procedure used

(extensive washing of the column and pH 2.5 elution) has resulted in the preferential isolation of the high affinity auto-IgG. Further characterization of the purified auto-IgG will permit a clarification of this point. The observation that purified autoantibodies are polyspecific (Fig. 4) is significant in terms of the efficiency of the possible formation of IC after intravenous injection of IVIg. It suggests that the autoantibodies could rapidly form auto-IC because the recognized antigens are heterogeneous. The high diversity of recognized plasma proteins also suggests that the formation of IC is less likely to result in immune depletion of certain plasma proteins in IVIg-treated patients. The purification results raise the interesting possibility of further fractionating the current IVIg preparations into two products. The flow-through of the column, which contains more than 90% of the starting IgG, is likely to represent IgG reacting with non-self structures and could be used to support immunodeficient patients. In this regard, the monthly infusion of IVIg in these patients is known to cause mild but significant adverse side effects in the first day following injection (3). A rare but serious adverse effect of IVIg injection is anemia resulting from the immune destruction of the patient red blood cells, generally caused by the uptake of circulating IC by the complement receptor present on red blood cells (23). It is tempting to speculate that the auto-IC formation reported here may play a role in this adverse side effect and that these patients may have a reduced ability to inhibit a portion of the infused auto-IgG, resulting in the formation of a higher amount of auto-IC. It remains to be seen if removal of autoantibodies in IVIg could reduce the severity of these side effects. The second fraction obtained by chromatography is the auto-IgG eluate, which represents <3% of the starting IgG. This fraction could be useful in the treatment of the diseases in which IVIg have immunomodulatory roles or inhibit phagocytosis. Further characterization of the biological activity of the purified auto-IgG fraction is needed to evaluate the therapeutic potential of this preparation in various inflammatory and autoimmune diseases.

The analysis of the auto-IC (Fig. 5) formed in human serum following the addition of auto-IgG indicated that the amount present in the blend was significantly higher than the sum of auto-IC present in the two separate fractions as evaluated by assay of either total IgG or anti-ferritin activity. Also, the added auto-IgG were present in the auto-IC as evaluated by the use of biotin-labeled auto-IgG. These results are qualitatively similar to those previously reported for IVIg (19). As expected from the increased specific activity (Table 1) and the change in the serum inhibition curve (Fig. 3), the amount of added auto-IgG necessary to obtain the auto-IC was much lower [6 μg/ml for auto-IgG (Fig. 3) compared to 50 μg/ml for IVIg (19)]. In additional experiments, we observed that the amount of formed auto-IC was much lower when the auto-IgG were added to IgG-depleted serum (data not shown) and that the auto-IC contained IgG originating from the serum. These results suggest the existence of a dynamic equilibrium between the endogenous and the added auto-IgG. Depletion of endogenous auto-IgG thus appears to increase the inhibitory capacity of serum and result in lower formation of auto-IC. On the other hand, the increase in the amount of endogenous auto-IgG present in auto-IC could be an indication that the added auto-IgG can replace the endogenous

auto-IgG interacting with the inhibitory anti-Id IgM in starting serum making the endogenous auto-IgG available for auto-IC formation. Additional work will be necessary to better characterize this equilibrium. The use in these studies of serum obtained from patients suffering from immunodeficiencies such as X-linked agammaglobulinemia (XLA), could permit further differentiation of the role of endogenous and exogenous immunoglobulins since XLA patients are known to have greatly reduced amounts of serum antibodies including auto-IgG (24).

The study of the mechanisms of action of IVIg is currently attracting much interest. A significant advance has been the recent report that the inhibition of phagocytosis by IVIg observed in an *in vivo* mouse model of arthritis was dependent on a subset of macrophages which appear to respond to the sudden increase in plasma IgG by inducing the expression of the inhibitory FcγRIIB on phagocytes (8). In this model, the inhibition of antibody-induced inflammation does not appear to be dependent on the formation of IC but rather to the large dose of injected IgG. Obviously, such a mechanism would indicate that the approaches based on the formation of competitive IC as reported here for auto-IgG would not have the desired clinical effect. However, it is now well demonstrated that injection of relatively low doses of anti-D can produce in ITP patients a rapid rise in platelet counts as efficiently as IVIg (4). This increase has recently been reproduced in the mouse ITP model by injection of anti-mouse red blood cells mAb (25). Additional work will be required to determine the relative importance of both mechanisms. One possibility is that the formation of competitive IC by anti-red blood cells antibodies and possibly by auto-IgG could represent the early mechanism of IVIg action in diseases such as ITP which would result in very rapid (within hours) inhibition of phagocytosis. The indirect control of phagocytosis by 'sensor' macrophages (8) may take more time to develop and could thus be responsible for the sustained inhibition of phagocytosis in the following days.

In conclusion, our work indicates that the auto-IC present in blends of human serum and therapeutic amounts of IVIg are induced by IVIg-derived auto-IgG which appear to over-saturate the capacity of serum anti-Id IgM to inhibit the auto-IgG. The convenient purification of the active auto-IgG raises the possibility of preparing from current IVIg preparations, two products for use in immunodeficient patients (column flow-through) and in autoimmune diseases such as ITP (column eluate). This additional fractionation step would have significant positive impacts on the availability and price of the current IVIg product. We are currently in the process of testing the importance of complement in the formation of auto-IC and the biological activity of the purified auto-IgG using *in vitro* assays and *in vivo* mouse models. This work will permit establishment of the potential of the purified auto-IgG to be developed into a novel plasma-derived therapeutic product.

Abbreviations

auto-Ag	autoantigen
auto-IC	autoimmune complex
FcγR	Fcγ receptor
IC	immune complex
Id	idiotype
IVIg	intravenous immunoglobulin
ITP	immune thrombocytopenia
NA	natural antibody

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