High Doses of Intravenous Ig Inhibit In Vitro Uptake of C4 Fragments Onto Sensitized Erythrocytes

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We have recently reported that intravenous Ig (IVlg) inhibits uptake of activated C3 fragments onto antibody-sensitized red blood cells (RBCs). To elucidate the mechanism by which IVlg exerts its effect on the complement system, we examined the possible interference with the C4 step of the classical complement cascade. We examined the capacity of autologous serum containing high concentrations of human IVlg to deposit C4 fragments onto model targets (guinea pig and/or human erythrocytes sensitized with rabbit anti-guinea pig/human erythrocyte IgG antibody). C4 binding was quantified with radiolabeled anti-C4. Guinea pig serum with added IVlg suppressed C4 uptake onto IgG-sensitized guinea pig erythrocytes at all time points (0, 5, 15, and 30 minutes). Using sera of guinea pigs treated with increasing doses of IVlg, this effect was shown to be dose-responsive. Serum from a patient treated with IVlg showed reduced C4 uptake onto sensitized homologous RBCs. In comparison with the serum from the same patient before IVlg therapy was administered, levels were decreased almost to background. C4 functional titers in those two samples were not different. C3 uptake was studied in parallel with C4 to compare the degree of inhibition using sera with increasing doses of IVlg in both the human and guinea pig system. C3 and C4 inhibition curves completely overlapped. Our findings suggest that IVlg is an effective inhibitor of deposition of early complement activation products (C4b, C3b) onto target surfaces and may indicate interference of IVlg with multiple sites of complement activation.

This is a US government work. There are no restrictions on its use.

MATERIALS AND METHODS

Animals. Female guinea pigs weighing 300 to 400 g (National Institutes of Health “multipurpose”–non-inbred) were obtained from the National Cancer Institute, Frederick, MD.

Antibodies. Rabbit IgM anti-guinea pig erythrocyte antibody was fractioned from the serum of a rabbit immunized IV with guinea pig red blood cells (RBCs). A 42.7% ammonium sulfate precipitate from the immune serum was redissolved in phosphate-buffered isotonic saline (PBS, pH 7.4) and chromatographed on a Sephadex G-200 (Pharmacia Fine Chemicals, Freehold, NJ) column. IgM-containing fractions were pooled and then passed over a concanavalin A (con A) agarose affinity column. IgM contained in the 0.5 mol/L glucose eluate from the con-A agarose affinity column was concentrated and further subjected to ultracentrifugation on a 10% to 40% sucrose gradient. Fractions containing IgM were pooled and stored directly in the sucrose solution at 4°C.

Rabbit polyclonal IgG anti-guinea pig erythrocyte antibody, anti-guinea pig C3, and anti-guinea pig C4 were purified from the sera of rabbits immunized with guinea pig erythrocytes, guinea pig C3, and guinea pig C4, respectively. IgG fractionation involved sequential ammonium sulfate precipitation, Sephacel G-200 sieving chromatography, and diethyloamino ethanol (DEAE) anion-exchange chromatography. Rabbit antihuman RBC IgG was purchased from Cappel (West Chester, PA). Antihuman C4 IgG polyclonal antibody was purified from a burro immunized with purified human C4. Antihuman C3 IgG polyclonal antibody was obtained by immunizing a goat with purified human C3. IgG fractionation was performed by octanoic acid precipitation.

Radiolabeling. Radiiodination of rabbit anti-guinea pig C3, rabbit anti-guinea pig C4, burro antihuman C4, and goat antihuman C3 was accomplished with iodobeads following the directions supplied by the manufacturer. Respective polyclonal IgGs, 300 μg, were incubated with 1 mCi of 125I and three iodobeads for 30 minutes at room temperature. Free iodine was separated from protein-bound isotope by passing the labeled material through a packed PD10 (Sephadex G-25M) column (Pharmacia Fine Chemicals, Freehold, NJ) in isotonic PBS, pH 7.4.

Complement components. Guinea pig fourth component of complement was purchased from Cordis Laboratories (Miami,
FL). Guinea pig C3 was purified to homogeneity by a modification of the procedure of Hammer et al.\textsuperscript{12} designed for human C3. Human C3 and human C4 were also purified by the method of Hammer et al.\textsuperscript{12}

**Complement titrations.** Hemolytic complement titrations of human C4 were performed using standard published procedures.\textsuperscript{13}

**Other reagents.** Human serum Ig for IV use in 10% maltose, pH 4.25 (Gammimune), and human serum albumin 25% solution for IV administration were purchased from Cutter Biologicals (Berkeley, CA). Maltose hydrate grade I was obtained from Sigma Chemical Co (St Louis, MO). Sodium (\textsuperscript{125}I) iodide was purchased from Amersham (Arlington Heights, IL). Iodobeads were purchased from Pierce Chemical Co (Rockford, IL).

**Buffers.** Isotonic Veronal-buffered saline containing 0.1% gelatin, 0.15 mmol/L Ca\textsuperscript{2+}, and 1 mmol/L Mg\textsuperscript{2+} (GVBS\textsuperscript{14}), isotonic Veronal-buffered saline containing 0.1% gelatin, and 10 mmol/L EDTA buffer were prepared as previously described.\textsuperscript{15}

**In vitro C4 uptake studies.** To obtain blood, a guinea pig was anesthetized by a subcutaneous injection of a cocktail containing 0.1 mL of Rompun (Xylazine; Mobay Corp, Shawnee, KS) and 0.5 mL of Vetalar (Ketamine HCl; Parke-Davis Co, Morris Plains, NJ). Cardiac puncture was then performed. RBCs were separated from EDTA plasma, washed, and standardized. Three tenths of 1 mL of guinea pig RBC suspension at 2.7 x 10\textsuperscript{9} cells/mL was incubated with 0.3 mL of diluted rabbit IgG or IgM anti-guinea pig erythrocytes antibody, or 0.3 mL of buffer for 30 minutes at 37°C. After two washes in GVBS\textsuperscript{16}, tubes were placed on ice. Pellets of aliquoted EA were resuspended in 500 µL of normal guinea pig serum (NGPS), NGPS substituted with IVlg (10 mg/tube), and NGPS substituted with human albumin in 10% maltose (10 mg/tube). E pellet was resuspended in NGPS. Immediately after the pellets have been resuspended, duplicate 60- µL samples were taken from each tube and transferred to corresponding tubes with 2 mL ice-cold EDTA buffer ("O" time points). The tubes were then placed in a water bath at 37°C. At 5-, 15-, and 30-minute time points, duplicate 60- µL samples were removed from each tube and transferred to EDTA wash tubes. The intact erythrocytes in these samples were pelletted and the extent of lysis was determined by measuring free hemoglobin in the corresponding supernatants spectrophotometrically. The pellets were then washed and resuspended in 200 µL of GVBS\textsuperscript{16}. Each tube received the amount of radiolabeled anti-C3 or anti-C4 equal to 2 x 10\textsuperscript{6} cpm and incubated on melting ice for 60 minutes with occasional shaking. After two further washes in ice-cold EDTA buffer, the pellets were counted in a µ counter. Where appropriate, counts were corrected for calculated percentage of lysis.

**RESULTS**

**C4 uptake onto IgG-sensitized erythrocytes.** Guinea pig erythrocytes sensitized with rabbit anti-guinea pig erythrocyte antibody were incubated with NGPS diluted 1:4, guinea pig serum substituted with 10 mg human IVlg, and guinea pig serum with added 10 mg of human albumin for IV use in 10% maltose, pH 4.25. At various time points, aliquots were removed to quantitate C4 binding by uptake of \textsuperscript{125}I anti-guinea pig C4 onto the sensitized erythrocytes. At all time points IVlg suppressed C4 uptake by 50% (Fig 1). Some C4 was deposited on antibody-sensitized cells incubated briefly in NGPS at 0°C, reflecting the known ability of guinea pig complement to allow uptake of C4 onto sensitized erythrocytes at 0°C. NGPS with added albumin in maltose had no effect on C4 uptake in comparison with the NGPS.

**C4 uptake onto IgM-sensitized guinea pig erythrocytes.** IgM-sensitized erythrocytes were used as targets and C4 uptake kinetics were quantified with anti-guinea pig C4 labeled with \textsuperscript{125}I. In the presence of NGPS diluted 1:4, C4 uptake reaches the maximum in 5 minutes and sharply decreases at the 15-minute time point. Over the next 15 minutes, C4 on the surface of targets shows tendency for a further decay. IVlg serum suppressed the C4 binding by 20% at all time points, but did not alter the shape of the curve (Fig 2). The effect was specific for IVlg, because albumin/maltose had no effect on C4 uptake relative to NGPS.
The effect of increasing doses of IVIg on C4 uptake onto IgG targets. Three guinea pigs were injected with increasing doses of IVIg (600 mg/kg, 1,200 g/kg, and 1,800 mg/kg). Three hours after the injection of IVIg, a sample of blood from each animal was obtained by cardiac puncture. Guinea pig erythrocytes were then sensitized with rabbit anti-guinea pig erythrocyte IgG antibody. Pellets of aliquoted EA were resuspended in undiluted NGPS and undiluted sera of animals treated with increasing doses of IVIg. After a 5-minute incubation at 37°C, aliquots of cells were removed for C4 uptake quantitation by radiolabeled anti-guinea pig C4 antibody. Stepwise increase of C4 uptake inhibition (50%, 76%, and 90%) was observed, proportional to the increase of IVIg dose in guinea pig serum, meaning that C4 uptake is inhibited in a dose-responsive manner (Fig 3).

C4 uptake inhibition by serum from an IVIg-treated patient. Human RBCs were sensitized with rabbit antihuman RBC IgG antibody and incubated with the undiluted serum from a patient treated with IVIg at 600 mg/kg for 3 consecutive days and the same patient’s serum sample before IVIg therapy. After 5-minute incubation at 37°C, aliquots of cells were taken for C4 uptake quantitation by antihuman C4 IgG antibody labeled with 125I. In comparison with the sample before IVIg therapy, posttreatment serum decreased C4 uptake onto sensitized homologous erythrocytes to almost background (Fig 4). C4 functional titer in those two samples were not different, meaning that therapy itself had no effect on complement levels in serum.

Parallel C3/C4 uptake studies. C3 uptake was studied in parallel with C4 to compare the degree of inhibition using sera with added increasing doses of IVIg in both the human and guinea pig system. Guinea pig erythrocytes were sensitized with rabbit anti-guinea pig RBC IgG antibody. Pellets of aliquoted antibody-sensitized erythrocytes were resuspended in NGPS and guinea pig serum substituted with increasing doses of IVIg (5 mg, 10 mg, 20 mg, and 25 mg/tube). After a 5-minute incubation at 37°C, aliquots of RBCs were taken for C3/C4 uptake quantitation using rabbit anti-guinea pig C3/C4 antibodies labeled with 125I. It was found that with increasing doses of IVIg, both C3 and C4 uptake inhibition increased to the same extent, so that their curves completely overlapped. Similar experiments were performed in the human system; human erythrocytes were sensitized with rabbit antihuman RBC IgG antibody. Pellets of sensitized erythrocytes were resuspended in autologous serum and the same serum substituted with increasing doses of IVIg (5 mg, 10 mg, 20 mg, and 25 mg/tube). After 5-minute incubation at 37°C, aliquots of RBCs were taken for C3/C4 uptake quantification with respective radiolabeled (125I) rabbit antibody. As in the guinea pig system, percent of uptake inhibition increased proportionally to the increase of added IVIg; uptake inhibition was the same both for C3 and C4 at all dose points, so that the inhibition curves were superimposable (Fig 5).

**DISCUSSION**

The propensity of IgG to serve as a covalent acceptor for nascent C3 fragments is well known. C3b produced during complement activation by soluble immune complexes or bacteria binds to IgG immune complexes or “innocent bystander” IgG molecules. Studies by Berger et al showed that IVIg-inhibited deposition of C3b onto antibody-sensitized erythrocytes in a system using serum diluted 40- to 50-fold, and the uptake of trypsin-generated C3 fragments onto particles has been efficiently blocked by fluid-phase IgG. C3b fragments coating antibody-sensitized targets are important for in vivo clearance of immune complexes. Using clearance of IgM-sensitized erythrocytes as an in vivo model free of IgG-Fc receptor interactions, we explored a potential in vivo action of IVIg through interference with
IVIG AND C4 UPTAKE

In previous studies we studied blockade of C3 fragment uptake on erythrocytes in detail, and examined binding of C3 to erythrocytes presensitized with antibody and C1 and C4 of the classical complement pathway. Specifically, pre-loading of targets with C1 and C1 + C4 did not affect the capacity of IVIg to block C3 uptake onto these particular targets. We concluded that IVIg exerts the major effect at C3 convertase step. The approach we used to address the issue was indirect, using limited amounts of C1 and C4 compared with what is available in plasma. Therefore, we felt that the effect of IVIg at early steps of complement activation by classical pathway requires careful re-examination. C4 was chosen for study because of many similarities between C3 and C4. Both are activated by cleavage of a small peptide from the α chain exposing a buried reactive internal thiolester bond. Both are capable of binding covalently to targets and both have a propensity to bind IgG. Similar to our previous C3 uptake studies, we found that IVIg had much more pronounced effect on C4 uptake by IgG-sensitized erythrocytes than IgM-coated targets. For that reason, in all subsequent experiments we used IgG-sensitized erythrocytes to evaluate C4 uptake. We showed that this process is inhibited by IVIg in a dose-responsive manner. As can be concluded from parallel C3/C4 uptake studies in both human and guinea pig systems, IVIg inhibits C3 and C4 uptake to the same degree. These findings imply that IVIg interferes with the classical complement cascade at multiple sites. It would be of interest to determine which of these steps is of greater importance in control of complement activation by this important therapeutic modality.

REFERENCES

12. Hammer CH, Wirtz GH, Renfer L, Gresham HD, Tack BFJ:

Fig 5. Parallel C3/C4 uptake studies in the human serum system. Human RBCs were sensitized with rabbit antihuman erythrocyte IgG antibody. Sensitized RBCs were incubated for 5 minutes at 37°C with undiluted autologous human serum and autologous human serum to which different doses of IVlg were added (5 mg, 10 mg, 20 mg, and 25 mg). C4 uptake was calculated as percent inhibition relative to the maximum uptake observed in autologous human serum without IVlg. Inhibition curves for C3(e) and C4 were identical in shape and magnitude.

complement fragment deposition onto targets. We found that the clearance of IgM-sensitized guinea pig erythrocytes was significantly retarded in guinea pigs treated with human IVIg preparation. In vitro C3 uptake studies suggested that IVIg produces a kinetic depression of C3 uptake onto IgM-sensitized targets. Next, we used Forssman shock, another model of complement-mediated tissue damage that is IgG dependent but free of IgG-Fc interactions. By treating the animals with high doses of IVIg we were able to prevent death in 40% of animals and prolong survival fivefold in those animals that eventually succumbed to an otherwise lethal dose of anti-Forssman antibody. No control animal, treated with human/guinea pig albumin and maltose vehicle survived Forssman shock. In vitro C3 uptake onto IgG-sensitized erythrocytes was almost completely inhibited in an IVIg-treated animal’s serum. We suggested that one of the mechanisms of action of IVIg is interference with complement fragment deposition onto antibody-coated targets.


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