High-Dose Intravenous Immunoglobulin Modifies Complement-Mediated In Vivo Clearance

By Milan Basta, Paul F. Langlois, Marisa Marques, Michael M. Frank, and Louis F. Fries

The mechanism of effect of high-dose intravenous immunoglobulin (IVIG) therapy in immune cytopenias is incompletely known. One of the leading theories ascribes the short-term effects of IVIG to the competition of infused IVIG for Fc receptors, thereby inhibiting IgG-mediated clearance. Using a system independent of IgG-Fc receptor interactions, we examined another potential mechanism of IVIG action. Guinea pigs were infused with a human IVIG preparation at 600 mg/kg/day for two consecutive days. Parallel groups of animals were treated with the same volume and/or concentration of saline and albumin. Clearance of IgM-sensitized guinea pig erythrocytes, which is wholly complement dependent, was significantly retarded in animals treated with high-dose IVIG. The effect was specific for IVIG, since human albumin (as a second foreign protein) failed to change the clearance of IgM-sensitized guinea pig erythrocytes. Experiments in which IVIG-treated animals were subjected to pre- and posttreatment clearance studies revealed heterogeneity among individual animals in respect to their response to IVIG infusions. Decrease of available plasma complement components did not account for the effect, since both C3 and CH50 values remained unchanged after IVIG treatment, despite rising levels of IVIG in sera of treated animals. The results of in vitro C3 uptake studies and the effect of IVIG on clearance of preopsonized cells suggest that IVIG produces a kinetic depression of C3 uptake and modifies the process of complement fragment deposition on erythrocytes. A generalized effect on mononuclear phagocytes is less likely but cannot be wholly ruled out. These studies establish another potential mechanism of IVIG action and suggest extension of its use to other complement-mediated diseases.

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

From the Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD.

Submitted August 30, 1988; accepted March 3, 1989.

Address reprint requests to Milan Basta, MD, Laboratory of Clinical Investigation, Bldg 10, Rm 11N228, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

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

0006-4971/89/7401-0041$0.00/0

colonies maintained by the National Cancer Institute at Frederick, Maryland.

Reagents. Human serum IgG for IV use, in 10% maltose, pH 4.25 (Gammune), was purchased from Cutter Biologicals (Berkeley, CA). Human serum albumin (HSA) 25% solution for IV administration was also obtained from Cutter Biologicals. Sodium 125I-iodide and sodium 9chromate were purchased from Amersham (Arlington Heights, IL). Iododecas were obtained from Pierce Chemicals (Rockford, IL).

Buffers. Isotonic veronal-buffered saline containing 0.1% gelatin, 0.15 mmol/L CaCl2 and 1 mmol/L MgSO4 (GVBS)24 and isotonic veronal-buffered saline containing 0.1% gelatin and 10 mmol/L EDTA buffer were prepared as previously described.25 Hank's balanced salt solution (HBSS) with and without divalent cations was also obtained from Biological Industries (Arlington, Virginia, CA). Human serum was purchased from Cutter Biologicals (Berkeley, CA). Human serum was redissolved in phosphate-buffered isotonic saline (PBS, pH 7.4), fresh guinea pig serum 1.5 mL was supplemented with 10 mmol/L EDTA and 25 μmol/L NPG and passed slowly over the column at 0°C. The pass-through was collected and concentrated to the original volume. C8-depleted serum was then dialyzed extensively at 0°C against veronal-buffered isotonic saline without Ca2+ or Mg2+, aliquoted and frozen at −70°C until used.

In vitro C3 uptake inhibition studies. One tenth of 1 mL guinea pig erythrocytes at 2.7 × 108 cells/mL were mixed with 0.1 mL

by percentage of the body weight representing the vascular space (estimated as 5%). Clearance of heat-damaged RBCs was studied in a similar manner, except that IgM sensitization was replaced by heating of the cell suspension at 49°C for 20 minutes according to the method of Bowring et al.26

IVIG treatment protocol: Pre- and post-clearance studies. Completion of each experiment took six days. On day 1, the "background" clearance of IgM-sensitized cells was performed as described above. On day 2, the animals were rested. On days 3 through 5, the animals were anesthetized; with a Pasteur pipette, samples of blood were then obtained from the retroorbital sinus and the animals were infused through a hind limb vein with the desired doses of immunoglobulin, albumin, or saline, respectively. On day 6, the second clearance of IgM-sensitized cells was performed in the same animals after a preliminary blood sample was obtained for complement titers, level of human IgG, and baseline radioactivity in blood remaining from the previous clearance study.

IVIG treatment protocol: Parallel studies. In another series of experiments, large groups of animals were treated simultaneously for two consecutive days (days 0 and 1) with IVIG, albumin, or saline, as described above. Before each infusion, blood samples for complement titers and concentration of human IgG were obtained by rotoorbital sinus puncture. On day 2, clearance of IgM-sensitized or heated guinea pig erythrocytes was studied.

C8 depletion of guinea pig serum. Rabbit IgG anti-guinea pig C8 was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. A 1 × 10-cm column was packed with this resin and equilibrated with isotonic veronal-buffered saline containing 10 mmol/L EDTA and the protease inhibitor p-nitrophenyl p-guanidino benzoate (NPG, 25 μmol/L). Fresh guinea pig serum 1.5 mL was supplemented with 10 mmol/L EDTA and 25 μmol/L NPG and passed slowly over the column at 0°C. The pass-through was collected and concentrated to the original volume. C8-depleted serum was then dialedyzed extensively at 0°C against veronal-buffered isotonic saline without Ca2+ or Mg2+, aliquoted and frozen at −70°C until used.

In vitro C3 uptake inhibition studies. One tenth of 1 mL guinea pig erythrocytes at 2.7 × 108 cells/mL were mixed with 0.1 mL

Fig 1. Effect of IVIG on clearance of IgM-sensitized guinea pig E. The experimental group of animals (---, n = 8) was treated for two consecutive days with human IVIG 600 mg/kg, while the parallel group of control animals (----, n = 8) received the same volume of saline. Twenty-four hours after the second treatment, the clearance study was performed. Data are means ± SEM. The intravascular survival of IgM-sensitized E in IVIG-treated guinea pigs was enhanced at all time points relative to saline-treated animals.
diluted rabbit IgM anti-guinea pig erythrocyte antibody or 0.1 mL buffer for 30 minutes at 37°C. Four separate reaction tubes (two containing unsensitized guinea pig erythrocytes (E) and two with sensitized guinea pig erythrocytes (EA)) were prepared, and the cells were washed and pelleted. Two hundred microliters undiluted normal guinea pig serum or serum from an animal treated for three days with IVIG and containing 8.5 mg/mL human IgG were each added to one pair of EA and E tubes. The tubes were placed in a water bath at 37°C. At one, two, five, and 15 minutes, 40-μL samples were taken from each tube and transferred to wash tubes with ice-cold EDTA buffer. The samples were pelleted, and the extent of lysis was determined by measuring free hemoglobin in the corresponding supernatants spectrophotometrically. The pellets were washed again and then resuspended in 200 μL GVBS². Ten microliters ¹²⁵I-labeled rabbit IgG anti-guinea pig C3 was added to each tube and incubated for 60 minutes at 0°C. After two further washes in ice-cold EDTA buffer, the pellets were counted in a γ-counter. When appropriate, counts were corrected for the calculated percentage of hemolysis. Alternatively, 200 μL C8-depleted serum, diluted at a ratio of four parts serum to one part HBSS and supplemented with Ca²⁺ and Mg²⁺ in doses calculated to replete these cations to physiologic levels, was used as a complement source and compared with a similar mixture containing IVIG (predialyzed against HBSS) at 10 mg/mL. In these latter studies, no detectable lysis occurred.

Complement titrations. Hemolytic complement titrations of CH50 and C3 were performed using standard published procedures.³²

Levels of human IgG in guinea pig sera. Concentrations of human IgG in guinea pig serum were determined using radial immunodiffusion plates (Diffu-gen, Tago, Burlingame, CA) and known calibrating standards. These plates gave no significant reactivity with guinea pig sera alone.

RESULTS

Effect of IVIG on clearance of IgM-sensitized guinea pig E. IV infusion of human IgG into guinea pigs (n = 8) at 600 mg/kg for two consecutive days increased the percentage of IgM-sensitized EA surviving at all time points relative to animals (n = 8) treated with the same volume of saline (Fig 1). The alterations of RBC clearance reflected changes in intact erythrocyte survival, since >95% of ⁵¹Cr counts in every sample segregated with the cell pellet. To test the significance of this difference, we calculated the area between the saline control mean curve and the clearance curve of each individual animal in the experimental group. We then tested the null hypothesis that this set of values did not differ significantly from zero, which would be the case were IVIG without effect relative to saline. The null hypothesis was readily rejected (t = 5.57, 7 df, P < .001). To examine the specificity of the IVIG effect on clearance of IgM-sensitized EA, a group of four animals was treated with IV human albumin at 600 mg/kg for two consecutive days. Their mean clearance curve is compared with that of the control group of saline-infused animals in Fig 2. Nonimmunoglobulin foreign protein infusion showed no net effect relative to saline. In such clearance studies, calculation of the zero time point is of crucial significance. Since this value is determined mathematically (as described in the Materials and Methods section), evaluation of the accuracy of our calculations was important. For that purpose, we injected 12 guinea pigs with unsensitized guinea pig erythrocytes subjected to sham in vitro manipulation (E) and obtained essentially flat clearance curves in all cases. Comparison of calculated zero time points with the multiple observed values in these studies revealed no significant difference and confirmed the accuracy of our method of zero time determination. IVIG treatment itself did not induce alteration in the survival of guinea pig E, nor did the infusions alter the accuracy of the zero time point, since clearance curves generated in guinea pigs studied with chromated E before and after two-day therapy with IVIG were likewise flat and demonstrated no alteration of the relationship between the calculated zero point and the observed circulating counts (Fig 3). These data suggested that IVIG was not altering the survival of normal guinea pig E per se. In addition, since intravascular volume for the zero time estimate was calculated as a constant fraction of total body weight, a selective expansion of the blood volume after IVIG would have led to observed values in unsensitized E studies which were consistently below the predicted time zero value; this was not true. Thus, IVIG probably did not exert its effect through simple expansion of the intravascular volume.

Heterogeneity of response to IVIG. In a second series of experiments, clearance was studied before and after IVIG infusion (ie, each animal served as its own control). After the first background clearance animals were rested for one day and then treated with IVIG at 400 mg/kg for three consecutive days. One day after the last IVIG infusion, the second clearance study was performed. By this method, we were able to detect heterogeneity of response to IVIG in individual guinea pigs. Two of six animals did not respond (ie, their pre- and posttherapy curves were virtually the same [Fig 4]). The mean area between the pre- and post-IVIG clearance curves was significantly different from zero among the animals in the "responder" group (t = 10.16, 3 df, P < .01), but not
among the animals in the "nonresponder" group. The responders and nonresponders clearly differed from one another ($t = -8.62, 4 \, df, \, P < .001$). Because of our nonrandom choice of these groups, we applied Bonferroni's inequality to allow for other possible separations of the data; the resulting level of significance remained $P < .01$.

During the course of these experiments, the animals were exposed to extensive handling and repetitive anesthesia. To assess resultant possible alterations in clearance behavior, we performed pre- and postclearance studies in animals sham treated with saline infusions for three days. The posttreatment curves showed slightly enhanced clearance (5% to 10%), at least at early time points (five and 15 minutes), relative to the background curve. Thus, the pre- and postclearance studies might have underestimated IVIG effects, especially at these early time points. Pre- and postclearance studies of albumin infusion also revealed slightly enhanced clearance at early time points, similar to the saline studies, but otherwise demonstrated no significant effect on clearance (data not shown).

In view of the observed animal-to-animal variations in IVIG response, we more carefully reexamined the data from the parallel cohort studies in search of similar heterogeneity. Figure 5 shows the eight individual clearance curves of our initial cohort of IVIG-treated animals as compared with the mean control curve. The animals readily fell into two groups, which did not overlap at any time point. By again analyzing the mean areas between these two groups of curves and the control curve, we detected a significant IVIG effect in both groups ($t = -22.3, 4 \, df, \, P < .001$ for the responder group and $t = -6.71, 2 \, df, \, P < .05$ for the poor responder group). These groups were also distinguishable from each other, despite small numbers, because of the limited within-group variability ($t = -10.3, 6 \, df, \, P < .001$). Again, application of Bonferroni's inequality resulted in a persistent level of significance of $P < .01$. The poor responder group represented a proportion of these animals (37.5%) similar to the nonresponders in the pre- and postclearance studies (33.3%), providing further support for the concept of heterogeneity in response to IVIG therapy.

Levels of human IgG obtained and failure to alter CH<sub>50</sub> and C3 titers. To address potential mechanisms for the IVIG effect, the concentration of human IgG, C3 titers, and CH<sub>50</sub> were determined in serum samples taken before (day 0), after one day of IVIG therapy (day 1), and on the day of clearance study (Fig 6). IVIG therapy at 600 mg/kg/day consistently produced serum human IgG levels ~9 mg/mL on day 2. Slightly lower levels were achieved consistently by three days of treatment at 400 mg/kg/day in the pre- and
postclearance studies (~8 mg/mL, data not shown). Neither CH₅₀ nor C3 titer was changed relative to preinfusion (day 0) values in the parallel cohort study (Fig 6), whereas in pre- and postclearance studies CH₅₀ was stable and mean C3 titer actually rose slightly on last day (not shown). Thus, complement consumption by infused aggregates in IVIG-treated animals was not responsible for the observed reduction in complement-mediated clearance.

**Clearance of preopsonized and heat-damaged RBCs.** To separate the effects of IVIG on complement activation from influences on reticuloendothelial function, we studied clearance of cells preopsonized in vitro in normal guinea pig serum. In this series of experiments, chromated E sensitized with IgM anti-guinea pig erythrocyte antibodies were incubated for five minutes with normal guinea pig serum diluted 1:4 as a source of complement, washed, and then infused into three IVIG-treated and three saline-treated guinea pigs. The clearance studies were performed as before. As shown in Fig 7A, differences between IVIG and saline clearance curves were suppressed at early time points (five and 15 minutes) but persisted at late time points. We then studied the effect of IVIG on clearance of heat-damaged RBCs, a target disposed of by the mononuclear phagocyte system in an antibody and complement-independent manner.²⁶,²⁷ Clearance of these targets by six animals treated with 600 mg/kg/day IVIG for two days was indistinguishable from that observed in five saline-treated animals and superior to that observed in two concurrent albumin-treated animals (Fig 7B).

**Effect of IVIG on C3 uptake by IgM-sensitized guinea pig E in vitro.** IgM-sensitized EA were incubated in vitro with undiluted normal guinea pig serum or serum from an IVIG-treated animal containing 8.5 mg/mL human IgG. Aliquots were removed at various time points, and C3 binding was quantified by uptake of ¹²⁵I anti-guinea pig C3 by the EA. At early time points (one and two minutes), IVIG markedly inhibited the extremely rapid uptake of IgM-sensitized E as compared with normal control. At five and 15 minutes, the differences in respect to C3 uptake disappeared (Fig 8A). These data were corrected for hemolysis by the formula of observed counts x 100/100-% lysis. This correction could be eliminated by using 80% C8-depleted guinea

---

Fig 5. Heterogeneity of response to IVIG in parallel cohort studies. Individual clearance curves of the eight IVIG-treated animals described in the legend to Fig 1 are shown in comparison with the control mean curve. The curves fell into two nonoverlapping groups: responders and poor responders.

Fig 6. Levels of human IgG achieved and failure to alter serum C3 titer and CH₅₀. C3 titers, CH₅₀, and the concentrations of human IgG in the serum samples taken before (day 0), after one day of IVIG therapy (day 1), and on the day of clearance study (day 2), were determined. IVIG treatment (600 mg/kg/day) consistently produced serum human IgG levels of 9 mg/mL on day 2, which was slightly greater than levels achieved by three days of treatment at 400 mg/kg in pre- and postclearance studies (not shown). Neither CH₅₀ nor C3 titer was changed relative to postinfusion (day 0) values in either protocol. Data are means and SEM of observations on eight animals.
pig serum supplemented with IVIG in vitro. This system, despite the complete absence of lysis, demonstrated similar displacement of the C3 uptake curve by IVIG (Fig 8B).

**DISCUSSION**

Erythrocytes sensitized with heterologous or homologous IgM antibodies are cleared by the liver in both human and guinea pig in vivo models. The clearance curve is typical: After initial hepatic sequestration at five- to 15-minute time points, which is presumably due to adhesion of the erythrocytes to Kupffer cells, a significant proportion of the detained cells are released back into the circulation and eventually appear to survive normally. The process is wholly complement dependent and requires intact complement components of the classical pathway through C3. A variable portion of the initially sequestered cells are not released and are presumably phagocytosed by the Kupffer cells. The release process, which is essentially complete at 60 to 90 minutes, is believed to reflect processing of target-bound C3 fragments to residues which no longer bind to mononuclear phagocyte receptors.

The results of the present study demonstrate that clearance of IgM-sensitized guinea pig erythrocytes is significantly depressed in animals treated with high-dose human IVIG. The effect is specific for IVIG since a second foreign protein, HSA, failed entirely to alter the clearance of IgM-sensitized erythrocytes. Relative to saline- and/or albumin-treated animals, the IgM clearance in IVIG-treated animals...
was affected both at the nadir (15% to 20% inhibition of sequestration) and later in the release phase, in which an average enhancement of crude RBC survival of 18.3% ± 3.3% (mean ± SEM) was observed at 90 minutes. Based on an average RBC survival of 51.2% at 90 minutes in controls, this represents a relative improvement in survival of 36% after IVIG treatment.

Our pre- and postclearance studies revealed heterogeneity in response to IVIG infusions. In two of six animals, the clearance was not affected by IVIG (ie, their pre- and postclearance curves overlapped). Reevaluation of the data from the initial parallel cohort studies confirmed a similar heterogeneity among those animals. The presence of some IVIG effect even among the poor responders in this first study may have reflected small systematic differences in the two treatment protocols (ie, IVIG plasma levels, extent of handling, variations in target preparation). Indeed, we were able to show a small but consistent acceleration of clearance in the second study of pre- and postclearance pairs after sham treatment, which might diminish apparent IVIG effects in that system yet remain undetected in the cohort studies. The source of the response heterogeneity is unclear; it may reflect genetic differences (these animals are not inbred) and may be comparable to heterogeneous clinical response to IVIG in disease states. Retrospective analysis of complement titers, human IgG levels, and pretreatment clearance curves revealed no distinctive features in the unresponsive subgroup which were reflected in these parameters. Detailed studies of such factors as Kupffer cell complement receptor activity or varying kinetics of C3 activation from animal to animal may be required to elucidate this issue.

As for the possible mechanism(s) of the IVIG effect, our data indicate that it is not due to a decrease of available plasma complement components, because both C3 and CH50 values in sera of treated animals remained unchanged after IVIG infusions. Saturation or modulation of complement fragment receptors on Kupffer cells also seems unlikely as an explanation, since initial binding and/or sequestration of cells preopsonized in normal serum was not significantly altered in animals treated with high-dose immunoglobulin (Fig 7A). At least two different mechanisms may be operative. First, IVIG produces a kinetic retardation of C3 uptake by target erythrocytes and modifies the process of complement fragment deposition. As demonstrated by our in vitro studies, C3 fragment deposition onto IgM-sensitized cells is very rapid and reaches peak levels two minutes after the beginning of the reaction; it is within that period of time that IVIG exerts its effect in terms of interference with C3 uptake. These results required a calculated correction for partial hemolysis but were verified in a nonhemolytic C8-depleted serum system. This finding probably represents the same phenomenon previously reported by Berger et al,21 differing in extent by virtue of the 40- to 50-fold higher concentration of serum (and hence complement components) used in our studies. Diversion of nascent C3 fragments to acceptor sites on high levels of ambient IgG is one attractive hypothesis, consistent with the known propensity of C3b to bind to IgG, which could explain this effect. In any case, reduced C3 binding resulting in impaired adherence of IgM-sensitized guinea pig erythrocytes to Kupffer cell complement receptors probably accounts for the reduced early sequestration which could be eliminated by preopsonization in normal serum. The failure to observe significant free chromium in vivo presumably reflects very rapid sequestration of cells that have the greatest extent of complement activation and hence are most likely to lyse. Chromium counts from cells intentionally lysed before infusion were cleared, but substantially more slowly than the rapid downstroke of the usual RBC clearance curve. Thus, major intravascular hemolysis should have been detected had it occurred. Infusion of targets preopsonized in normal serum into IVIG-treated animals also suggested a second distinct process—enhancement of the release phase. This could imply a depression of Kupffer cell phagocytic activity toward the targets, and thus parallel the in vitro finding of impaired Fe receptor-mediated phagocytosis by cultured monocytes from IVIG-treated humans.9 We therefore considered the possibility that high-dose IVIG might induce a generalized phagocytic abnormality, at least in mononuclear phagocytes. However, the clearance of heat-damaged cells, which is opsonin independent and involves both splenic and hepatic function, was unaltered by IVIG. Thus, alteration in the physiologic state of fixed mononuclear phagocytes, if it occurs, must be relatively specific for opsonic receptor-mediated phagocytosis. The extent to which ongoing cycles of C3 activation and deposition on IgM-coated targets condition the shape of the release phase is not explicitly known. Thus, an alternative explanation for enhancement of release of preopsonized cells would feature inhibition of such late-occurring C3 fragment deposition in vivo by IVIG. These studies suggest that IVIG could be of utility in diseases characterized by complement-dependent tissue damage. The levels of IgM sensitization of the erythrocytes studied were quite high, with very rapid and efficient complement activation. Although this was necessary to study clearance on a convenient time scale, such conditions may not obtain in clinical disease. In situations in which complement activation and C3 fragment deposition may proceed less rapidly and efficiently (ie, targets bearing IgG or smaller amounts of IgM), IVIG may be a more effective complement modulator. Preliminary in vitro studies using IgG-bearing targets suggest that this is indeed true, and we are currently developing an Fc-receptor-independent in vivo model of IgG and complement-mediated tissue damage which will permit assessment of IVIG effects. The extent to which inhibition of complement opsonization is active in IVIG therapy of immune cytopenias is not predictable from our data. An important role of IVIG in modulating IgG Fe-receptor-mediated clearance is established.88 Nonetheless, complement is clearly a synergistic enhancer of antibody effects in this setting,22,28 and suppression of C3 fragment binding could have clinical utility. In addition, reports of aggressive IVIG use in complement-dependent IgM-mediated hemolysis are very few. Our findings suggest that IVIG in large doses may merit trial in this often intractable disease state.
REFERENCES


High-dose intravenous immunoglobulin modifies complement-mediated in vivo clearance

M Basta, PF Langlois, M Marques, MM Frank and LF Fries