Functional Capacity of Immunoglobulin G Preparations and the F(ab′)_2 Split Product

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Five immunoglobulin G preparations, including one 5S F(ab′)_2 split product, were compared for activity against common bacterial, viral, and protozoan pathogens. Standard assays were used to quantitate antibodies to tetanus, diphtheria, cytomegalovirus, herpes simplex virus types 1 and 2, rubella virus, and Toxoplasma gondii. Opsonization and killing of bacteria were examined by chemiluminescence methods using Streptococcus pneumoniae types 5, 12F, and 14 and Staphylococcus aureus. Antibodies to the viral pathogens and T. gondii were not detectable for the 5S immunoglobulin even at high concentrations (50 mg/ml) but were present in all 7S preparations at immunoglobulin concentrations of 10 mg/ml. Relatively lower activities for tetanus and diphtheria antibody were also seen with the F(ab′)_2 product. Opsonizing capacity against all pneumococcal serotypes and Staphylococcus aureus was lowest for the 5S product and highest for the commercially available intravenous immunoglobulin product that is purified by using a pH 4.25 formulation. These data do not support potential clinical usefulness of immunoglobulin G split products and suggest wide variations of specific antibody among commercial intravenous immunoglobulin preparations.

There are 4 intravenous immunoglobulin (IVIG) preparations commercially available in the United States, while 15 to 20 additional products are being used in Europe and Japan. These have largely replaced intramuscular immunoglobulin for the maintenance therapy of patients with primary or secondary hypogammaglobulinemia. IVIG is also approved for the management of acute and chronic idiopathic thrombocytopenic purpura. In addition, controlled clinical trials have demonstrated efficacy for the treatment of Kawasaki disease and for the prevention of the following infections: sepsis in preterm neonates, sepsis in infants with acquired immunodeficiency syndrome, and cytomegalovirus (CMV) infection in transplant recipients. Open (uncontrolled) studies have suggested benefit in the treatment of neonatal sepsis, chronic Epstein-Barr virus infection, and a number of autoimmune diseases. These indications have been reviewed recently (13).

There was no distinct therapeutic advantage for a native IVIG product over a modified preparation in human efficacy trials (12), although there are theoretical reasons to use formulations which have utilized methodology to preserve the molecular integrity of immunoglobulin G (IgG). There are also theoretic advantages for F(ab′)_2 split products, including a reduced release of inflammatory mediators, decrease in potential immune suppression, short serum half-life, faster tissue penetration, and synergistic effects with antibiotics (11).

Most important in evaluating potential efficacy for any new IVIG material is first to determine the in vitro biologic effects which are most likely to correlate with clinical protection. Few investigations of this kind have been undertaken (2, 11, 15). The present study was therefore designed to compare the functional capacities of five IVIG preparations against important microbial pathogens. These products were selected because various methods for purifying and stabilizing them are utilized. One is an investigational 5S F(ab′)_2 split immunoglobulin prepared by enzymatic digestion.

MATERIALS AND METHODS

Total IgG levels were determined by the standard method of rate nephelometry by using an automated immunochemical system (Beckman Instruments, Inc., Fullerton, Calif.) as described in detail previously (3). Specific antibodies to herpes simplex virus types 1 and 2 (HSV-1 and -2), cytomegalovirus, rubella virus, and Toxoplasma gondii were measured by an automatic analyzer (model PR50; Gilford Instrument Laboratories, Inc., Oberlin, Ohio) using commercially available enzyme-linked immunosorbent assay kits (Microbiological Associates Bioproducts, Walkersville, Md.) (16). The anti-human IgG reagent in this assay is directed against the whole IgG molecule. Tetanus and diphtheria antibodies were measured by using a tannic acid hemagglutination microtechnique (4). Titers are expressed as the reciprocal of the highest dilution which demonstrated agglutination.

IVIG preparations. Five IgG products were examined in these in vitro investigations: (i) 7S-IVIG, purified with a pH 4.25 formulation (Gamimmune-N; Cutter Laboratory, Berkeley, Calif.); (ii) 7S-IVIG, treated at acid pH in the presence of trace amounts of pepsin (Sandoglobulin; Sandoz AG, Basel, Switzerland); (iii) sulfonated 7S-IVIG, fully reconstituted under physiologic conditions (Venimunun; Behring Werke GmbH H., Marburg a.L., Federal Republic of Germany); (iv) 5S-IVIG F(ab′)_2 treated with pepsin (Gamma-Venin; Behring Werke); and (v) an investigational 7S-IVIG, nearly unmodified, treated with low concentrations of pepsin (BI 61 011; Behring Werke). For the two commercially available IVIG preparations, Gamimmune-N and Sandoglobulin, three different lots were combined for analysis.

Each IVIG product was diluted in Gey’s balanced salt solution (GBSS) to IgG concentrations ranging from 2.8 to 50 mg/ml. Most experiments were performed at 2.8 mg/ml since this represents the final concentration of IVIG when given at the usual recommended dose of 200 mg/kg of body weight. CL. For chemiluminescence (CL) assays, venous blood
was collected from a single adult volunteer, and polymorphonuclear leukocytes (PMN) were isolated by a dual Ficoll-Hypaque gradient separation technique. The double-layer gradient was prepared by placing 2 ml of monoply resolving medium (specific gravity, 1.114 g; Flow Laboratories, Inc., McLean, Va.) in a plastic centrifuge tube (16 by 100 mm) and carefully layering 2 ml of Histopaque (specific gravity, 1.077; Sigma Chemical Co., St. Louis, Mo.) over the first. Four milliliters of blood were gently layered on top of the gradients and centrifuged at 250 × g for 35 min in a swinging bucket rotor at 20°C. The top fraction was discarded, and the lower fraction (PMN) was collected and washed once with Hank's balanced salt solution by centrifugation at 400 × g for 10 min. To remove contaminating erythrocytes, the cell pellet was suspended in 0.84% ammonium chloride and incubated for 5 min in a 37°C water bath. The PMNs were washed three additional times before adjusting the concentration to 5 × 10^9/ml in GBSS.

*Staphylococcus aureus* bacteriophage type 502A and three serotypes of *Streptococcus pneumoniae* were obtained from the American Type Culture Collection, Rockville, Md. These are Danish types 5 (6305), 12F (6312), and 14 (6414). Organisms were inoculated into several tubes of Todd-Hewitt broth and incubated at 37°C for 72 h. The suspensions were centrifuged at 1,000 × g for 10 min at 25°C and then washed and suspended in GBSS to a concentration of 5 × 10^9/ml. Bacteria were aliquoted and stored at −70°C until used.

On the day of each assay, *Staphylococcus aureus* and the three *S. pneumoniae* serotypes in volumes of 0.2 ml each were opsonized with 0.2 ml of IVIG-0.05 ml of guinea pig complement for 30 min at 37°C. After incubation, 0.55 ml of GBSS was added to each opsonized bacterial suspension. This 1-ml volume was added to the reaction mixture vial for a final bacteria/PMN ratio of 100:1.

IVIG opsonizing antibodies to bacteria were tested by methods described in detail previously (1). CL was measured at room temperature in dark-adapted polypylene scintillation vials, with the photomultiplier tube of a Beckman LS 9000 liquid scintillation spectrophotometer set in the out-of-coincidence mode. The vials contained a total of 5 × 10^5 PMN in 4 ml of GBSS and were equilibrated in the scintillation counter until a stable background was reached. CL was initiated by adding 1 ml of previously opsonized bacteria (10^8 to 10^9) to each vial. All vials were counted for 30 s at approximately 7-min intervals for 90 min. CL was expressed as peak activity during the reaction time. Maximum or peak counts per minute usually occurred between 35 and 60 min.

Initial assays were designed to examine nonspecific opsonization by determining results of CL activity by using bacterium-absorbed serum. For these experiments, 10^8 killed organisms were added to 2.0 ml of serum, incubated at 30°C for 2 h, and centrifuged at 3,000 × g. Serum was decanted and reincubated in a similar fashion twice more with additional aliquots of bacteria. The resulting absorbed serum was reconstituted with guinea pig complement prior to the CL assays. Guinea pig complement alone was shown not to generate any CL activity. In addition, serum from patients with congenital agammaglobulinemia was incubated by the same method to determine nonspecific opsonization. This serum had measured immunoglobulin concentrations as follows: IgG, 60 mg/dl; IgA, 0 mg/dl; and IgM, 32 mg/dl. Data indicate that maximum counts per minute representing nonspecific opsonization were 10 × 10^3 for type 5 *S. pneumoniae*, 8 × 10^3 for type 12F, and 10 × 10^3 for type 14. Pooled normal human sera yielded peak CL responses at least twice as high, thereby defining positive specific antibody activity. For presentation of the present data, nonspecific opsonization was subtracted from total counts per minute recorded in each assay.

**RESULTS**

At the expected in vitro concentration of IVIG (2.8 mg/ml) following a 200-mg/kg infusion, there was great variation in specific antibody among the five preparations (Tables 1 and 2; Fig. 1 and 2). Most consistent was that the 5S immunoglobulin showed lower activity than those of all 7S preparations.

**Antibodies to viral and protozoan pathogens.** Gamimune demonstrated the highest activity in enzyme-linked immunosorbent assays for antibody to CMV, HSV-1, HSV-2, rubella

<table>
<thead>
<tr>
<th>Prepn (conc [mg/ml])</th>
<th>CMV</th>
<th>HSV-1</th>
<th>HSV-2</th>
<th>Rubella</th>
<th>T. gondii</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamimune (2.8)</td>
<td>0.87 (MP)</td>
<td>0.49 (LP)</td>
<td>0.44 (LP)</td>
<td>0.68 (HP)</td>
<td>0.50 (MP)</td>
</tr>
<tr>
<td>Venimun (2.8)</td>
<td>0.49 (MP)</td>
<td>0.35 (LP)</td>
<td>0.16 (N)</td>
<td>0.10 (N)</td>
<td>0.08 (N)</td>
</tr>
<tr>
<td>BI 61 011 (2.8)</td>
<td>0.60 (MP)</td>
<td>0.25 (LP)</td>
<td>0.25 (LP)</td>
<td>0.24 (MP)</td>
<td>0.11 (N)</td>
</tr>
<tr>
<td>Gamma-Venin (2.8)</td>
<td>0.07 (N)</td>
<td>0.07 (N)</td>
<td>0.07 (N)</td>
<td>0.02 (N)</td>
<td>0.00 (N)</td>
</tr>
<tr>
<td>Sandoglobulin (50)</td>
<td>0.22 (N)</td>
<td>0.35 (N)</td>
<td>0.21 (N)</td>
<td>0.12 (N)</td>
<td>0.11 (N)</td>
</tr>
<tr>
<td>Venimun (10)</td>
<td>0.40 (LP)</td>
<td>0.51 (LP)</td>
<td>0.42 (MP)</td>
<td>0.27 (MP)</td>
<td>0.26 (LP)</td>
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<tr>
<td>Gamma-Venin (10)</td>
<td>0.71 (MP)</td>
<td>0.96 (MP)</td>
<td>0.69 (HP)</td>
<td>0.56 (HP)</td>
<td>0.28 (LP)</td>
</tr>
<tr>
<td>Sandoglobulin (10)</td>
<td>0.08 (N)</td>
<td>0.22 (N)</td>
<td>0.16 (N)</td>
<td>0.13 (N)</td>
<td>0.01 (N)</td>
</tr>
<tr>
<td>Gamma-Venin (50)</td>
<td>0.20 (N)</td>
<td>0.28 (N)</td>
<td>0.25 (N)</td>
<td>0.10 (N)</td>
<td>0.10 (N)</td>
</tr>
<tr>
<td>Sandoglobulin (50)</td>
<td>0.60 (MP)</td>
<td>1.06 (MP)</td>
<td>0.62 (HP)</td>
<td>0.54 (HP)</td>
<td>0.54 (MP)</td>
</tr>
</tbody>
</table>

* Optical density readings. HP, high range positive; MP, midrange positive; LP, low range positive; N, negative.

**TABLE 1.** Specific antibody in five IVIG preparations as measured by enzyme-linked immunosorbent assay.

<table>
<thead>
<tr>
<th>Prepn*</th>
<th>Activity* against:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tetanus</td>
</tr>
<tr>
<td>Gamimune</td>
<td>1:2,187</td>
</tr>
<tr>
<td>Sandoglobulin</td>
<td>&gt;1:10,000</td>
</tr>
<tr>
<td>Venimun</td>
<td>1:243</td>
</tr>
<tr>
<td>Gamma-Venin</td>
<td>1:729</td>
</tr>
<tr>
<td>BI 61 011</td>
<td>1:2,187</td>
</tr>
</tbody>
</table>

* Each preparation was made up to a standard concentration of 2.8 mg/ml.

* Expressed as the highest dilution with a positive hemagglutination response.

**TABLE 2.** Tetanus and diphtheria antibody in five IVIG preparations
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IgG level, the detectable antibody and T. gondii (Table 1). Venimmun and HSV-1 virus, and T. gondii (Table 1). At concentrations of 2.8 mg/ml, antibody was detectable for all five pathogens. At this IgG level, the investigational 7S-IVIG, Bl 61 011, lacked detectable antibody to T. gondii but was positive for all four viral agents, while Venimmun had activity only in assays for CMV and HSV-1 antibody. Both Sandoglobulin and Gamma-Venin were negative in all assays with IgG concentrations of 2.8 mg/dl. At 10 mg/ml, Sandoglobulin was positive in all five assays. Even at 50 mg/ml, Gamma-Venin remained negative.

Antibodies to tetanus and diphtheria. Assays for antibodies to these two bacterial pathogens were positive with all IVIG preparations at a concentration of 2.8 mg/ml (Table 2). Sandoglobulin yielded the highest titers at levels above the maximum dilutions performed. The 5S product (Gamma-Venin) yielded hemagglutination responses at lower dilutions as contrasted with other products.

Bacterial opsonizing antibodies. Comparative results for pneumococcal and staphylococcal opsonizing antibody as measured by CL techniques were consistent in that all 7S preparations yielded high opsonizing activity while the 5S split product was always deficient. Data were similar for all pneumococcal serotypes studied; those for type 12 are presented in Fig. 1. Results for opsonizing experiments with Staphylococcus aureus are summarized in Fig. 2. Measurements above 8,000 cpm with this pneumococcal strain and above 10,000 cpm for Staphylococcus aureus are higher than what was defined as nonspecific opsonization, i.e., CL responses with agammaglobulinemic or absorbed serum.

DISCUSSION

Intramuscular immune serum globulin has been used for the prevention and treatment of infectious diseases since the late 1930s (7). Diseases previously treated for which this therapy is no longer recommended include polio, mumps, rubella, and varicella-zoster virus infections. Currently, the intramuscular preparation is used for replacement therapy of patients with antibody deficiency states and for the prevention of hepatitis A, hepatitis B, and measles. Hyperimmune human serum globulin is also available for the prevention of varicella-zoster virus infections, hepatitis B, and rabies.

IVIG was introduced for general clinical use in 1981 and has since been the preferred maintenance therapy for immune deficiency disorders. Intravenous therapy is of course better tolerated by antibody-deficient patients since it avoids the excessive pain accompanying intramuscular injections with large volumes of material. The major limiting factor for routine selection of IVIG is the high cost for these products.

Initially, it was assumed that modified IVIG, i.e., with split IgG or altered Fc portions and absence of the IgG3 subclass, would prevent optimal functional capacity. The first product commercially available, Gamimune, which was reduced and alkylated, was indeed chemically modified in this fashion. However, both in vitro and in vivo examination indicated that such modification did not alter functional capacity. In spite of these findings, this product was replaced by one purified with a pH 4.25 formulation, Gamimune-N, which retained the native IgG molecule. The three other formulations commercially available in the United States are likewise processed to maintain molecular integrity.

At the present time, there are 15 to 20 IVIG preparations under investigation for clinical application. Among these are split immunoglobulin products obtained by enzymatic digestion yielding F(ab')2, or Fab antibodies. The theoretic advantage of split over native immunoglobulins is that when immune complexes are formed, their inability to interact with Fc receptors reduces the potential for release of inflammatory mediators (10). Thus, systemic reactions from IVIG infusions might be prevented. Another consequence of the inability of split IgG to bind to Fc receptors is avoidance of immunosuppression which may occur with complexes (8).
It has been reported that F(ab')\textsubscript{2} molecules are superior to unsplit IgG in the elimination of haptens (9) and are equal in their antibacterial and antiviral effects (6). These preliminary observations prompted the present investigations, which better defined in vitro activity of split immunoglobulin. Such studies are essential before clinical application can be recommended.

These experiments were designed to compare and contrast functional capacity of various IVIG formulations. Included were two commercially available in the United States and Europe (Gamimmune-N and Sandoglobulin), one commercially available in Europe but not the United States (Venimun), an investigational 7S-IVIG reported to have exceptional antibacterial activity (BI 61 011), and one 5S-IVIG F(ab')\textsubscript{2} split product (Gamma-Venin) also commercially available in Europe. There were three important findings in these investigations. (i) The 5S immunoglobulin appeared inferior to native material in its activity against a wide spectrum of pathogens. (ii) There was great variation among 7S preparations for individual assays. (iii) No 7S product was consistently superior to others.

Other studies have suggested that F(ab')\textsubscript{1}, is equivalent to native IVIG in functional activity (5, 13). One which examined in vitro opsonic and in vivo protective activity against three enterobacterial strains found that protection by Fab fragments varied among in vivo models (14); they concluded that although there is a consistent loss in phagoctyosis-enhancing activity, this deficiency appears to be of relatively greater importance in eliminating more virulent organisms. Another study also suggested that native immune serum is comparable to the F(ab'), fragment in augmenting the phagoctyosis rate of bacteria (2). In these experiments the Fab/Fc, Fab, and Fc fragments did not enhance neutrophil function. Moreover, mice given fatal intraperitoneal inoculations of bacteria could be protected with either whole IgG or F(ab')\textsubscript{2}. These investigators concluded that the Fc region of the IgG molecule is not predominantly responsible for opsonization.

Direct evidence has suggested that F(ab')\textsubscript{2} fragments can opsonize bacteria since this does not require the Fc portion of the molecule (2). However, the present studies do not support this contention, as CL responses were markedly reduced following opsonization of pneumococci with 5S split IVIG.

Although there were differences among the 7S-IVIG products in individual assays, it is unlikely that such differences would indicate a therapeutic advantage. We and others have been unable to show that exceptionally high in vitro antibody responses for a particular IVIG preparation predict an increase in clinical efficacy (12). It appears important to demonstrate only that antibody to a particular pathogen is present in consistently measurable concentrations.

LITERATURE CITED