Circulating Cytomegalovirus (CMV) Neutralizing Activity in Bone Marrow Transplant Recipients: Comparison of Passive Immunity in a Randomized Study of Four Intravenous IgG Products Administered to CMV-Seronegative Patients


Forty-two cytomegalovirus (CMV)-seronegative bone marrow transplant (BMT) recipients were randomized in a double-blind fashion to receive one of four commercially available intravenous Ig (IVIgG) products (Gamimmune N, Immune Globulin Intravenous, Gammagard, or Sandoglobulin) at a dose of 500 mg/kg every other week. The four treatment groups were similar in distribution of patient ages, weights, autologous versus allogeneic donor type, and underlying diseases. Every other week administration of IVIgG provided total serum IgG levels within the physiologic range for age. CMV titers by latex agglutination were stable (average geometric mean titer of 18.4 after the second IVIgG dose), with no statistically significant differences among the four treatment groups. CMV neutralizing activity (CMVNA) and CMV enzyme-linked immunosorbent assay (ELISA) titers were determined on a subset of sera from 27 study patients representing the four product groups. Patient serum samples obtained before IVIgG infusions and 2 weeks after the second IVIgG dose (ie, 3 weeks post-BMT) were assayed for CMVNA and CMV ELISA titers. Geometric mean titers of CMVNA and CMV ELISA varied among the product groups. The highest mean 50% CMVNA was 1:43 for product B, whereas the lowest mean 50% CMVNA was 1:14 for product A; two of the IVIgG product groups showed intermediate 50% mean titers of 1:27 (product C) and 1:28 (product D) for an overall P = .02. CMV ELISA titers (expressed as Paul Ehrlich International units [PEI U]) also showed the highest mean of 2.95 PEI U/mL for product B and the lowest mean of 1.34 PEI U/mL for product A. Intermediate mean values of 2.27 PEI U/mL and 2.03 PEI U/mL were obtained with products C and D, respectively (overall P = .003). The CMV ELISA titers show a minimal correlation (r = .566) to the observed CMVNA titers. We conclude that commercially available IVIgG products passively exert CMVNA, and that the level of circulating CMVNA is affected by the IVIgG product used.

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cally available IVIgG products commonly used in the United States. In the first phase of the study, patients were treated on a weekly basis with IVIgG, resulting in the accumulation of IgG with all product groups. 

The current report describes antibody studies performed on sera from CMV-seronegative patients receiving 500 mg/kg IVIgG every other week. This dosing schedule maintained stable IgG levels within the physiologic range for age and stable CMV titers by latex agglutination after the second dose. Trough serum levels obtained after the second IVIgG dose during the third posttransplant week were analyzed for CMVNA and CMV enzyme-linked immunosorbent assay (ELISA) titers, providing the first published comparison of circulating, passively transferred functional CMV antibody levels observed with four different commercial IVIgG products.

**MATERIALS AND METHODS**

**Patients and IVIgG administration.** Forty-two consecutive CMV-seronegative marrow transplant recipients were entered into this study between July 1988 and August 1989. Patients were randomized to receive one of four commercially available IVIgG products (product A, Gammimmune N [Miles, Cutter, Elkhart, IN]; product B, Gammagard [Baxter Hyland, Glendale, CA]; product C, Sandoglobulin [Sandoz, East Hanover, NJ]; product D, Immune Globin Intravenous [ARC-Hyland, Glendale, CA]) at a dose of 500 mg/kg every other week for three doses beginning 1 week before BMT. All patients in a given product group received the same lot of IVIgG. IVIgG lots were obtained commercially during the study period and were not preselected in any way. Patients were stratified according to age (≥18 years of age) and autologous versus allogeneic BMT, and randomized to receive products A through D by the BMT database personnel, who were blinded to product identity. Products were dispensed confidentially by pharmacy personnel. Both investigators and patients were masked with respect to the products infused. IVIgG was infused over 2 hours after premedication with diphenhydramine and acetaminophen. All patients received CMV-seronegative blood product support during this study.

**IgG levels.** Serum levels of total IgG and IgG subclasses were determined before each IVIgG infusion in all patients using standard nephelometric methods.

**CMV antibody measurements.** All patients had baseline serum CMV antibody titers determined within 3 weeks before BMT by latex agglutination (Becton Dickinson, Cockeysville, MD). CMV titers of less than 4 by latex agglutination were required for entry onto study. Subsequently, sera were collected at the following time points: (1) on the day of but before the first IVIgG infusion (week −1); (2) before the second IVIgG infusion (week +1); (3) before the third IVIgG infusion (week +3); and (4) 2 weeks after the third IVIgG infusion (week +5), if the patient was still in the hospital. Blood samples were processed within 4 hours of receipt in the laboratory. CMV latex agglutination studies were performed on fresh serum samples in keeping with the standard CMV surveillance protocol at our BMT program. The remaining sera were coded, frozen, and stored at −70°C in the University of Minnesota Clinical Virology Laboratory. At a later date, paired sera representing all patients for whom adequate sterile sera were available both before the first IVIgG infusion (negative control) and before the third IVIgG infusion (week +3 post-BMT) were shipped on dry ice to Baxter Healthcare Corporation (Duarte, CA), where CMV neutralization and ELISA assays were performed on coded samples by M.K. Bechtel and S.A. Strauss, respectively. Coded samples from known CMV-seropositive BMT patients were also submitted for analysis.

Sera from normal CMV-seropositive and CMV-seronegative controls were included in the assays.

**CMV neutralization assay.** The in vitro neutralization bioassay evaluated the CMVNA present in the patient sera by measuring the reduction of cytopathic effect with a laboratory strain of human CMV in human foreskin fibroblast (HF) cells. HF cells obtained from Baxter Bartels Division (Bellevue, WA) were propagated in Minimum Essential Medium (MEM; Whittaker Bioproducts, Walkersville, MD) and 10% heat-inactivated fetal bovine serum (FBS; Hyclone Labs, Logan, UT) in the absence of antibiotics. All HF cells were evaluated for growth rate characteristics and virus sensitivity and then used at passage level 10 in the assay. Cells were seeded in a 24-well plate with 1 × 10⁶ cells/well and incubated at 37°C in a humidified 5% CO₂ atmosphere for 2 days before the assay.

A laboratory strain of human CMV (Towne Strain, ATCC VR-977; American Type Culture Collection, Rockville, MD) was used to grow a stock CMV suspension. The stock suspension was prepared by adding CMV at a multiplicity of infection of approximately 0.05 to freshly split HF cells and by feeding the cells every 3 to 4 days until a 90% cytopathic effect was observed. A cell-free virus suspension was obtained by disruption of the infected cells followed by centrifugation. The suspension was stored in MEM plus 30% FBS and 5% dimethylsulfoxide (Sigma, St Louis, MO) and placed in liquid nitrogen storage. The virus stock used throughout the study had a titer of 5.47 ± 0.2 plaque forming units (PFU)/mL, as measured by plaque assay on HF cells (n = 25).

All serum samples were filtered through 1.2- and 0.22-µm syringe filters (Millipore, Bedford, MA) before use to remove lipid (frequently found in BMT patients receiving IV alimentation) and ensure sterility. The serum samples were heated at 50°C for 30 minutes to inactivate complement. Patient samples from each of four treatment groups were included each time an assay was performed to reduce the potential for bias due to assay to assay variability.

The neutralization plaque assay was performed in HF cells incubated in MEM without FBS with a target range of 10% to 90% viral reduction. Serum samples were prepared in six to eight twofold serial dilutions. Complement was not used in the assay after initial studies showed that the presence of guinea pig complement resulted in variable CMVNA in this bioassay.

Briefly, the neutralization plaque assay was performed by preparing six to eight twofold serial dilutions of each serum sample using MEM without FBS (MEM-SF) to target a range of 10% to 90% virus neutralization. The CMV stock was diluted to obtain approximately 1,500 to 3,500 PFU/mL. Equal volumes of the serum predilutions (0.2 mL) were combined with the diluted CMV (0.2 mL) and incubated for 1 hour at 37°C. After this incubation, the HF cell monolayers were washed with 1 mL MEM-SF, and 0.1 mL of each serum-virus mixture was added to triplicate wells of HF cells. After 2 hours of incubation at 37°C, the fluid was aspirated from the cell monolayers, replaced with MEM containing 3% FBS plus 0.4% agarose (FMC Bioproducts, Rockland, ME), and incubated at 37°C for 7 days. After plaque development was observed, the agarose layers were removed and the monolayers fixed for 30 minutes at room temperature with 1.3% crystal violet stain (Sigma) containing 50% ethanol and 4% formaldehyde in phosphate-buffered saline (PBS). Monolayers were air dried and plaques counted at 13 to 17× magnification. A percent neutralization value was derived for each serum dilution using the formula:

\[
\text{Mean Virus Control Plaque Count} - \frac{- \text{Mean IGV Dilution Plaque Count}}{\text{Mean Virus Control Plaque Count}} \times 100
\]
Assay reproducibility was ensured by using two lots of commercially available IVlgG as controls for each assay. Each control sample had been reassayed at least 20 times to obtain an assigned 50% neutralization titer. A set of assays in this study was considered acceptable if the 50% neutralization titer for the control IVlgG, which were assayed simultaneously with the serum samples, was within an assigned range (mean ± 25%) and the virus control had a demonstrated human CMV (HCMV) titer of 5.27 to 5.67 log_{10} PFU/mL.

**CMV ELISA assay.** Serum antibody titers to CMV were determined using the Cytomegalovirus II Test kit (Whitaker Bioproducts, Inc, Walkerville, MO) in microtiter plates. No significant differences between ELISA values were obtained in test runs using either heated (56°C for 30 minutes) or unheated clinical serum samples. All calibrators were assayed in at least triplicate determinations and all control and test sera were assayed in at least duplicate. When using dilutions in addition to those specified in the kit, no values needed to be extrapolated beyond the standard curve.

**Statistical analysis.** The mean total IgG concentration at each study time point was generated and compared among IVlgG product groups using the Kruskal-Wallis one-way analysis of variance statistic. Similarly, CMV latent agglutination titers expressed as the average geometric mean titers were compared at each time point among IVlgG product groups.

Statistical analyses involving CMV neutralization bioassays and comparison with ELISA titers were performed using the R/Explore statistical software program (BBN Software Products Corp, Cambridge, MA). A neutralization curve was generated from each serum sample by plotting serum dilutions versus the plaque count, and a 50% neutralization titer, expressed in dilution values, was predicted from this curve using curve fitting least squares regression analysis. Results of regression analysis were subsequently analyzed by one-way analysis of variance comparing multiple categorical variables (different IVlgG products) against a single continuous response variable (50% neutralization titer).

**RESULTS**

A summary of patient demographics of each IVlgG product group is presented in Table 1. There were no significant differences among groups in terms of patient age, weight, or pretransplant diagnoses. Eight of the 23 allogeneic recipients had CMV-seropositive donors: product A, 1; product B, 2; product C, 0; and product D, 5. All of the patients were monitored weekly while hospitalized using rapid detection with fluorescent monoclonal antibody for CMV intermediate-early antigen (CMV-IE) in the urine in shell vial cultures and routine CMV cultures in tissue culture of the urine, throat, and stool. All patients were cultured at 100 days posttransplant for CMV when they returned to the clinic for routine follow-up. None of the patients showed evidence of CMV infection during the first 3 months posttransplant. One patient who received product A had a positive urine culture for CMV 1 year posttransplant and two patients who received product D developed CMV from bronchoalveolar lavage and blood at 3.5 months and 11 months posttransplant, respectively; all three had CMV-seropositive donors. Patients who received products B and C remained free of CMV up to 1 year posttransplant, when routine monitoring for this infection ceased.

**Serum IgG levels.** The mean total serum IgG levels before the initial IVlgG infusion and serum levels obtained just before the second and third infusions at weeks +1 and +3 and 2 weeks after the third infusion at week +5 were calculated and compared among recipients of the four IVlgG products (Fig 1). IgG levels were obtained before the first infusion and at weeks 1 and 3 for all study participants. Fewer samples were obtained at week +5 because some patients had been discharged from the hospital or had died. Because only two patients who received product A had week +5 levels drawn, no mean data is presented for this product group at the final comparison point. Patients in all product groups showed evidence of modest IgG accumulation during the study. No significant differences were identified among product groups (P = .2). Additional analyses comparing recipients of autologous versus allogeneic transplants and young (< 18 years of age) versus older patients (≥ 18 years of age) according to product infused also did not show any statistically significant differences.

**CMV antibody titers.** Geometric mean CMV antibody titers determined by latex agglutination at each study time for each product were also compared (Fig 2). A sustained fourfold increase in titers was achieved after the second dose with each product. Although product B produced consistently higher titers at each analysis time (as it had in the earlier IVlgG comparison study using a weekly infusion schedule), there were no statistically significant differences among the study products.

Comparisons of a representative subset of sera for CMVNA and CMV ELISA titers did indicate statistically significant differences referable to the specific products infused.

Median preinfusion CMVNA were less than 1:4 for all product groups (Fig 3). Simultaneous studies of CMVNA
on paired preinfusion and postinfusion sera showed that the median preinfusion CMVNA for all products was less than 1:4, with the following exception: for products A and B, one of the eight pairs of sera from each product tested showed CMVNA in the preinfusion specimen (1:18 and 1:36, respectively). These titers were similar to week +3 CMVNA titers of 1:12, and 1:23, respectively, obtained from the same patients. In addition, two other preinfusion samples tested during the preliminary phase of the study (but for whom postinfusion samples were not available) also showed positive 50% CMVNA of 1:37 and 1:18, thereby showing that 4 of 24 preinfusion samples tested had quantifiable CMVNA. Otherwise, all paired samples showed preinfusion antibody activity below the level of detection and positive CMVNA titers at week +3. Mean 50% CMVNA titers were highest for product B at 1:43 (with a predicted range at 95% confidence level of 1:26 to 1:61), whereas the lowest mean 50% CMVNA was 1:14 for product A (95% confidence level, 1:6 to 1:22); two of the IVlgG product groups showed intermediate 50% mean titers of 1:27 (product C) (95% confidence level, 1:14 to 1:39) and 1:26 (95% confidence level, 1:14 to 1:38) for an overall \( P = .02 \). Fifty percent CMVNA titers in sera from normal CMV seropositive controls ranged from 1:17 to 1:595.

CMV ELISA values were determined on the subset of 25 sera (representing the 4 IVlgG product groups) that was tested for CMVNA: 7 patients who received product A, 8 patients who received product B, 4 patients who received product C, and 6 patients who received product D. Median CMV ELISA for 21 preinfusion samples was 0.13 Paul Ehrlich International Units (PEI U)/mL. The four patient sera that showed CMVNA before IVlgG infusion also showed significant preinfusion CMV ELISA titers: 1:18 (2.76 PEI U/mL), 1:18 (1.78 PEI U/mL), 1:36 (2.87 PEI U/mL), and 1:37 (3.45 PEI U/mL). All patient samples that were negative for CMVNA before IVlgG also had negligible CMV ELISA titers ranging from 0.03 to 0.19 PEI U/mL. Figure 4 illustrates the comparison of postinfusion CMV ELISA titers versus the IVlgG product. Product B again showed the highest CMV titer with a mean of 2.95 ± 1.17 PEI U/mL. The lowest mean of 1.34 ± 0.55 PEI U/mL was obtained with product A, with intermediate mean values obtained with product C (mean 2.27 ± 0.87 PEI U/mL) and product D (mean 2.03 ± 0.75 PEI U/mL) for an overall \( P = .003 \).

DISCUSSION

This clinical study represents the first quantitative comparison of circulating functional anti-CMV activity after passive administration of Ig products into CMV-seronegative individuals. This analysis was performed as part of a larger study investigating the kinetics of two different IVlgG dosing schedules and the comparative antibody titers against other viral pathogens achieved by IVlgG administration to children and adults undergoing BMT.
IVIgG is now widely used in the management of BMT patients for a variety of indications. At our institution, the cost of IVIgG often exceeds the cost of all other medications used in BMT added together. Yet, little information is available regarding the in vivo kinetics of IVIgG in this patient population, the in vivo biologic activity of the antibodies, nor the in vivo comparability of different preparations.

The major objective of this study was to perform an in vivo quantitative comparison of passively transferred functional anti-CMV antibody (ie, viral neutralizing activity) among commercially available IVIgG products manufactured from pooled human plasma by different techniques. The study period was purposely limited to the first few weeks posttransplant. During this period, endogenous IgG synthesis is markedly depressed after cytoreductive therapy and the potential contribution of passively transferred CMV immunity for the subset of recipients with CMV-seropositive donors (3 of 27 recipients: 1 with product A and 2 with product B) is still minimal. The assays were performed on sera obtained at the “trough” of the passive IgG infusion schedule and thereby represent the lower end of the range of passively transferred functional antibody concentrations.

The experimental data obtained indicate that the individual IVIgG products can affect the titer of passively transferred functional antibody. The donor pool (eg, prevalence of seropositivity for CMV and mean titer of CMV neutralizing antibody in the donor population), as well as the manufacturing procedure, may affect the concentration of functional antibody in the final IVIgG product. As commercial products continue to be developed, it is possible that differences within products and among products will continue. In our study, two of the products were manufactured in an identical manner, but from varying plasma sources (products B and D). We cannot draw any conclusions regarding the impact of lot to lot variability on circulating antibodies.

Reports of pharmacokinetic studies of IgG levels and CMV-specific IgG antibodies after IVIgG administration for BMT patients have indicated a wide range of half-life estimates for both parameters, which lends credence to the possibility that host factors, such as nutritional and infectious status, and changes in function of the reticuloendothelial system after cytoreductive therapy may further alter the fate of passively infused antibodies. Our findings have established the feasibility of detecting functional anti-CMV antibodies in the circulation of BMT patients who receive passive IgG transfusion and of detecting differences in relatively small subsets of patients. However, no conclusions as to the protective efficacy can be drawn from this relatively small study.

We conclude that all commercially available IVIgG products provide functional anti-CMV activity that can be detected in vivo, but that discernable differences in titers of neutralizing activity can be related to the product administered. Additional studies to determine optimal dosing schedules based on the determination of functional CMV antibody titers in seronegative or seropositive patients receiving either standard polyclonal IVIg products, hyperimmune sera, or monoclonal anti-CMV antibodies should be performed in vivo and correlated with clinical outcomes.

REFERENCES

Circulating cytomegalovirus (CMV) neutralizing activity in bone marrow transplant recipients: comparison of passive immunity in a randomized study of four intravenous IgG products administered to CMV-seronegative patients

AH Filipovich, MH Peltier, MK Bechtel, CL Dirksen, SA Strauss and JA Englund