In vitro evaluation of cytomegalovirus-specific hyperimmune globulins vs. standard intravenous immunoglobulins

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Background and Objectives To evaluate standard intravenous immunoglobulin (IVIG) as an alternative to intravenous cytomegalovirus hyperimmune immunoglobulin (CMVIG) for prophylaxis and therapy of cytomegalovirus (CMV) disease, we measured the ELISA and neutralizing titres of CMV-specific antibodies in CMVIG and IVIG preparations.

Materials and Methods Anti-CMV-IgG ELISA and neutralizing titres (fibroblast-based test) in CMVIG CG (Cytogam®, n = 20), CMVIG CT (Cytotect®, CP, n = 3), IVIG P (Privigen®, n = 32) and IVIG K/G (Kiovig®/Gammagard®, n = 5) were compared, and IgG subclasses 1–4 were determined by nephelometry.

Results Cytomegalovirus hyperimmune immunoglobulins contained more than fourfold higher CMV ELISA and CMV-neutralizing activity per gram of IgG than the standard IVIGs. Pooled data for all four products showed a significant correlation between anti-CMV-IgG ELISA and neutralizing titres (r = 0.93, P < 0.001). There was a good correlation between the IgG3 content and CMV-neutralizing antibodies amongst lots of CMVIGs (r = 0.91, P = 0.01), but this did not extend to the IVIGs. CMVIG CG contained the highest CMV-neutralizing activity (3497 ± 395 PEIU/g IgG) of any product tested.

Conclusion The higher anti-CMV neutralization capacity of CMVIG per gram of IgG vs. standard IVIG suggests that standard IVIGs are not equivalent to or interchangeable with CMVIG.

Key words: antibodies, cytomegalovirus, hyperimmune globulin, immunoglobulins, neutralizing, transplantation.

Introduction

Human cytomegalovirus (CMV) is a ubiquitous herpesvirus that infects 60–100% of humans [1]. Immunocompromised individuals, such as those receiving organ or tissue transplants, are at increased risk of severe CMV disease. CMV is one of the most important post-transplant pathogens, causing disease in up to 50% of solid organ transplant recipients (depending on the organ) and resulting in significant morbidity and mortality [2–4]. At greatest risk are CMV-sero-negative recipients of organs from CMV-sero-positive donors (D+/R−). Antiviral agents, such as intravenous ganciclovir and oral valganciclovir, are the first-line drugs for prevention and treatment of CMV infection and disease in transplant recipients [4–7], but the development of drug resistance often limits their usefulness [8]. Other problems with antiviral chemotherapy include toxicity, such as bone marrow suppression and renal failure [9–11], multidrug interactions and lapses in patient adherence to treatment [11].

There is thus a need for other approaches for preventing and treating CMV. Although not currently used as a first-line antiviral agent in transplant patients, intrave-
nous CMV hyperimmune globulin (CMVIG), derived from human plasma with high anti-CMV antibody titres, is a valuable option [9, 10]. In the USA, Cytogam® (CMVIG CG; CSL Behring, Bern, Switzerland) is licensed for the prophylaxis of CMV disease associated with transplantation of kidney, lung, liver, pancreas and heart [12]. In Europe, Cytotect® CP (CMVIG CT; Biotest, Dreieich, Germany) is licensed for the prophylaxis of CMV infection in patients receiving immunosuppressive therapy, particularly transplant patients [13]. In 2008, a meta-analysis of 11 randomized trials involving 698 solid organ transplant recipients found that prophylactic CMVIG improved overall graft and patient survival and reduced CMV-associated disease and deaths [14]. More recently, analyses of large data sets from the Scientific Registry of Transplant Recipients have shown an association between the use of CMVIG and increased patient survival and reduced CMV-associated disease and deaths [14]. More recently, analyses of large data sets from the Scientific Registry of Transplant Recipients have shown an association between the use of CMVIG and increased long-term survival of patients and grafts after liver transplantation [15] and after heart transplantation [16].

Previous studies compared titres of CMV-specific antibodies in CMVIG and standard intravenous immunoglobulin (IVIG) preparations [17, 18]. Planitzer et al. [18] reported that one CMVIG preparation contained higher CMV-IgG concentrations, as measured by ELISAs, than did standard IVIG preparations. Surprisingly, however, the standard IVIG preparations contained significantly higher titres of CMV-neutralizing antibodies than did the CMVIG preparation [18]. Furthermore, Planitzer et al. reported that the proportions of IgG3, which appears to be the most relevant subclass for neutralizing CMV [17, 19], were also higher in the standard IVIG preparations [18]. Planitzer et al. [18] concluded that standard IVIGs were suitable alternatives to CMVIG. However, Gupta et al. [17] had previously reported that a different CMVIG preparation contained higher CMV-neutralizing titres than standard IVIG. Many investigators and regulators consider neutralizing activity more relevant than binding assay results, so these differences may be important.

To identify the reasons for the differences between these reports, we analysed ELISA titres, CMV-specific neutralizing activity and IgG3 content in multiple lots of currently available CMVIG preparations and two widely used standard IVIG preparations. We used ELISA and fibroblast (MRC-5)-based neutralization assays similar to those used by Planitzer et al. [18] and Gupta et al. [17].

Materials and methods

Immunoglobulin preparations

The CMV-IgG reference standard [12/1996], with a potency of 110 U/ml determined by ELISA and a protein concentration of 49.3 mg/ml, was obtained from the PEI (Paul Ehrlich Institute, Langen, Germany). As no international reference standard is currently available for CMV-neutralization assays, this ELISA reference standard is also used for the neutralization assays and is assigned 110 NT-units (U_{NT})/ml.

Cytomegalovirus hyperimmune immunoglobulin preparations used were Cytogam® (CMVIG CG; CSL Behring) [12] and Cytotect® (CMVIG CT; Biotest) [13], both 5% liquid IVIG preparations prepared from donated plasma with high titres of antibodies against CMV. CMVIG CG contains 5% sucrose and 1% albumin as stabilizers, in addition to the labelled content of IgG. CMVIG CT contains glycine as stabilizer.

Standard IVIG preparations used were Privigen® (IVIG P; CSL Behring) [20] and Kiovig®/Gammagard® (IVIG K/G; Baxter AG, Vienna, Austria) [21], both 10% liquid IVIG preparations. IVIG P contains 250 mmol/l l-proline as stabilizer. IVIG K/G contains 0.25 mol/l l-proline and 2 mol/l H2SO4. DRG Instruments GmbH). As no international reference standard is currently available for CMV-neutralization assays, this ELISA reference standard is also used for the neutralization assays and is assigned 110 NT-units (U_{NT})/ml.

Anti-CMV-IgG ELISA

Quantitative determination of anti-CMV-IgG antibody titres in the Ig preparations was carried out using a commercially available ELISA (DRG Diagnostics Cytomegalovirus IgG ELISA; DRG Instruments GmbH, Marburg, Germany), as per the manufacturer’s instructions. Twofold dilutions (ranging from 1:800 to 1:6400) of the IgG preparations, including a CMV negative control serum, were added to microtiter plate wells precoated with an inactivated CMV antigen mixture (strain AD-169), containing CMV antigens from all parts of the virus cycle of replication including early and late antigens, nuclear antigen, cytoplasmic antigen, structural and non-structural proteins, and incubated for 60 min at 37°C. Following removal of unbound antibodies by washing the wells five times with Wash Solution (DRG Instruments GmbH), anti-human IgG antibodies conjugated to horseradish peroxidase (HRP) were added and incubated for 30 min in the dark at room temperature. The wells were washed as before, and complexes were detected by addition of HRP-substrate [tetramethylbenzidine (TMB)]. After 10 min incubation at room temperature, protected from light, the reaction was stopped (Stop Solution, 0.2 mol/l H2SO4; DRG Instruments GmbH) and the absorbance of the product was measured at 450 nm in a TECAN Sunrise Reader (Tecan, Männedorf, Switzerland). A standard curve was prepared by sigmoidal curve fit using a secondary standard calibrated against the anti-CMV PEI standard. For samples, results in PEIU/ml are calculated from sample OD values obtained at different dilutions, by comparison with the standard curves. The coefficient of variation was calculated from replicate measurements.
Anti-CMV neutralization test

A fibroblast-based neutralization test was used to quantify the anti-CMV potency of each Ig preparation. This approach has been described in detail elsewhere [22]. Serial dilutions (1:38-fold dilutions ranging from 1:105 to 1:1904) of each Ig preparation or control were added to microtiter plates along with 2500 TCID50/well of human CMV strain AD-169 (American Type Culture Collection – ATCC VR-538), and the plates were incubated at 37°C in a 5% CO2 incubator for 4 h. Human fetal lung fibroblasts (15 000 MRC-5 cells/well, ATCC CCL-171) were added, and the plates were incubated as above for 3–4 days. The cells were fixed in 4°C methanol for 20 min at room temperature and washed with Tris-buffered saline (TBS) three times. Non-specific binding sites were blocked for 45 min at room temperature with 0.1% Triton-X-100/1% BSA in TBS. The fixed cells were incubated for 90 min at room temperature with two anti-CMV antibody clones DDG9 + CCH2 (Dako, Glostrup, Denmark) directed against viral antigens (early nuclear protein of 76 kDa) and washed four times with TBS/0.1% Triton-X-100. Secondary antibody coupled with HRP was then added, and following 90 min incubation at room temperature, the wells were washed as before. The complex was detected by the addition of HRP-substrate (TMB One Component Microwell Substrate; Southern Biotech, Birmingham, AL, USA) and incubation at room temperature in the dark for 20 min, which yielded a blue coloration in the presence of HRP. By addition of TMB Stop Solution (Southern Biotech), a stable yellow end product was formed which was quantified by absorbance at 450 nm (TECAN Sunrise Reader).

The 50% neutralization dose (ND50) was calculated using a four-parameter logistic mixed effects model, one parameter of which was the ND50 value. Parallelism was assessed by means of equivalence testing (equivalence limits for upper and lower plateaus and slope of the curve at the ND50). The relative potency for preparations with statistically parallel curves was determined by comparison with an in-house standard reference preparation calibrated against the PEI reference standard.

IgG nephelometry

For each of the CMVIG and standard IVIG preparations, the content of IgG subclasses 1–4 was determined by nephelometry, using the BN Prospec automated nephometer system and reagents as per the manufacturer’s instructions (Siemens’s Healthcare Diagnostics AG, Zurich, Switzerland). Briefly, IgG subclass antibodies contained within randomly selected samples of each Ig preparation were agglutinated through mixing in a cuvette with specific antisera to human IgG1 or IgG2 or with polystyrene particles coated with anti-human IgG3 or IgG4 antibodies. Calculation of individual IgG subclass concentrations was based on standard curves prepared with a commercial standard (N Protein Standard SL No. 001M13; Siemens Healthcare Diagnostics AG) calibrated against an International Standard (CRM-470; Siemens Healthcare Diagnostics AG).

Statistical methods

All results are presented as mean ± standard deviation (SD), and the number of different lots (n) of each Ig preparation analysed is stated. Statistical analyses were performed using Excel™ (Microsoft, Washington, DC, USA). Anti-CMV-IgG and neutralizing antibody titre were compared between CMVIG and standard Ig preparations using Student’s t-test (unpaired, two-tailed), and P-values are stated (significance level < 0.05). All association analyses were determined through Pearson’s correlation; the correlation coefficient r and P values are stated (significance level < 0.05).

Results

All results are expressed per gram of IgG as indicated in the product label, to allow comparison of potency of products with different concentrations, and are presented as mean ± SD, with ‘n’ indicating the number of different lots of each Ig preparation analysed.

Anti-CMV-IgG ELISA titres

Cytomegalovirus-IgG concentrations were determined for each CMVIG and standard IVIG preparation with a commercially available CMV-IgG ELISA, using plates precoated with CMV antigen, with reference to a calibration curve derived with a standard obtained from the PEI. Both of the hyperimmune CMVIG products – CMVIG CG and CMVIG CT – contained more than fivefold higher CMV-specific antibodies per gram of IgG by ELISA assay than did either standard IVIG product (Fig. 1): CMVIG CG (2141 ± 323 PEIU/g IgG; n = 20) and CMVIG CT (2304 ± 255 PEIU/g IgG; n = 3) vs. IVIG K/G (309 ± 60 PEIU/g IgG; n = 5) and IVIG P (386 ± 108 PEIU/g IgG; n = 31; CMVIG products vs. standard IVIG products; P = 0.002, Student’s t-test, unpaired, two-tailed). The CMV binding ELISA titres of CMVIG CG and CMVIG CT were not significantly different from each other (P = 0.42).

Anti-CMV neutralization titres

Anti-CMV-neutralizing IgG titres (NT) were determined for each Ig preparation using a fibroblast-based neutralization test. Contrary to the findings previously reported
by Planitzer et al. [18], who compared the previously marketed product CMVIG CT with IVIG K/G and Gammar S/D; we found that the CMVIG products, CMVIG CG (CMV NT = 3497 ± 395 PEIU/g IgG; \( n = 20 \)) and CMVIG CT (CMV NT = 2613 ± 568 PEIU/g IgG; \( n = 3 \)), contain significantly more CMV-neutralizing antibodies per gram of IgG than either IVIG K/G (CMV NT = 742 ± 167 PEIU/g IgG; \( n = 5 \)) or IVIG P (CMV NT = 663 ± 154 PEIU/g IgG; \( n = 32 \); CMVIG products vs. standard IVIG products; \( P = 0.034 \), Student’s t-test, unpaired, two-tailed) (Fig. 2). CMVIG CG contained the highest amount of CMV-neutralizing antibodies, with mean titres five times higher than IVIG P, the standard IVIG made by the same manufacturer. This content of neutralizing antibodies in CMVIG CG was also higher than found in CMVIG CT (\( P = 0.0025 \)), but both hyperimmunes had much higher titres than either standard IVIG product (\( P < 0.001 \) vs. IVIG K/G and IVIG P). Pooled data for all four products suggest a strong correlation between anti-CMV-IgG neutralizing titres and specific anti-CMV-IgG concentrations as measured by ELISA (Fig. 3: correlation coefficient \( r = 0.93, P < 0.001, n = 59 \)). However, this calculation may be heavily weighted by the clustering of results for the standard and hyperimmune preparations, respectively. In any case, it seems clear that the different mean neutralizing titres in CMVIG CG vs. CMVIG CT do not correlate with differences in their ELISA titre results, which overlap with each other.

IgG subclass distribution

Human IgG subclasses have different physiochemical and biological properties, and IgG3 has been reported to be the most relevant subclass for CMV-neutralizing capacity in vitro [17, 19]. Distributions of IgG subclasses 1–4 were therefore determined by nephelometry for each preparation. Comparison amongst products indicated that CMVIG CG contained a higher proportion of IgG3 (as percentage of total IgG), than did CMVIG CT (\( P = 0.0027 \), although the proportion of IgG3 was similar to that in IVIG K/G (\( P = 0.17 \)) (Fig. 4). However, although there was a

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Fig. 4  IgG3 content (% of total IgG) of cytomegalovirus (CMV) hyperimmune globulins and standard intravenous immunoglobulin (IVIG) preparations. Student’s t-test (unpaired, two-tailed): CMVIG CG vs. CMVIG, $P = 0.0027$; and CMVIG CG vs. IVIG K/G, $P = 0.17$. *The IgG3 level for IVIG P is mean value [20]. Data are for total IgG3 levels, not anti-CMV IgG3 alone. $n$, number of different lots of each Ig preparation; SD, standard deviation.

Discussion

The data presented clearly demonstrate that the CMV hyperimmune globulin products CMVIG CG and CMVIG CT have higher titres of anti-CMV antibodies per gram of IgG than the standard IVIG products IVIG K/G and IVIG P, by both ELISA and neutralization assays. These results mirror the findings of Gupta et al. [17] but not those of Planitzer et al. [18], although similar assay methods were used in all three studies. Planitzer et al. [18] reported that a previously marketed CMVIG preparation contained higher concentrations of IgG which bound to CMV, as measured by ELISAs, than did standard IVIG preparations with equivalent total IgG concentrations. Surprisingly, however, the standard IVIG preparations contained significantly higher titres of CMV-neutralizing antibodies when compared to the CMVIG preparation [18]. We analysed CMVIG CT (50 mg/ml IgG and 100 PEIU/ml) whereas Planitzer et al. analysed the previously marketed preparation, Cytotect® (CMVIG C – 100 mg/ml IgG and 50 PEIU/ml). Differences in the plasma sources, initial fractionation, subsequent purification and formulation procedures may account for some of the differences in the results. CMVIG CT contained a somewhat higher ELISA titre of anti-CMV antibodies but CMVIG CG demonstrated better CMV-neutralizing activity. The protective activity of neutralizing antibody titres remains to be fully defined, but these have been considered as criteria for the adequacy of antiviral activity of human immune globulin products [23, 24]. Therefore, the neutralizing antibody titre may be more clinically relevant than the ELISA titre.

The cellular substrate for the neutralizing antibody assays in our study and the Planitzer [18] study was the same: MRC-5 fibroblasts. This assay mainly measures inhibition of entry of free virus into the cells rather than cell–cell spread, but was selected to allow comparisons between the studies. Cell–cell spread may be more important in epithelial cell-based assays [24, 25] and in treatment rather than prophylaxis in vivo. Whilst infection of fibroblasts is mediated by gB, gH/gL/gO and gM/gN glycoprotein complexes, infection of epithelial and other cell types requires the pentameric gH/gL/UL128/UL130/UL131 complex [24, 26–28]. Indeed, it has been shown that epitopes within gB can comprise up to 50% of the fibroblast-based neutralizing activity [29]. Recent results indicate that the majority of CMV-neutralizing antibodies in CMVIG CG are directed against the pentameric gH/gL/UL128/UL130/UL131 complex rather than glycoprotein gB [26]. CMV can also enter myeloid cells via gB complexes and establish a latent non-productive infection [30]. Reactivation of latent CMV causes a significant proportion of CMV disease in transplant recipients, and the risk is highest in D+/R− patients, especially those who
have received heart, lung or heart–lung transplants [4]. Even at subclinical levels of infection, CMV may cause allograft damage by activating immune-evasion mechanisms and pro-inflammatory pathways leading to endothelial cell injury [4].

In our study, the CMV-neutralizing activity was not tested in epithelial cells; however, the high CMV-neutralizing activity of CMVIG demonstrated herein, together with the high potency of these preparations in preventing infection of epithelial and endothelial cell types previously shown, is believed to be particularly important, given the ability of the virus to infect multiple cell types [24, 26, 31]. The broad cellular tropism of CMV may facilitate transmission of the virus from the graft or passenger lymphocytes to the host and may favour systemic spread and efficient replication, leading to wide dissemination of CMV which can result in infection of multiple organs in the immunosuppressed recipient [31].

Cytotoxic T lymphocytes have been identified as the key host defence against CMV infection in transplant recipients [32–34]. Although humoral immunity cannot prevent infection when cell-mediated immunity is severely impaired [33, 34], the value of prophylactic CMVIG therapy has been demonstrated in several studies briefly described below. The most widely accepted clinical use of CMVIG is for the prophylaxis of CMV infection in recipients of solid organ transplants involving kidney, lung, liver, pancreas or heart [12]. There is a long history of use of CMVIG for CMV prophylaxis in transplantation and this continues today, usually in combination with ganciclovir. A 50% reduction in primary CMV disease in kidney transplant patients who received CMVIG has been reported [10]. In liver transplant patients who received CMVIG there was a 56% reduction in serious CMV disease [9], and CMVIG prophylaxis was associated with increased survival [32]. In D+/R– transplants of organs other than kidneys, prophylactic CMVIG should be considered in combination with ganciclovir [12]. Current guidelines for the management of CMV in transplant recipients recognize that there is limited data for the prophylactic use of CMVIG alone [4]. However, in some settings, such as heart and lung transplantation, guidelines recommend CMV prophylaxis with valganciclovir or ganciclovir, with or without CMVIG [6]. CMVIG is also used as a therapy during hematopoietic stem cell transplantation [35] and in countries other than the USA to prevent or treat fetal CMV infection [36–38]. In a recent study involving 123 women, it was shown that treatment with CMVIG CT did not significantly modify CMV infection during pregnancy [39]. Fetal CMV infection is the leading cause of mental retardation and sensorineural deafness [40, 41], and the NIH is currently conducting a multicenter study in the USA of the use of CMVIG CG to prevent fetal infection [42].

Human IgG subclasses have different physiochemical and biological properties, and IgG3 has been reported to be the most relevant subclass for CMV-neutralizing capacity in vitro [17, 19]. The physiological distribution of the IgG subclasses in CMVIG CG is nearly that of normal serum [43–45]. Our nephelometric analyses indicate that this preparation contains the highest proportion of IgG3 of any of the products tested. Planitzer et al. [18] suggested that improved manufacturing procedures may ‘better preserve the physiological IgG subclass distribution’ as an explanation for the higher neutralizing titres they observed with IVIG K/G and Gammagard S/D® [18]. However, in our study, the standard IVIG products demonstrated lower CMV-neutralizing antibody titres per gram of IgG than did either of the CMV hyperimmune globulin preparations. The anti-CMV-IgG neutralizing titres of the CMVIG CG and CMVIG CT preparations seem to correlate with IgG3 content in different lots of those products, but that relationship did not include IVIG K/G. The latter preparation has a much lower neutralizing titre than its IgG3 content might suggest, in comparison with CMVIG CG and CMVIG CT. This suggests that selection of plasma donors with high CMV titres (>6–7 PEIU/ml as determined by ELISA, in comparison with a reference standard from PEI) has more impact on the activity of the final product than the manufacturing procedures [12, 45]. Importantly, the results show that preservation of IgG3 levels in the final product cannot provide assurance of high CMV titres or neutralizing activity.

The small number of samples, especially for IVIG K/G and CMVIG CT, is a limitation of our study, but the degree of lot-to-lot variation was not large compared to the differences between the standard and hyperimmune products. The actual assay methods used by Planitzer et al. [18] were not identical to ours. Furthermore, we analysed the current product, CMVIG CT (50 mg/ml IgG; 100 PEIU/ml), whereas the Planitzer study [18] used the previously marketed product, CMVIG C (100 mg/ml IgG; 50 PEIU/ml). Both studies are small in vitro investigations whose clinical relevance is uncertain. Although ideally, randomized controlled trials of CMVIG vs. standard IVIG should be performed, this is unlikely, and in vitro comparisons may continue to provide the only available results.

In summary, our results clearly demonstrate higher anti-CMV neutralization capacity per gram of IgG for CMVIG preparations as compared to standard IVIG products, even though titres of different products may vary [46]. Thus, the conclusions that standard IVIG products are functionally equivalent to CMVIG preparations and that administering equivalent amounts of CMV antibodies based on ELISA titres will have equivalent clinical efficacy lack validation. The use of much higher doses of
relatively low titered standard IVIG preparations to achieve adequate protection against CMV may increase the risks of adverse effects associated with IVIG therapy, which are more likely when very high doses are administered [20, 21, 47].

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Conflict of interests

DRS received consulting fees from CSL Behring, Millennium, Genzyme, Genentech, Chimerix, Merck, AstraZeneca, Seres Health and Microbiotix within the past year. SMM, TMH, MK, PL, JLV and MB are employees of CSL Behring.

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