Antibodies against the gH/gL/UL128/UL130/UL131 Complex Comprise the Majority of the Anti-Cytomegalovirus (Anti-CMV) Neutralizing Antibody Response in CMV Hyperimmune Globulin

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Anti-cytomegalovirus (anti-CMV) hyperimmune globulin (HIG) has demonstrated efficacy in preventing CMV disease in solid-organ transplant patients as well as congenital disease when administered to pregnant women. To identify the neutralizing component of cytomegalovirus hyperimmune globulin (CMV-HIG), we performed serial depletions of CMV-HIG on cell-surface-expressed CMV antigens as well as purified antigens. Using this approach, we demonstrate that the major neutralizing antibody response is directed at the gH/gL/UL128/UL130/UL131 complex, suggesting little role for anti-gB antibodies in CMV-HIG neutralization.

The mechanism by which naturally acquired immunity confers protection against cytomegalovirus (CMV) infection is unknown, but antibody response is likely to play an important role. Nigro et al. reported that in a nonrandomized prospective study, passive immunization with anti-CMV hyperimmune globulin (CMV-HIG) showed promising results for the treatment and prevention of congenital infection (8). In addition, preconception maternal immunity to CMV provides considerable protection against vertical transmission, likely due to the presence of neutralizing antibodies (3). Administration of CMV-HIG has also been shown to be effective in preventing CMV disease in solid-organ transplant (SOT) patients (14, 15, 20). Many studies have shown that human sera can react with a broad spectrum of CMV proteins from either purified virus or virus-infected cells (1, 2, 10, 13). However, binding to viral components does not necessarily confer protection. CMV uses two different entry mechanisms to infect fibroblasts, epithelial cells, endothelial cells, and macrophages. Fibroblast entry is mediated by glycoprotein complexes gB, gH/gL/gO, and gM/gN, whereas entry into epithelial cells, endothelial cells, and macrophages requires the addition of the gH/gL/UL128/UL130/UL131 complex (5, 6, 12, 17–19). While it has been reported that the anti-gB response correlates with neutralizing activity, these studies have been restricted to study of fibroblast entry using fibroblast-tropic strains lacking a functional locus containing genes encoding UL128 to UL131 (UL128–131). In addition, these studies drew conclusions from a small number of seropositive individuals, whose neutralizing immune responses may not be representative of the population as a whole (2, 7). More recently, several studies have suggested a positive correlation between a highly potent neutralizing antibody response and the presence of antibodies in serum against the UL128–131 gene products (4, 16).

With the aim of reconciling these different observations and identifying the neutralizing component of CMV-HIG, we performed serial depletions of CMV-HIG on cell-surface-expressed CMV antigens as well as purified antigens and evaluated the neutralizing potency of depleted CMV-HIG. In this study, we used CMV-HIG (Cytogam; CSL Behring) pooled from >1,000 infected individuals. Due to the size of the pool, we believe that this IgG preparation captures the anti-CMV

immune response of the infected population. Our results showed that the most significant portion of the neutralizing activity derives from antibodies against the gH/gL/UL128/UL130/UL131 complex and not gB.

Our key goal was to assess the potency contribution of specific anti-CMV antibody populations on different cell types. Consistent with others, we have found that CMV-HIG has a much higher potency on epithelial cells, endothelial cells, and monocyte-derived macrophages than on fibroblasts (Fig. 1) (4, 16). Epithelial cells and fibroblasts (ARPE-19 and MRC-5, respectively) (American Type Culture Collection [ATCC], Manassas, VA) and P0 human umbilical vein endothelial cells (HUVECs) (Lonza, Basel, Switzerland) were grown using the manufacturer's suggested conditions. Human monocyte-derived macrophages (MDM) were generated from monocytes isolated from a human donor using Rosettesep human monocyte enrichment cocktail (Stemcell Technologies, Vancouver, Canada) and differentiated into macrophages by overnight treatment with lipopolysaccharides (LPS). In this *in vitro* neutralization assay, CMV-HIG was mixed with the low-passage-number clinical CMV strain VR1814 (11) such that the final virion concentration resulted in approximately one infectious virus per cell (i.e., multiplicity of infection [MOI] of 1). The MOIs for the different cell types were ascertained by determining the titer of the same viral stock on each cell type (e.g., epithelial infected cells were infected with an MOI that was determined from the titer on epithelial cells). Infection proceeded for 18 h at 37°C prior to fixation and staining for the immediate-early (IE) nonstructural antigen using an anti-IE antibody (Mab810; Millipore) and Hoechst nuclear counterstain. In this assay, CMV-HIG was greater than 100-fold more potent (comparing 50% effective concentrations [EC₅₀s]) at blocking viral entry into epithe-

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	EC ₅₀ µg/ml	EC ₉₀ µg/ml
Fibroblasts	157	590
Epithelial cells	1.3	9.1
Endothelial cells	1.3	9.1
Macrophages	1.1	7.4

FIG 1 Neutralization of VR1814 by CMV-HIG on 4 cell types. CMV-HIG was mixed with the low-passage-number clinical CMV strain VR1814 (11) such that the final virion concentration resulted in approximately one infectious virus per cell (i.e., an MOI of 1). The four cell types studied were fibroblasts, epithelial cells, endothelial cells, and macrophages. At 18 h postinfection, the cells were fixed in ethanol, blocked with PBS containing 2% bovine serum albumin (BSA), and stained with anti-IE antibody (Mab810; Millipore), anti-mouse Alexa Fluor 488, and Hoechst stain (Invitrogen). The cells were imaged and counted using the ImageXpress Micro and MetaXpress software (Molecular Devices). Data from two independent experiments were graphed using Prism (GraphPad Software). The concentration of CMV-HIG (in micrograms per milliliter) is shown on the x axis of the graph. EC₅₀ and EC₉₀ values were calculated from the best-fit curves for each experiment using Prism EC₅₀ curve-fitting algorithm and then averaged.

lial cells, endothelial cells, and macrophages than blocking viral entry into fibroblasts (Fig. 1). Given that CMV utilizes different modes of entry into epithelial cells, endothelial cells, and macrophages versus fibroblasts, we hypothesized that the discrepancy in neutralizing potency may be due to antibodies in CMV-HIG against the gH/gL/UL128/UL130/UL131 complex.

To address this hypothesis, we expressed gH/gL or gH/gL/UL128/UL130/UL131 or gB on the surfaces of human primary epithelial cells (ARPE-19) using adenoviral gene delivery. We utilized antigens displayed on cells for the serial depletion of CMV-HIG rather than soluble gH/gL/UL128/UL130/UL131 complex due to the challenges of making significant quantities of homogeneous complex. In addition, the literature suggests that conformational epitopes of viral glycoprotein complexes may be more native on cells than soluble antigens (9). To express antigen for the depletions, epithelial cells were transduced with adenovirus expressing gB or gH/gL or with a vector (mock transduced) or cotransfected with gH/gL and UL128/130/131 for 72 h resulting in >50% of cells expressing gB and >80% of cells expressing gH/gL or gH/gL/UL128/UL130/UL131 (Fig. 2).

To deplete CMV-HIG of specific antibody populations, CMV-HIG (Cytogam) (50 mg/ml stock concentration) was diluted to 40 µg/ml in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and was serially incubated (4 times) with 4.5×10^6 to 6×10^6 transduced, dissociated ARPE-19 cells expressing CMV glycoproteins (gB, gH/gL, gH/gL/UL128/UL130/UL131 or mock depleted) for 1-h

incubations. The extent of the depletion of CMV-HIG was determined by an enzyme-linked immunosorbent assay (ELISA) (Table 1). Analysis of the absorbed serum showed that most of the antibodies reactive with gB were absorbed by this procedure (91% depletion on cells expressing gB versus 0% on control cells [Table 1]). Absorption of the gH/gL or gH/gL/UL128/UL130/UL131 antibodies from CMV-HIG was not as efficient (additional incubations did not deplete antibodies further) but still depleted the majority of the specific antibodies from the serum (67% and 79% depletion on cells expressing gH/gL or gH/gL/UL128/UL130/UL131, respectively, versus 0% on control cells [Table 1]). We compared the ELISA results with the results of quantitative fluorescence-activated cell sorting (FACS) (e.g., depletions analyzed by binding to cells expressing specific glycoproteins) and found that the results were consistent (data not shown).

The depleted CMV-HIG was evaluated for potency by neutralization assay on epithelial cells and compared with the mock depletion (cells not expressing CMV antigens) to control for non-specific antibody loss (Fig. 3A). CMV-HIG that was depleted of gB antibodies or gH/gL antibodies did not affect its neutralizing activity on epithelial cells (Fig. 3A). In contrast, removal of gH/gL/UL128/UL130/UL131-specific antibodies decreased the neutralization EC₅₀ titer 85% when tested on epithelial cells, despite partial depletion (Fig. 3A and Table 1).

Evaluating the potency of this material on fibroblasts was not possible because of the very high concentration needed to detect neutralization; the EC₅₀ of CMV-HIG is approximately 500 µg/ml on this cell type (refer to Fig. 1). To make sufficient antigen, we utilized the baculovirus expression system. Since the UL128, UL130, and UL131 proteins are not required for entry into fibroblasts and gH/gL/gO could not be expressed at sufficient levels, soluble gB or gH/gL was used for these depletions. Both soluble gB and gH/gL bound antibodies that recognize nonconformational and conformational epitopes, suggesting that these proteins were folded properly (data not shown). Purified recombinant proteins (1 to 2 mg of soluble gB or gH/gL) were chemically coupled to Sterogene ALD Superflow resin via sodium cyanoborohydride (0.2 ml of 1 M solution) and dialyzed against PBS overnight. The individual resins were loaded into columns and extensively washed with PBS to remove any unbound protein. Two milligrams of CMV-HIG (in a volume of 800 µl) was loaded onto the individual columns, and the flowthrough was collected in 0.5-ml aliquots, concentrated to 100 µl, and analyzed by a neutralization assay (Fig. 3B). With nearly complete depletion of anti-gB antibodies (95% depletion of gB antibodies on the gB column versus 0% depletion of gB antibodies on the gH/gL column), no reduction in EC₅₀ was observed on fibroblasts (Table 2 and Fig. 3B). However, with the majority of anti-gH/gL antibodies depleted (84% depletion of gH/gL antibodies on the gH/gL column versus 0% depletion of gH/gL antibodies on the gB column), a 65% reduction in EC₅₀ was seen on fibroblasts (Fig. 3B and Table 2).

These data strongly suggest that the major neutralizing antibodies for epithelial cell entry in CMV-HIG are directed at the gH/gL/UL128/UL130/UL131 complex and the gH/gL antibodies in CMV-HIG have a dominant role in inhibition of viral entry into fibroblasts. While it is accepted that the majority of the antiglycoprotein response is directed toward gB, these ex-

FIG 2 Epithelial cells express adenovirus-encoded CMV glycoproteins on their surfaces. ARPE-19 cells were transduced with adenovirus encoding either gB or gH/gL (at a multiplicity of infection [MOI] of 150) or cotransfected with adenoviruses encoding gH/gL (at an MOI of 100) or UL128/130/131 (at an MOI of 150). After 3 days, the cells were dissociated using Accutase, stained with the following primary antibodies: anti-gH (α -gH) (MSL-109), anti-gB (α -gB) (ITC-88), affinity-purified rabbit anti-UL130 (SPWFTLTANQNPPWSKLTYKPHDC), or affinity-purified rabbit anti-UL131 (TAEKNDYYRVP HYWDACSRALPDQTRYK). The cells were stained with the appropriate secondary antibody conjugated to allophycocyanin (APC) (Jackson ImmunoResearch). Fluorescence of individual cells was measured using FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star). Adenoviral vectors were constructed to contain CMV gB, gH and gL, or UL128, UL130, and UL131. gH and gL were cloned into a pRK tk Neo vector separated by a “self-cleaving 2A peptide” (15a). The entire transcript was then cloned into pShuttle-UBC-TO-EV and recombined into pAd-pl-DEST using the ViraPower system (Invitrogen). UL128, UL130, and UL131 were amplified from cDNA and cloned into pRK tk Neo vector, each gene separated by a “self-cleaving 2A peptide.” The entire coding sequence of UL128, UL130, and UL131 and that of gB were cloned into adenoviral vectors and stocked by Vector Biolabs.

periments show little role for anti-gB antibodies in CMV-HIG neutralization. One caveat of this approach is that depletion was incomplete. The remaining potency on epithelial cells may be due to gH/gL/UL128/UL130/UL131 antibodies that recognize only epitopes present on the virus, and thus, cannot be captured on cells. Additionally, the remaining potency on fibroblasts may be accounted for by antibodies against gO or gM/gN. Ultimately, this study may help provide a basis for developing new vaccines and immunotherapies for CMV infec-

TABLE 1 Depletion of CMV-HIG, assayed on surface-expressed protein, for neutralization on epithelial cells^a

CMV protein(s) expressed on the cell surface	% depletion of CMV-HIG		
	gB lysate	gH/gL lysate	gH/gL/128/130/131 lysate
All (mock depletion)	0	0	0
gB	91	8	19
gH/gL	0	67	73
gH/gL/128/130/131	5	68	79

^a Percent depletion of specific antibody populations in CMV-HIG (normalized to the values for mock depletion). Percentages are transformed from ELISA data. Maxisorb (Nunc) plates were coated with lysate made from epithelial cells (ARPE-19) expressing gB, gH/gL, or gH/gL/UL128/UL130/UL131. The plates were blocked and incubated with depleted CMV-HIG prior to washing and detection using anti-human IgG (Fcγ fragment-specific) antibody conjugated to horseradish peroxidase (HRP) (Jackson Laboratory).

FIG 3 Neutralization of CMV VR1814 by CMV-HIG, depleted of specific CMV glycoprotein antigens. To evaluate neutralization potency, depleted CMV-HIG was serially diluted and incubated with virus at an MOI of 1 for 1 h prior to addition to epithelial cells (ARPE-19) (A) or fibroblasts (MRC-5) (B). At 18 h postinfection, cells were fixed, stained, and analyzed by immunofluorescence for anti-IE antibody and Hoechst stain. The concentration of CMV-HIG (in micrograms per milliliter) is shown on the x axes of the graphs.

TABLE 2 Depletion of CMV-HIG, assayed on purified protein, for neutralization on fibroblast cells^a

CMV protein(s)	% depletion of CMV-HIG	
	Purified gB	Purified gH/gL
All (mock depleted)	0	0
gB	95	0
gH/gL	0	84

^a Percent depletion of specific antibody populations in CMV-HIG (normalized to the values for mock depletion). Percentages are transformed from ELISA data. Maxisorb (Nunc) plates were coated with soluble gB or gH/gL protein. The plates were blocked and incubated with depleted CMV-HIG prior to washing and detection using anti-human IgG (Fcγ fragment-specific) antibody conjugated to horseradish peroxidase (HRP) (Jackson Laboratory).

tion by contributing to the understanding of the immune response to specific CMV glycoproteins.

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