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Strain-Specific Neutralizing Antibody Responses against Human Cytomegalovirus Envelope Glycoprotein N

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The human cytomegalovirus (HCMV) gM-gN complex is a major target of virus-neutralizing activity, and gN subtypes induce strain-specific antibodies. However, the biological significance of HCMV gN polymorphisms is not known. Neutralizing antibody responses against HCMV gN recombinant viruses were investigated at study entry in 80 healthy HCMV-seropositive women who were monitored for the appearance of new antibody specificities against linear strain-specific epitopes on glycoproteins gH and gB as evidence of HCMV reinfection. Neutralizing activity against all four gN recombinant viruses was seen in 74% of subjects, and 61% of subjects had strain-specific responses. Significantly fewer women (9/39 subjects [23%]) with serological evidence of reinfection had strain-specific neutralizing responses than the women without reinfection (21/41 subjects [51%]). Women with antibodies against at least one of the four linear gB and gH antigens at study entry had higher neutralizing titers against gN-1 ($P = 0.006$) and gN-2 ($P = 0.007$). Neutralizing titers of ≥400 against gN-3 ($P = 0.043$) and gN-4 ($P = 0.049$) at study entry were associated with longer times to serological evidence of reinfection. The findings demonstrate that HCMV gN elicits strain-specific neutralizing antibody responses and that broader anti-gN neutralizing activity may provide some protection from reinfection with a different virus strain.

Human cytomegalovirus (HCMV) is a frequent cause of congenital infection and an important pathogen in immunocompromised individuals, including allograft recipients and patients with AIDS. HCMV clinical strains have been shown to be highly diverse, and the implications of this diversity among circulating viral strains with respect to pathogenicity and effective immune responses have not been defined (6–8, 14, 15, 17–19, 22). Although there is no consensus definition for distinguishing different HCMV strains, polymorphisms in some or all of the glycoproteins, including gB, gH, gL, gO, and gN, have been used to identify genomic variants or genotypes of HCMV (6–8, 15, 17, 19). Studies have documented the presence of multiple HCMV strains or genotypes in a variety of clinical settings, including immunocompetent hosts, transplant recipients, HIV/AIDS patients, and congenitally infected infants (12, 15, 18, 22). Infection with multiple HCMV strains could be due to simultaneous infection with multiple virus strains or to acquisition of virus strains over time. In allograft recipients, reinfection with a genetically distinct donor virus was associated with a higher risk of developing severe HCMV disease than that with reactivation of the endogenous virus (9). In renal transplant recipients, reinfection with a different HCMV strain was associated with adverse outcomes, including transplant rejection and CMV disease (10). In HCMV-seropositive women, reinfection with a different virus strain can lead to intrauterine transmission and symptomatic congenital infection (2, 27).

HCMV envelope glycoproteins, including gB, the gH-gL-gO complex, and the gM-gN complex, have been shown to be important targets of neutralizing antibodies and can induce strain-specific antibodies (4, 11, 23, 25). A recent report demonstrated strain-specific antibody responses, including virus-neutralizing activity, against gN (5). However, the role of strain-specific immune responses in providing protection from HCMV disease and resolution of HCMV infection has not been understood. The definition of strain-specific immune responses is important in the development of an effective HCMV vaccine. The objective of our study was to determine the occurrence and frequency of strain-specific antibody responses against gN in healthy seropositive individuals and to examine the association between strain-specific anti-gN responses and HCMV reinfection.

(This research was presented in part at the 36th Annual International Herpesvirus Workshop, Gdansk, Poland, 24 to 28 July 2011 [16a].)

MATERIALS AND METHODS

Subjects and specimens. The study subjects were derived from a previously described cohort of HCMV-seropositive women that included predominantly young, unmarried black women enrolled in the postpartum period in an HCMV reinfection study (21). The study participants enrolled in the reinfection study between February 2000 and January 2002 constituted the study population for the current study. There were no differences in demographic and exposure characteristics between the 80 study women and the entire cohort of 205 participants of the HCMV reinfection study (data not shown).

Cells and viruses. Recombinant HCMV strains containing different genomic variants of UL73 (gN) coding sequences (gN genotypes 2, 3, and 4) were constructed using the AD169 bacterial artificial chromosome (BAC) as described previously (5). The four gN recombinant viruses differed only with respect to the gN genotype. The gN congenic viruses were propagated in human foreskin fibroblast (HFF) cells cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum. To obtain recombinant gN infectious virus stocks, infected cells were harvested, sonicated, and pelleted by centrifugation

Received 13 February 2012 Returned for modification 13 March 2012
Accepted 31 March 2012
Published ahead of print 4 April 2012
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doi:10.1128/CVI.00092-12

at 1,600 rpm at 4°C for 10 min. Supernatant containing the cell-free virus was collected and stored at −80°C.

Neutralization analysis. The assay for the determination of virus-neutralizing antibody titers was adapted from a previously described technique (11). Briefly, individual gN recombinant viral titers were adjusted to give 100 to 150 infectious foci per well in a 96-well microtiter plate. Serial serum dilutions (in the range of 1:100 to 1:6,400) in MEM with 2% guinea pig complement were incubated with individual gN recombinant virus preparations for 1.5 h at 37°C and plated in 96-well microtiter plates with confluent HFF cells. Infected HFF cells were stained with a monoclonal antibody (MAb) directed against the immediate-early protein 1 of HCMV (3). Neutralizing titer was expressed as the highest dilution of serum resulting in a 50% reduction in infectivity compared to that in wells without sera.

Strain-specific antibodies against linear epitopes within gH and gB. HCMV strain-specific antibody responses were determined on the basis of polymorphisms in antibody binding sites within envelope glycoproteins gH and gB between the two prototypical laboratory strains of HCMV, i.e., AD169 and Towne (13, 16, 24). The detection of new antibody specificities to either epitope (AD169 or Towne) on gH or gB in follow-up serum samples was considered evidence of infection with a new virus strain (reinfection) during the study.

Data analyses. Statistical significance was determined using the χ^2, Fisher exact, or Wilcoxon rank sum test where appropriate. All data analyses were performed using SAS software (version 9.1; SAS Institute, Cary, NC).

RESULTS

Study population. Most of the study participants were black (88%) and unmarried (90%). The mean age of the subjects was 18.35 ± 1.54 years. There were no differences in demographic and exposure characteristics between the 39 women with serologic evidence of HCMV reinfection during the study and the remaining 41 women without evidence of reinfection (data not shown).

Virus-neutralizing activity. Overall, neutralizing antibodies against all four recombinant viruses containing individual gN coding sequences were observed at study entry in 59/80 (74%) seropositive individuals. The neutralizing titers against the recombinant viruses ranged from 100 to 6,400. The proportions of individuals with detectable neutralizing responses against gN-1, gN-2, gN-3, and gN-4 recombinant viruses were 75%, 87.5%, 91.2%, and 81.2%, respectively. Forty-nine study individuals had strain-specific anti-gM/gN neutralizing antibodies at study entry, and of those, 27 had serological evidence of reinfection as defined by the appearance of new antibody specificities against the strain-specific gB or gH antigen during follow-up. No significant differences in the median neutralizing antibody titers at study entry were observed between women with evidence of HCMV reinfection and those without reinfection during follow-up.

Strain-specific neutralizing antibody responses. A strain-specific neutralizing antibody response was defined as a >3-fold difference in neutralizing titers against at least two different gN recombinant viruses in a serum specimen (5). Overall, sera from 61% (49/80 women) of the study women showed evidence of strain-specific virus neutralization activity against recombinant viruses. When the responses against individual recombinant viruses were analyzed, more subjects had strain-specific neutralizing antibodies against the gN-2 virus (45%), followed by the gN-3 virus (40%). Only 16% and 11% of samples showed strain-specific responses against the gN-1 and gN-4 viruses, respectively. Strain-specific responses against more than one gN recombinant virus were seen in about a third (30/80 women) of the study women. When strain-specific neutralizing activity against any of the four gN recombinant viruses was examined at study entry in women with and without serologic evidence of HCMV reinfection during follow-up, significantly fewer women with reinfection (9/39 women [23%]) had strain-specific responses against recombinant gN viruses than women without reinfection (21/41 women [51%]; $P = 0.009$). In addition, 56% (23/41 women) of women without serologic evidence of reinfection had strain-specific neutralizing antibodies against gN-3, compared to 23% (9/39 women) of those with reinfection ($P = 0.003$).

TABLE 1 Comparison of neutralizing titers against gN and the presence or absence of antibodies against linear strain-specific epitopes within gH and gB

gN recombinant virus	Median (range) neutralizing titer		*P* value
	Antibodies against one or more gH or gB antigens ($n = 39$)	No antibodies against gH or gB antigens ($n = 41$)	
gN-1	600 (<100–3,200)	300 (<100–2,400)	0.006
gN-2	600 (<100–6,400)	400 (<100–3,200)	0.007
gN-3	600 (<100–3,200)	400 (<100–3,200)	0.106
gN-4	400 (<100–3,200)	300 (<100–1,600)	0.169

Association between neutralizing titers and antibodies against linear strain-specific epitopes on gB and gH. In a previous study of HCMV reinfections in 205 healthy seropositive women, including the 80 women examined in the current study, responses against the strain-specific linear antibody binding sites within envelope glycoproteins gB and gH of the two prototypic laboratory strains of HCMV, AD169 and Towne, were determined (21). We compared neutralizing responses against the four gN recombinant viruses with the presence of antibodies against one or more (39/80 women) of these antigens or against none of the four strain-specific linear epitopes on gB and gH at study entry (Table 1). Those with antibodies against at least one of the four gB and gH antigens at study entry had higher median neutralizing titers against gN-1 ($P = 0.006$) and gN-2 ($P = 0.007$) viruses (Fig. 1). Significantly more women with antibodies against one or more strain-specific gB and gH antigens had neutralizing titers of ≥800 against gN-1 (17/39 women versus 6/41 women; $P = 0.009$) and gN-2 (17/39 women versus 8/41 women; $P = 0.03$) recombinant viruses than those with antibody responses against none of the gB or gH antigens. The mean duration to serologic evidence of reinfection (appearance of new antibody specificities) was significantly longer for women with neutralizing titers of ≥400 against ADgN-3 ($P = 0.043$) and ADgN-4 ($P = 0.049$) at study entry (Table 2).

DISCUSSION

The importance of antiviral neutralizing antibodies in protective immune responses against infectious agents, including viruses, has been shown in experimental systems (28). Envelope glycoproteins of HCMV, including gB, gH, and gN, have been shown to be important targets of antiviral antibody responses, including virus neutralization activity (4, 23, 25). Both strain-common and strain-specific epitopes within gB and gH have been described (13, 24). In a more recent study, Burkhardt et al. used recombinant viruses that differed only in the gN genotype to demonstrate that sera from about 30% of seropositive individuals contained

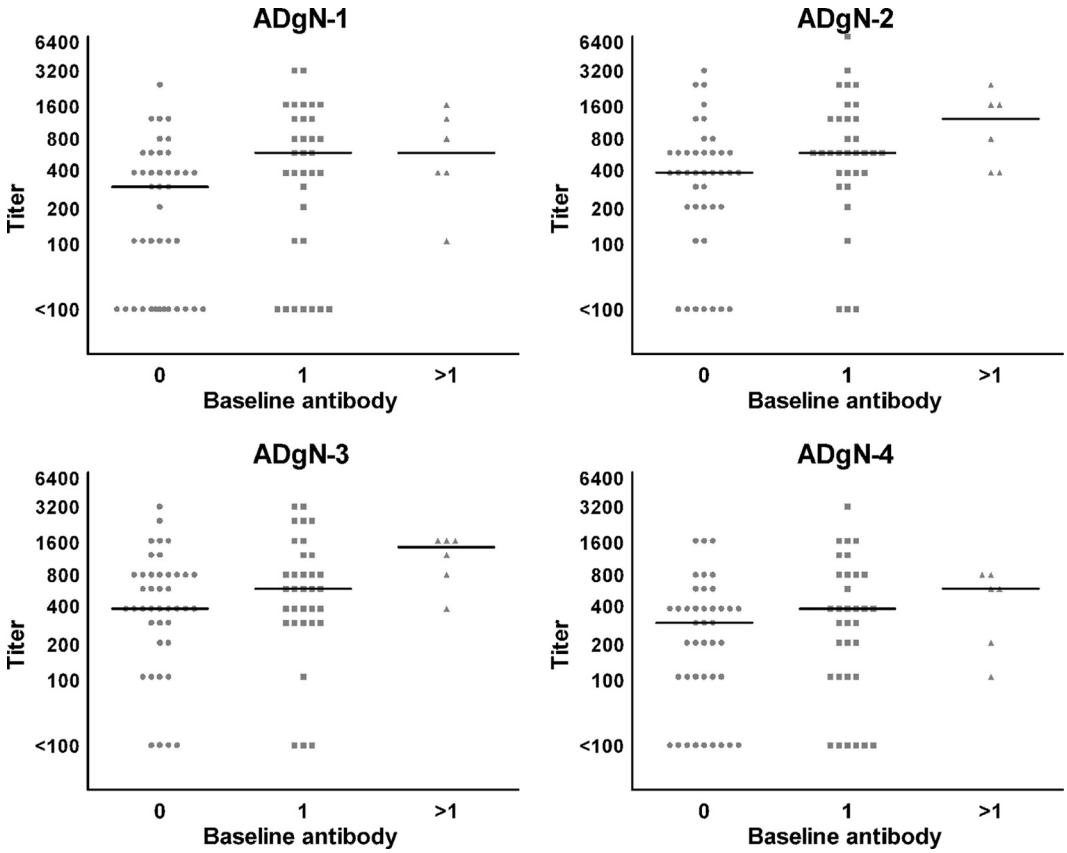

FIG 1 Neutralizing titers against gN recombinant viruses according to the presence of strain-specific antibodies against the four linear epitopes within gH and gB at study entry (baseline). 0, absence of antibodies against strain-specific linear epitopes; 1, presence of antibodies against at least one of the four antigens; >1, presence of antibodies against more than one antigen.

HCMV strain-specific neutralizing antibodies directed against gN (5). Our study, utilizing the same recombinant viruses, demonstrated that the majority of HCMV-seropositive individuals exhibited strain-specific neutralizing antibody responses against gN. The larger proportion of individuals with strain-specific neutralizing antibodies in our study could be due to differences in the study population. In a previous study from our laboratory, we demonstrated that the majority of study subjects were infected with multiple viruses (15). In addition, the current study included a larger number of HCMV-seropositive individuals than the 20 subjects in the study of Burkhardt et al. (5). We found that sera from some seropositive women neutralized all four recombinant viruses with comparable efficiencies, while clear differences in neutralizing capacity against individual gN recombinant viruses were seen in sera from others. This finding confirms that the gM-gN complex contains strain-common as well as strain-specific epitopes. Of the sera from the 80 women in this study, most (74%) showed evidence of neutralizing activity against all four gN recombinant viruses. However, responses against individual gN recombinant viruses varied, indicating strain-specific neutralizing activity.

TABLE 2 Neutralizing titers against gN recombinant viruses at study entry and time to appearance of new antibody specificities against linear epitopes within gH and gB

gN type	Mean time (mo) (±SD) to serological evidence of reinfection (*n*)		*P* value
	Neutralizing titers of ≥400	Neutralizing titers of <400	
gN-1	18.3 ± 10.9 (23)	13.6 ± 10.0 (16)	0.185
gN-2	18.2 ± 10.5 (28)	11.7 ± 10.3 (11)	0.09
gN-3	18.8 ± 11.2 (26)	11.5 ± 8.2 (13)	0.043
gN-4	19.0 ± 11.3 (24)	12.1 ± 8.4 (15)	0.049

A strain-specific neutralizing response was defined as a >3-fold difference in the titers of neutralizing activity against at least two different gN recombinant viruses in a serum sample. Overall, 61% (49/80 samples) of samples showed a strain-specific response to at least one of the four recombinant viruses. An examination of the responses against individual gN recombinant viruses showed that the highest neutralizing titers were seen against gN-2 (45%), followed by gN-3 (40%). It was also observed that women without serological evidence of acquisition of a different HCMV strain (reinfection) had higher strain-specific responses against multiple gN types than did women with evidence of reinfection. Since the recombinant viruses differed only with respect to gN, the findings of our study suggest that gN constitutes one of the major determinants for induction of a strain-specific neutralizing antibody response.

When the neutralizing antibody response was compared to the presence of antibodies against the linear strain-specific epitopes on gH and gB, we found that those with antibodies to at least one

of the four antigens had significantly higher neutralizing titers against gN-1 and gN-2 recombinant viruses. In addition, individuals with antibodies against more than one of the gH and gB antigens had higher titers of neutralizing antibodies against gN-1, gN-2, and gN-3 recombinant viruses. Since the presence of antibodies against two or more strain-specific linear epitopes within gH and gB indicated infection with multiple HCMV strains, our finding suggests that infection with multiple HCMV strains results in higher titers of neutralizing antibodies.

It is known that variations in surface glycoproteins in influenza virus (1) and HIV (26) have profound effects on antibody binding and neutralization. Since HCMV is a DNA virus, the sequence variation is assumed to be lower than that observed in RNA viruses. However, a recent study by Renzette et al. demonstrated that the genomic variability in HCMV is comparable to that in many RNA viruses (20). Since gN is a surface glycoprotein, it is possible that immune pressure may result in genomic variation. It was suggested in the study by Burkhardt et al. that the difference in neutralization activity against gN genomic variants could be due to the amount of glycosylation in the gN protein (5). Although the appearance of new antibody specificities against strain-specific gB and gH epitopes has been considered serologic evidence of HCMV reinfection, it is possible that the evolution of new virus strains over time may lead to the generation of new antibodies (2, 21). Extensive virus diversity in infants with congenital infection and in allograft recipients has been demonstrated by utilizing deep-sequencing technology. Explanations for the extent of virus diversity could include coinfection with multiple virus strains, sequential reinfection with different viruses over time, and/or evolution of new virus strains from the original virus over time.

The mean time to serologic evidence of reinfection was significantly shorter for women with neutralizing titers of <400 against gN-3 and gN-4 recombinant viruses at study entry than for those with titers of ≥ 400, suggesting that a larger neutralizing antibody response against gN delays infection with a different virus strain in seropositive individuals. However, identification of reinfection with a different HCMV strain was based on the appearance of new antibody specificities against strain-specific epitopes within gH and gB. Therefore, it is possible that the frequency of reinfection could have been underestimated in the study population because of the use of a limited number of antigens. Although higher neutralizing titers against gN-2 and gN-3 were observed, the distribution of gN genotypes of the circulating virus strains in the population is not known. Since neutralizing antibody responses against gN recombinant viruses were examined only at study entry, it is unknown whether virus-neutralizing activity against gN changes with respect to the titer and specificity at the time of HCMV reinfection.

In summary, the findings of our study demonstrate the presence of strain-specific neutralizing antibodies in a majority of HCMV-seropositive individuals. In addition, higher titers of neutralizing activity against gN in women with broadly reactive antibody responses, as measured by the presence of antibodies against strain-specific linear epitopes within gH and gB, suggest that infection with multiple HCMV strains leads to higher neutralizing activity. Furthermore, the longer time to serological evidence of reinfection for women with higher neutralizing titers against gN-3 and gN-4 recombinant viruses at study entry suggests that neutralizing antibodies against gN may provide protection against infection with a different HCMV strain in seropositive individuals. Although the exact protective nature of the antibody response against gN needs to be defined, inclusion of gN as one of the antigens in HCMV subunit vaccines could enhance protection.

ACKNOWLEDGMENTS

This work was supported by the National Institute on Deafness and Other Communication Disorders (R01 DC04163 to S.B.B.) and by the General Clinical Research Center (M01 R00032).

No authors have any conflict of interest to disclose.

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