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Cell Surface Expression of Human Cytomegalovirus (HCMV) gp55-116 (gB): Use of HCMV-Recombinant Vaccinia Virus-Infected Cells in Analysis of the Human Neutralizing Antibody Response

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Cell surface expression of the human cytomegalovirus (HCMV) major envelope glycoprotein complex, gp55-116 (gB), was studied by using monoclonal antibodies and an HCMV gp55-116 (gB) recombinant vaccinia virus. HCMV-infected human fibroblasts and recombinant vaccinia virus-infected HeLa cells expressed three electrophoretically distinct proteins of M_r 170,000, 116,000, and 55,000 on their surface. These species have been previously identified within infected cells and purified virions. Two unique neutralizing epitopes were shown to be present on the cell surface gp55-116 (gB). Utilizing HeLa cells infected with the gp55-116 recombinant vaccinia virus as a specific immunosorbent, we have shown that approximately 40 to 70% of the total serum virus-neutralizing activity of a group of individuals with past HCMV infections was directed against this single envelope glycoprotein. The implications of this finding for vaccine development are discussed.

Human cytomegalovirus (HCMV) is the most common cause of congenital viral infection in humans, with a reported incidence of 1% in the United States (1). Although most infants with congenital HCMV exhibit no evidence of infection, approximately 10% will have severe infections with multiple organ involvement and up to 80% of these infants will have central nervous system sequelae resulting from this chronic intrauterine infection (1). HCMV is also an important pathogen in immunocompromised patients and accounts for significant morbidity and mortality in organ allograft recipients (21, 24, 29, 36). More recently, HCMV has been shown to be an important cause of disease in patients with human immunodeficiency virus (HIV) infection (11, 26). Because all of these patient populations have abnormalities in their immune systems and because HCMV rarely causes symptomatic infections in normal children and adults, resistance to severe HCMV infection appears to depend on an intact host immune system.

The host-derived immune responses responsible for limiting the severity of HCMV infections have been studied in several different patient populations. A number of studies of organ allograft recipients have suggested that cellular functions, specifically T lymphocytes, may be critical for recovery from HCMV infections (20, 26). Other studies have shown that passive transfer of anti-HCMV antibodies could limit disease in both organ allograft recipients (23, 37) and newborn infants (38). Finally, some evidence has been presented suggesting that passively administered lymphokines also play a role in modulating HCMV infection (16, 19). In contrast, resistance to fetal HCMV infection is poorly understood, but is thought to be dependent on the maternal immune response to HCMV (30–32). Because congenital HCMV infection results from fetal infection acquired in utero, maternal cellular elements are likely excluded from the fetal circulation. However, maternal antibodies directed against HCMV readily enter the fetal circulation and play an important role in curtailing severe fetal infections.

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Although the nature of protective immune response in HCMV infections remains unknown, several different antibody-mediated antiviral functions have been described. These mechanisms may include cell surface-reactive antibodies, including antibodies mediating complement-dependent cytolysis (2, 22) and neutralizing antibodies. The neutralization response to HCMV is the best studied, and at least five electrophoretically unique glycoproteins have been shown to be targets of this response (3, 14, 15, 17, 27, 28, 33). Because of the large number of envelope proteins in HCMV and the diversity of individual responses to these proteins as measured in immunoblot and immunoprecipitation assays, no correlation between antibody level and neutralizing activity has been described. More recently, several reports have defined the location of epitopes on one HCMV glycoprotein (gB) which are targets of neutralizing antibodies (18, 34). To date, however, no studies have addressed the importance of the individual glycoproteins within the envelope in the overall HCMV neutralizing antibody response of humans. To investigate this question directly, we utilized a recombinant vaccinia virus containing the gene encoding HCMV gp55-116 (gB) to study the human antibody response to a single HCMV envelope glycoprotein. Our initial studies were aimed at characterizing the expression of HCMV gp55-116 (gB) on the surface of infected cells. In the second part of this study, we utilized recombinant vaccinia virus-infected cells as specific immunosorbents to determine the contribution of anti-gp55-116 (gB) antibodies to the overall neutralizing response in different human serum specimens. Our results indicate that, not infrequently, the majority of neutralizing antibodies in human serum HCMV were directed against the product of this single envelope glycoprotein gene (gB).

MATERIALS AND METHODS

Cells and viruses. Human fibroblast (HF) cells were propagated as described before (3). HeLa cells were obtained from C. Morrow, University of Alabama, Birmingham, and propagated in Dulbecco modified Eagle medium supple-

mented with 10% fetal bovine serum. Human TK-143, CV-1, and BSC-1 cell lines also were grown in that same medium (8). HCMV strain AD169 was utilized in all experiments (3). Vaccinia virus strain IHD-J was grown in CV-1 cells and purified as described previously (8).

Enzymes and chemicals. T4 DNA ligase, Klenow DNA polymerase, and all restriction endonucleases were obtained from New England BioLabs, Beverly, Mass., and used as specified by the supplier. 5-Bromo-2-deoxyuridine obtained from Sigma Chemical Co., St. Louis, Mo., was prepared at a stock concentration of 25 mg/ml in H_2O, filtered, and stored at −20°C until used.

Construction of the vaccinia virus recombination vector and generation of recombinant vaccinia viruses expressing the gp55-116 (gB) glycoproteins. We constructed a vaccinia virus recombination vector that utilized the vaccinia virus p11 promoter to express the gp55-116 gene. We chose to utilize this promoter instead of the conventional p7.5 promoter because significantly higher levels of gp55-116 were expressed when the p11 promoter region was used. The vector, pED2, is a vaccinia virus recombination vector that contains a unique *Sma*I site that allows expression of foreign genes from the vaccinia virus p11 promoter. The details of the vector construction will not be presented but are available upon request. Recombinant vaccinia viruses expressing gp55-116 were constructed by subcloning a *Sma*I fragment from pCMVS (8) containing the intact gp55-116 gene into the *Sma*I site of pED2. A vaccinia virus recombination plasmid that contained the intact gp55-116 gene of HCMV in the correct orientation was isolated and used to generate recombinant vaccinia viruses. Several vaccinia virus recombinants were isolated that expressed the gp55-116 of HCMV. One of the recombinants, VVPED8, was chosen for subsequent biochemical analyses.

Cell surface expression of gp55-116. For cell surface immunofluorescence assays, cover slips containing viable HCMV-infected HF cells or VVPED8-infected HeLa cells were washed extensively and fixed in 0.5% paraformaldehyde in phosphate-buffered saline for 30 min. Following washing in Tris-buffered saline (0.05 M Tris, 0.15 M NaCl, pH 7.4), monoclonal antibody was added and the cover slips were incubated for 60 min at 4°C. After washing and incubation with fluorescein-conjugated goat anti-mouse immunoglobulin G (Tago, Burlingame, Calif.) for 30 min at 37°C, the cover slips were mounted and examined with a Leitz epifluorescence microscope. Identical results were obtained when viable VVPED8-infected cells were reacted with monoclonal antibody 7-17 or 27-39 prior to paraformaldehyde fixation.

To determine what proteins of the gp55-116 complex were expressed on the cell surface, infected cells were radioiodinated by the Iodogen method (Pierce Chemical Co., Rockford, Ill.) as described previously (6). Following iodination, the cells were disrupted in immune precipitation buffer consisting of phosphate-buffered saline, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholate, and 1% Nonidet P-40 and processed as described before (3). Procedures used for immunoprecipitation with gp55 monoclonal antibodies, analysis by SDS-polyacrylamide gel electrophoresis (PAGE), and immunoblotting have been described in detail elsewhere (3, 5). All samples were reduced with 2-mercaptoethanol prior to application to the gels.

Neutralization assays. Neutralizing activity was assayed by a rapid (16-h) immunofluorescence assay (1a). Briefly, 0.2 ml of human serum diluted in medium was added to 0.2 ml of titered virus containing 10% (vol/vol) guinea pig complement. Following a 60-min incubation at 37°C, 0.1 ml of the mixture was added to replicate wells of a 96-well microdilution tissue culture plate containing HF cells. After an approximately 2-h absorption period, the inoculum was removed, the monolayers were washed once with medium, fresh medium was added, and the plate was incubated overnight at 37°C. The following day the medium was removed, and monolayer was washed once with phosphate-buffered saline (pH 7.4) and fixed in absolute ethanol. Following fixation, the monolayers were stained with monoclonal antibody P63-27, which is reactive with the major HCMV immediate protein, pp72, and the number of antigen-positive cells was quantitated by immunofluorescence. Results are expressed as the mean percent reduction in fluorescent nuclei (percent infectivity reduction). The standard error of the mean of this assay is at most 10%, with usual values of 5%. The results obtained with this rapid assay were equivalent to results obtained with a conventional 14-day plaque reduction assay (1a).

Absorption of neutralizing antibodies. Titers of human serum samples were determined prior to use of the samples in the absorption experiments to achieve a preabsorption neutralizing activity of approximately 50% reduction in input infectivity. A 1-ml amount of appropriately diluted sera was then mixed with a pellet of HeLa cells infected with either VVPED8 or a recombinant vaccinia virus expressing the HIV *gag* proteins. The mixture was briefly vortexed, and the absorption was allowed to proceed on ice for 60 min. Following centrifugation, the serum was carefully removed, heated at 56°C, filtered through a 0.2-μm filter, and assayed for neutralizing activity. The heat treatment and filtration were shown to remove residual vaccinia virus infectivity. Infectivity reduction was calculated as follows: {1.0 − [infectivity (experimental)/infectivity (control)]} × 100%, where experimental represented immune serum and control represented serum from a seronegative individual at the same dilution subjected to the same treatments as experimental serum. The standard error of the mean was <10% in all experiments.

RESULTS

Cell surface expression of gp55-116 complex (gB). The cell surface expression of the gp55-116 (gB) complex was initially examined by immunoprecipitation of radioiodinated, infected-cell surface proteins followed by SDS-PAGE. Preliminary experiments in which cell surface expression of the gp55-116 (gB) was assayed by immunofluorescence suggested that maximal expression occurred approximately 60 h postinfection (data not shown). In addition, electron microscopic examination of infected cells at this time indicated that few, if any, progeny viruses were passively absorbed to the cell surface (data not shown). For these reasons, viable HCMV-infected cells were radioiodinated at 60 h postinfection to optimize labeling of cell surface proteins but to minimize the chance of labeling passively absorbed progency virions. Three polypeptides associated with the gp55-116 (gB) complex were present on the cell surface, with M_rs of 170,000, 116,000, and 55,000 (Fig. 1A). These forms have been identified previously in extracellular virions and infected cells (Fig. 1B) (3, 5, 7). The migration of the diffusely migrating gp116 was arbitrarily designated 116,000 based on the comigration of its most slowly migrating boundary with the molecular weight standard β-galactosidase (8). To verify that these forms were indeed products of the gene encoding the gp55-116 (gB) complex, we carried out identical radioiodinations of viable HeLa cells infected with a recombinant

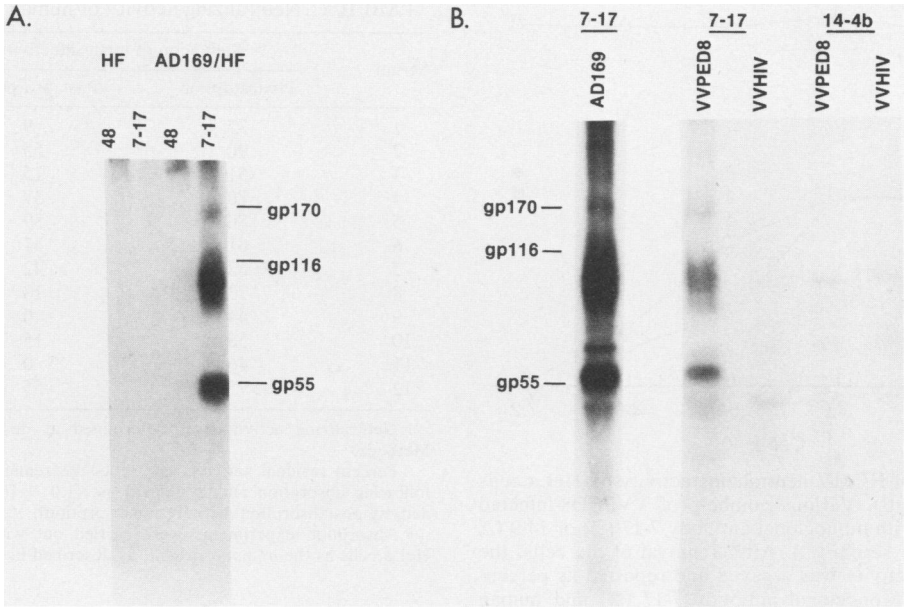

FIG. 1. Cell surface expression of gp55-116 (gB). (A) Viable uninfected HF or HCMV AD169-infected HF cells were radioiodinated, solubilized, and immune precipitated as described in Materials and Methods. Precipitated proteins were analyzed under reducing conditions by SDS-PAGE. Control monoclonal antibody 48 is reactive with murine leukemia virus gp70 and HCMV-specific antibody 7-17 reacts with gp55 of the gp55-116 complex (34). (B) Viable HeLa cells infected with either VVHIV or VVPED8 recombinant vaccinia virus were radioiodinated, solubilized, and immune precipitated with either antibody 7-17 or control antibody 14-4b, and precipitated proteins were analyzed under reducing conditions by SDS-PAGE. Gradient-purified AD169 virions were radioiodinated and subjected to immune precipitation and SDS-PAGE as described above.

vaccinia virus encoding the gp55-116 (gB) complex (VVPED8). We could demonstrate three proteins of M_rs 170,000, 116,000, and 55,000 on the surface of HeLa cells infected with VVPED8 but not on that of HeLa cells infected with a recombinant vaccinia virus expressing the HIV *gag* gene product (VVHIV) (Fig. 1B). These forms comigrated with gp170, gp116, and gp55 immunoprecipitated from radioiodinated AD169 virions (Fig. 1B) (7). These results indicated that at least three forms of the gp55-116 (gB) complex were present on the surface of infected cells and also provided definitive evidence that the higher-molecular-weight forms, most notably, the gp116, were products of the gene encoding the gp55-116 (gB) complex.

The presence of multiple forms of the gp55-116 (gB) complex on the surface of infected cells raised the possibility that neutralizing epitopes present on mature infectious virions were also expressed on the surface of infected cells. We examined this question by using viable HCMV-infected HF cells and the VVPED8-infected HeLa cells in immunofluorescence assays. Two previously characterized neutralizing monoclonal antibodies, 27-39 and 7-17, which recognized different epitopes (34) were used to detect cell surface gp55-116 (gB). Both antibodies reacted with the surface of infected cells, although the VVPED8-infected cells displayed quantitatively more antigen on the cell surface (data not shown). Thus, it appeared that neutralizing epitopes on cell surface gp55-116 (gB) were accessible to these antiviral antibodies and suggested that neutralizing antibodies may interact not only with infectious virions but also with viable virus-infected cells.

Characterization of the human neutralizing antibody response to HCMV. The availability of human cells expressing a single HCMV glycoprotein allowed us to investigate the relative contribution of antibodies against this glycoprotein to the overall HCMV neutralizing antibody response in humans. Initially, we utilized increasing numbers of VVPED8- or VVHIV-infected HeLa cells as immunosorbents to deplete the neutralizing activity of an anti-gp55-116 (gB) neutralizing monoclonal antibody, 7-17 (3). Absorption with increasing numbers of VVPED8 recombinant virus-infected cells reduced the neutralizing activity of antibody 7-17 until, at the highest cell concentration utilized, the neutralizing activity was completely eliminated (Fig. 2). When antibody 7-17 was absorbed with 7.2×10^6 VVHIV-infected cells, there was no reduction in neutralizing activity as compared with unabsorbed antibody (Fig. 2). Absorption of monoclonal antibody 14-9, which is directed against an unrelated HCMV glycoprotein, gp62 (4), with VVPED8-infected cells failed to alter its neutralizing activity appreciably (Fig. 2). These experiments indicated that the VVPED-infected cells could be used as a specific immunosorbent to deplete anti-gp55-116 (gB) neutralizing antibodies. Utilizing VVPED8- or VVHIV-infected cells, we examined the neutralizing activity of a single human serum sample. Absorption with the 7.2×10^6 VVHIV-infected cells failed to reduce neutralizing activity of the sera significantly as compared with unabsorbed sera, whereas absorption with increasing numbers of VVPED8-infected cells reduced the neutralizing activity of the sera (Fig. 3). Interestingly, at the highest cell concentration, the residual neutralizing activity was only 14% of the value following absorption with VVHIV-infected cells (Fig. 3). These results indicated that, in this serum, antibodies reactive with the HCMV gp55-116 (gB) glycoprotein complex were the major constituent of the HCMV neutralizing response.

Additional sera were then examined in similar experiments. Again, we noted a progressive decline in the neutralizing activity of individual serum samples following absorp-

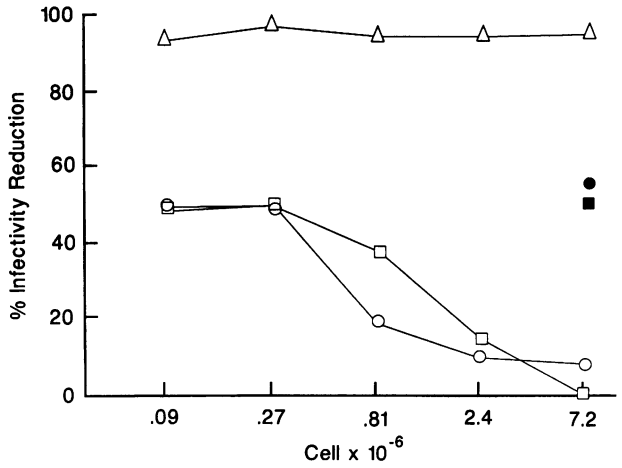

FIG. 2. Depletion of HCMV neutralizing activity by HeLa cells expressing gp55-116 (gB). Various numbers of VVPED8-infected cells were incubated with monoclonal antibody 7-17 (□) or 14-9 (△ [4]) or human immune serum (○). After removal of the cells, the residual neutralizing activity was assayed and reported as percent infectivity reduction. Monoclonal antibody 7-17 (■) and human immune serum (●) were also absorbed with 7.2×10^6 VVHIV-infected cells, and residual neutralizing activity was assayed as described above.

TABLE 1. Neutralizing activity of human convalescent serum[a]

Serum	% Reduction of input infectivity		% Residual activity[b]
	Preabsorption	Postabsorption[c]	
1	37	9	24
2	90	55	61
3	51	15	39
4	70	37	53
5	80	39	49
6	61	41	67
7	62	42	68
8	73	18	25
9	44	0	0
10	58	15	26
11	46	0	0
12	75	55	74

[a] Neutralizing activity was determined as described in Materials and Methods.

[b] Percent residual activity represents the remaining neutralizing activity following absorption and is defined as: {1.0 − [(activity preabsorption − activity postabsorption)/activity preabsorption]} × 100.

[c] Absorption experiments were carried out with 10^7 VVPED8-infected HeLa cells as the immunosorbent, as described in Materials and Methods.

tion with increasing numbers of VVPED8-infected cells (Fig. 3). In this group of sera, absorption of anti-gp55-116 (gB) antibodies with 10^7 VVPED8-infected cells resulted in a 45 to 100% reduction of the total neutralizing activity of the sera (Fig. 3). Additional evidence of the importance of anti-gp55-116 (gB) antibodies in the neutralizing activity of human convalescent sera was obtained by absorbing sera from a group of laboratory workers known to be HCMV seropositive. In these experiments, we used only a single concentration of VVPED-infected cells as an immunosorbent. Following absorption, all sera had less neutralizing activity, ranging between 0 and 74% of their original activity (Table 1). These results supported our previous results and, taken together, suggested that in some individuals the majority of neutralizing antibodies were directed against a single envelope gene product, while in others a smaller but significant component of the HCMV neutralizing response was directed against the gp55-116 component of the virion envelope. It

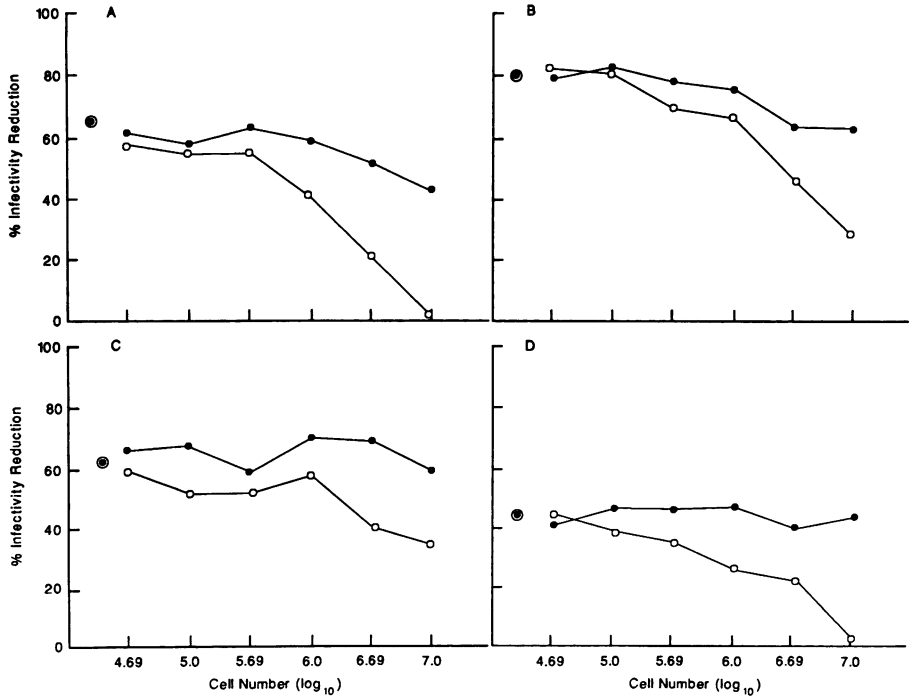

FIG. 3. Reduction of neutralizing activity by HeLa cells expressing gp55-116 (gB). Serum samples from four patients with past HCMV infection were absorbed with various numbers of VVPED8 (○)- or VVHIV (●)-infected cells, and residual neutralizing activity was assayed as described in Materials and Methods. Neutralizing activity of unabsorbed serum (⊙) is shown.

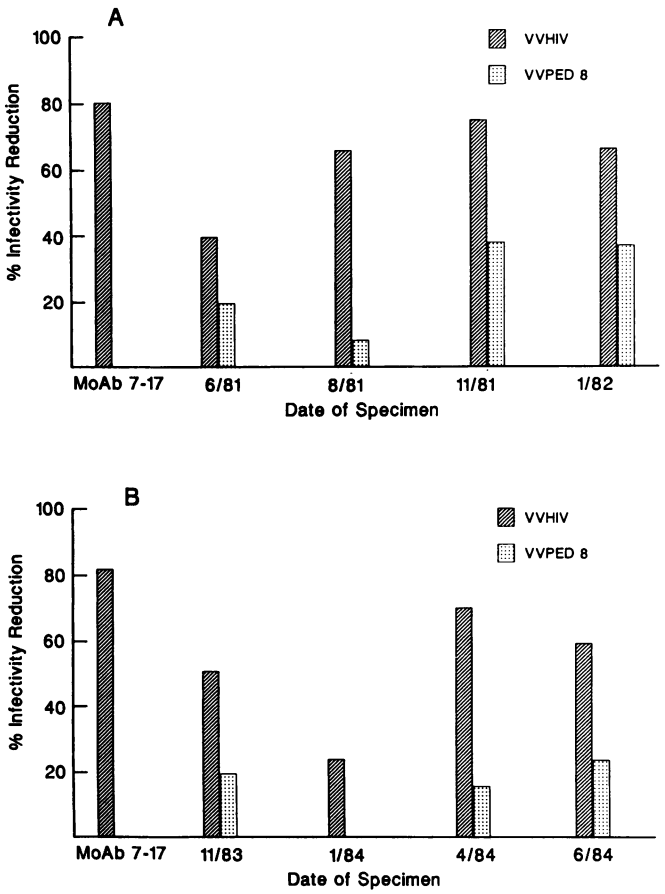

FIG. 4. Reduction of neutralizing activity by HeLa cells expressing gp55-116 (gB). Serum samples from two patients (A and B) with primary HCMV infection during pregnancy were absorbed with either VVHIV- or VVPED8-infected HeLa cells as described in Materials and Methods. Residual neutralizing activity is expressed as percent infectivity reduction. Specimens 1/82 and 6/84 represent the serum obtained at delivery of the infected infant. Monoclonal antibody 7-17 was included as a control in each experiment.

must be noted, however, that glycoproteins other than gp55-116 (gB) likely elicit neutralizing antibodies in humans and in some individuals might be the major target of neutralizing antibodies.

In a final series of experiments, we examined the neutralizing response in three women with primary HCMV infection during pregnancy. The results with sera from two of these patients are presented in this report, although all three exhibited similar responses. Serial serum samples were absorbed with VVPED8-infected cells, and the resulting neutralizing activity was compared with the activity of serum absorbed with VVHIV-infected cells. The serum-neutralizing activities of the initial seropositive specimens and subsequent specimens were reduced significantly (40 to 100%) following absorption with VVPED8 (Fig. 4). As a control, monoclonal antibody 7-17 was also absorbed with the same number of VVPED8-infected cells. This treatment completely eliminated its neutralizing activity (Fig. 4). These results confirmed our previous findings and indicated that, during acute HCMV infection in pregnancy, at least in this small number of patients, a significant component and in some cases the major component of the serum-neutralizing activity were directed against the gp55-116 (gB) complex.

DISCUSSION

The detection of gp55 on the surface of infected cells was anticipated because previous studies utilizing monoclonal anti-gp55 antibodies have shown that these antibodies reacted with the surface of infected cells (35). However, the finding of gp116 and gp170 on the cell surface was somewhat unexpected. Although gp116 has been detected in mature virions in quantities similar to gp55, our previous studies have failed to detect significant quantities of this protein within infected cells (5, 7). A likely explanation for these discrepant results is the low-level incorporation of [^{35}S]methionine which has been used to intrinsically radiolabel infected-cell proteins, whereas in the experiments described in this report the proteins were radioiodinated. The finding of gp170, the fully glycosylated but uncleaved precursor of the gp55-116 (gB) complex (5, 7), was also of interest. Because this protein represented a minor component within preparations of extracellular virion proteins, its relative abundance on the cell surface suggested that its transport to the surface was more efficient than its incorporation into virions. Furthermore, the presence of this uncleaved form on the cell surface indicated that proteolytic cleavage of the complex was unnecessary for cell surface transport. This finding has been shown for other viral glycoproteins. For example, previous studies have indicated that the glycosylated but uncleaved precursor, gp85, of the Friend mink cell focus-inducing virus was efficiently transported to the surface of infected cells (12). Finally, the levels of cell surface gp170, -116, and -55 were similar in both HCMV-infected HF and VVPED8-infected HeLa cells, suggesting that processing and transport of the gp55-116 (gB) complex were likely authentic in the recombinant vaccinia virus-infected cells.

The expression of biochemically and antigenically authentic gp55-116 (gB) on the surface of recombinant vaccinia virus-infected cells provided a useful reagent for characterization of human immune responses to HCMV. Our results utilizing recombinant vaccinia virus-infected cells as immunosorbents have shown that a significant, and often the majority of, serum-neutralizing activity was directed against this single envelope glycoprotein complex. In fact, in some individuals, serum-neutralizing activity could be abrogated by absorption with cells expressing gp55-116 (gB). Such preferential reactivity of human serum for one virion component was surprising in view of the fact that the HCMV genome can potentially encode as many as 17 glycoproteins, and at least 5 electrophoretically unique glycoproteins have been shown to be targets of neutralizing antibodies (3, 14, 15, 17, 22, 27, 28, 33). Several explanations may account for this biased neutralizing response against gp55-116 (gB), including the relative abundance of this protein complex within the virion envelope as compared with other glycoproteins, its expression on the surface of infected cells, and its immunogenicity as evidenced by the routine isolation of anti-gp55-116 (gB) monoclonal antibodies from experimental animals following immunization with crude HCMV-infected cell immunogens (W. J. Britt, unpublished observation). The structural properties of this protein complex which lead to its immunogenicity remain undefined but could result from its extensive glycosylation (7). Nonglycosylated gp55-116 (gB) produced in recombinant procaryotic systems has been less immunogenic than glycosylated protein produced in eucaryotic systems (8; Britt, unpublished observation). Whether this was secondary to increased stability arising from extensive glycosylation or important carbohydrate-dependent

conformational determinants is unknown. Regardless of the mechanism which accounts for the human antibody reactivity against the protein, it was apparent that it represents a major target of serum-neutralizing activity. Finally, it must be kept in mind that additional HCMV-encoded glycoproteins also elicit neutralizing antibodies and in some cases, possibly in some of the individuals described in this study, might account for more potent neutralizing responses than the gp55-116 (gB) complex.

Protective immunologic responses which modulate HCMV infections have been poorly characterized. Previous studies have correlated cellular immunity with survival from severe HCMV infections as well as resistance to virulent infection (26). Other investigations have indicated that antiviral antibodies might protect the host from virulent HCMV infection (23, 37, 38). Epidemiologic studies have as yet failed to clarify the role of various immunologic effector functions in prevention of severe intrauterine HCMV infections, although it has been well documented that preexisting serologic immunity does preclude virulent intrauterine and perinatal infections (30, 32). Our studies have focused on neutralizing antibodies because this antibody activity can reduce virus infectivity and passive transfer of neutralizing monoclonal antibodies or immune serum can prevent virus-induced disease in several animal models, including other herpesvirus infections in humans (9, 10, 13). In addition, the presence of at least two neutralizing epitopes on the surface of infected cells suggested that neutralizing antibodies may also mediate clearance of infected cells, thus providing additional mechanisms for reduction of virus load. Although the importance of neutralizing antibodies to the in vivo pathogenesis of HCMV remains undefined, the presence of neutralizing epitopes on the surface of infectious virions and infected cells suggested that antibodies reactive with these sites could play an important role in virus clearance.

Perhaps the most interesting result of our study was the characterization of the HCMV neutralizing response of women with primary acquisition of HCMV during pregnancy. Throughout their pregnancy, including the postpartum period, a significant component of their serum-neutralizing antibody response was directed against a single virus envelope protein complex. This result indicated that the neutralizing antibody response directed against the gp55-116 (gB) complex was generated early in the course of the infection and persisted for over 1 year following virus acquisition. Because these neutralizing antibodies were likely transferred transplacentally to the fetus, this antibody response might represent an early and persistent protective mechanism which could modulate the severity of intrauterine HCMV infections. Although at present this hypothesis is speculative, we hope that future studies will clarify these issues and provide directions towards development of effective immune prophylaxis against virulent intrauterine HCMV infection.

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