Induction of Complement-Dependent and -Independent Neutralizing Antibodies by Recombinant-Derived Human Cytomegalovirus gp55-116 (gB)

WILLIAM J. BRITT,^{1*} LEONE VUGLER,¹ AND EDWARD B. STEPHENS²

*Department of Pediatrics, School of Medicine, University of Alabama at Birmingham, Birmingham, Alabama 35294,¹ and
Department of Infectious Diseases, School of Veterinary Medicine, University of Florida, Gainesville, Florida 32611²*

Received 30 October 1987/Accepted 10 May 1988

The human cytomegalovirus (HCMV) envelope glycoprotein complex gp55-116 was expressed in both *Escherichia coli* and cells infected with a recombinant vaccinia virus. *E. coli* produced a single protein of M_r 100,000 which approximated the size of the nonglycosylated gp55-116 precursor found in HCMV-infected cells. Cells infected with the recombinant vaccinia virus contained three intracellular forms of M_r 160,000, 150,000, and 55,000 which were detected by a monoclonal antibody reactive with gp55. Comparison of the immunological properties of these recombinant proteins indicated that several of the HCMV gp55-116 monoclonal antibodies and sera from patients infected with HCMV reacted with the vaccinia virus-derived proteins whereas a more restricted group of monoclonal antibodies recognized the *E. coli*-produced protein. Immunization of mice with either *E. coli* or vaccinia virus recombinant HCMV gp55-116 resulted in production of virus-neutralizing antibodies. In contrast to the almost exclusive production of complement-dependent neutralizing antibodies following immunization with recombinant vaccinia virus, the *E. coli*-derived protein induced complement-independent neutralizing antibodies.

Human cytomegalovirus (HCMV) represents a serious and often life-threatening infectious complication in patients undergoing allograft transplantation (18). Recently, HCMV has emerged as an important cause of disease in patients with acquired immunodeficiency syndrome (25). Although less widely appreciated, HCMV is the most common cause of intrauterine viral infection (1). Not infrequently, this chronic viral infection leads to significant central nervous system damage in the developing fetus (1). In each of these clinical situations, an underlying defect in the host immune system is thought to lead to HCMV-associated disease. Even though most investigators would agree that the immune system is required to limit HCMV infection, little is known about what constitutes a protective response to disease associated with HCMV. Furthermore, the virus with its numerous virally encoded structural and nonstructural proteins has hindered attempts to define target antigens of potentially protective responses. Several reports have implicated the viral envelope glycoproteins as likely targets of protective responses based on their immunogenicity (12, 22). In addition, several of the envelope glycoproteins can be readily detected on the surface of infected cells and infectious virions, providing accessibility for effector functions of the host immune system. Finally, protective immunity to many animal viruses, including other human herpesviruses, has been shown to be directed against envelope glycoproteins (7, 32). Thus, it would seem likely that protective immunity to HCMV might include responses directed against specific envelope glycoproteins.

The envelope of HCMV contains at least eight electrophoretically unique glycoproteins, of which only five have been analyzed by immunological techniques (2, 3, 13, 14, 19, 23, 28, 33). The most widely studied and most abundant HCMV envelope glycoprotein is a complex of proteins variously termed gA (23), gp58 (19), gp55-116 (2), and, more recently,

gB (6). Within the mature virion, this protein complex exists as a heterodimer containing two proteins of M_r 116,000 and 55,000 covalently linked by disulfide bonding (4). Intracellular forms of this complex include the two mature forms as well as two glycosylated intermediates of M_r 150,000 and 160,000 (4). Several previous studies have demonstrated that monoclonal antibodies and human sera containing antibodies reactive with this protein complex neutralize infectious virus (2, 22, 27). In addition, two reports have demonstrated that immunoaffinity-purified HCMV gp55-116 can induce neutralizing antibodies in experimental animals (10, 26). Although these investigators could not rigorously exclude the possibility that the immunoaffinity-purified material contained copurifying viral proteins, expression of the gene encoding this protein in a recombinant vaccinia virus confirmed these earlier findings (6). Because of the immunological responses evoked by HCMV gp55-116 (gB), as well as its suggestive homology to herpes simplex virus (HSV) gB (6), a glycoprotein which plays an essential role in virus infectivity as well as host-derived protective immunity, further studies on the structure, function(s), and immunogenicity of this HCMV protein complex seem warranted.

To investigate more fully the immunological characteristics of this envelope protein complex as well as to begin studies on its role(s) in virus infectivity, we have expressed the gene encoding HCMV gp55-116 (gB) in *Escherichia coli* and mammalian cells by utilizing a recombinant vaccinia virus. These recombinant proteins were then utilized to study several aspects of the immunogenicity of this protein complex. Our results indicated that both native HCMV gp55-116 as expressed in mammalian cells and the procaryote-derived, nonglycosylated protein could induce neutralizing antibodies in mice. Interestingly, the *E. coli*-produced protein induced complement-independent neutralizing antibodies, whereas the vaccinia recombinant virus-immunized animals generated complement-dependent neutralizing antibodies almost exclusively. Both immunogens primed ani-

* Corresponding author.

FIG. 1. Construction of procaryotic expression plasmid pCMVS-1 and shuttle vector pVVCMV. Details of the construction of these plasmids are described in Materials and Methods.

imals for an augmented response following a booster immunization. These initial findings suggested that HCMV gp55-116 may have multiple functions in the infectious cycle of the virus and that antibody responses to the molecule appeared to be strongly influenced by the presence of carbohydrate modifications.

MATERIALS AND METHODS

Cells and viruses. Cell lines HEP-2 and CV-1 were maintained in Dulbecco minimal essential medium supplemented with 10% fetal bovine sera, 100 U of penicillin per ml, and 50 µg of gentamicin per ml. Human TK-143B cells were maintained in the above medium containing 25 µg of 5-bromo-2-deoxyuridine per ml. Human foreskin fibroblast cells were prepared and maintained as described previously (2). Stocks of wild-type vaccinia virus, strain IHD-J, or recombinant vaccinia viruses were prepared in HEP-2 cells and titrated in CV-1 cells. HCMV strain AD169 was passaged, titrated, and gradient purified as described before (2).

Monoclonal antibodies and human sera. The characteristics of HCMV-specific murine monoclonal antibody 7-17 have been described in a previous report (2). Additional murine monoclonal antibodies specific for HCMV gp55-116 used in these studies have also been described (4). Sera from children with serologic evidence of HCMV infection were kindly provided by Robert Pass, University of Alabama at Birmingham.

Construction of the recombinant vaccinia viruses. The strategy for cloning the gp55-116 envelope glycoprotein gene into vaccinia virus is shown schematically in Fig. 1. Although previous studies have shown that the gene encoding the gp55-116 glycoprotein is located within the *Hind*III F fragment of HCMV strain AD169 (15), at the time this project was initiated the sequence of the entire gene was unknown. However, the approximate size of the gene was known, and based on limited sequence analysis and restriction endonuclease mapping of the *Hind*III F fragment, we chose two convenient restriction endonuclease sites, *Bam*HI and *Sac*I, which were outside the putative coding region. A subsequent publication of the sequence of the gene has verified the position of these sites (6). To isolate the gp55-116 envelope gene, the *Hind*III F fragment of HCMV strain

AD169 in pUC-19 was digested to completion with restriction endonucleases *Hind*III and *Bam*HI, and the 5.0-kilobase fragment containing the gp55-116 envelope gene was isolated by agarose gel electrophoresis and electroelution. The isolated 5.0-kilobase fragment was ligated into pUC-19 cleaved with restriction endonucleases *Bam*HI and *Hind*III, using T4 DNA ligase, and used to transform competent JM109 cells. The resulting transformants were screened for inserts by insertional inactivation of the β -galactosidase gene of pUC-19 and confirmed by restriction endonuclease analysis of plasmid preparations. The resulting plasmid, pCMVBH, containing the 5.0-kilobase *Bam*HI-*Hind*III fragment was digested with *Sac*I, and the fragment containing the envelope gene was inserted into the *Sac*I site of pUC-19 by the methods described above. The resulting plasmid, pCMVS1, contains the intact glycoprotein gene which is flanked by *Sma*I sites. To insert the gp55-116 envelope gene into vaccinia virus, pCMVS1 was digested with *Sma*I and the envelope gene was separated from the pUC-19 vector by agarose gel electrophoresis and electroelution. The isolated envelope gene was ligated into the *Sma*I site of the pSC11 vaccinia virus recombination vector, using T4 DNA ligase, and the ligated DNA was then used to transform competent JM109 cells. The resulting transformants were then screened with an envelope gene probe constructed from a gp55-116 cDNA by colony blot hybridization. The orientation of the envelope gene within the pSC11 vector was determined by restriction endonuclease analysis.

For construction of infectious recombinant vaccinia viruses, CV-1 cells (approximately 80% confluent) were infected with wild-type vaccinia virus at a multiplicity of infection (MOI) of 0.05. At 2 h postinfection, cells were transfected with a calcium phosphate precipitant of 20 µg of plasmid (purified by two cycles of CsCl gradient centrifugation), 10 µg of wild-type vaccinia virus DNA, and 15 µg of salmon sperm DNA per ml of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline. Stocks of TK-vaccinia virus were prepared from TK-143B cells as described previously (30). To select for recombinants, monolayers of TK143B cells were infected with TK vaccinia virus in the presence of 5-bromo-2-deoxyuridine (25 µg/ml). At 48 h postinfection, the monolayers were overlaid with 1% low-melting agarose containing 300 µg of 5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside per ml. At 4 to 6 h, blue plaques (recombinant vaccinia viruses) were picked and further purified by two additional rounds of plaque purification. The vaccinia virus analyzed in this study, VVCMVV containing the HCMV gp55-116 glycoprotein gene, was grown in HEP-2 cells.

Construction of HCMV gp55-116 expression plasmid pCMVS-1. Plasmid pCMVS1 described in the previous section was cleaved with restriction endonucleases *Sca*I and *Sma*I, and the resulting 3.0-kilobase fragment containing the sequence encoding gp55-116 was isolated by agarose gel electrophoresis (Fig. 1). This fragment was then ligated by standard techniques (17) into the Klenow-repaired *Nco*I site of expression plasmid pkk233-2 (Pharmacia, Inc., Piscataway, N.J.). The ligation mixture was used to transform *E. coli* JM105, and the resulting colonies were screened by colony hybridization with the envelope probe described in the previous section. Orientation of the insert was determined by restriction endonuclease mapping.

Analysis of recombinant-derived HCMV gp55-116. Expression of HCMV gp55-116 in bacteria was achieved by induction of *E. coli* carrying the pCMVS-1 plasmid with 5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 30 min. The

bacteria were collected by centrifugation and lysed by two cycles of rapid freezing and thawing. After brief vortexing, the samples were heated to 100°C for 3 min in Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.4) containing 2% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol. Following electrophoretic separation by SDS-polyacrylamide gel electrophoresis (PAGE), the recombinant-derived proteins were identified by Coomassie blue staining or Western immunoblotting as described previously (4).

Expression of HCMV gp55-116 with recombinant vaccinia virus was performed in HEP-2 cells. Briefly, confluent monolayers of HEP-2 cells were infected with VVCMV or wild-type vaccinia virus at an MOI of 10. At 8 h postinfection, the monolayers were either pulse-radiolabeled with 50 μ Ci of [35 S]methionine per ml for 4 h or scraped from the flasks and analyzed by Western immunoblotting (4). Radiolabeled infected cell proteins were immune precipitated with monoclonal antibodies or human sera, and the immune precipitated proteins were separated by SDS-PAGE as described before (4).

Cell-binding assay. CV-1 cells were grown on 13-mm glass cover slips and infected with VVCMV at 10^{-1} and 10^{-2} dilutions. Eight hours later, one set of cover slips was fixed in acetone at -20°C. The duplicate set was left unfixed. Both sets of cover slips were then reacted with monoclonal antibody 7-17 followed by reaction with 125 I-labeled protein A and autoradiography to detect antibody binding. A specificity control consisted of CV-1 cells which were infected with a 10^{-1} dilution of wild-type vaccinia virus and processed in an identical manner.

Immunogenicity of recombinant-derived HCMV gp55-116 in experimental animals. Two-month-old BALB/c mice were immunized with 10^7 PFU of VVCMV by intradermal tail scratch. Littermates were initially immunized with 100 μ g of *E. coli* lysate emulsified in complete Freund adjuvant followed 14 days later by 100 μ g of lysate emulsified in incomplete Freund adjuvant. The *E. coli* lysate was prepared from a 50-ml IPTG-induced culture of pCMVS-1-containing cells. The cells were collected and lysed in 1 M NaCl in Tris-buffered saline by repeated freeze-thawing, followed by heating to 100°C for 3 min. Boosting of the primary immunization described above was done at 60 days post primary inoculation. Mice initially immunized with VVCMV were injected intravenously with 10^6 viable NIH 3T3 mouse fibroblasts infected 8 h earlier with VVCMV at an MOI of 10. Animals initially inoculated with *E. coli*-derived HCMV gp55-116 were boosted with 10^7 PFU of VVCMV by intradermal tail scratch. A control immunization was performed by inoculating mice (3) with 100 μ g of *E. coli* lysate, strain y1089, which was lysogenized with a λ bacteriophage containing the cDNA encoding the HCMV pp49 DNA-binding protein (5). These lysogeny were induced by heating to 42°C followed by the addition of 10 mM IPTG. The bacteria were collected, processed, and used in a primary immunization protocol identical to that described above.

Mouse sera were obtained at various times after immunization and assayed for anti-HCMV antibodies in virus-binding assays or neutralization tests (2). The binding assay utilized gradient-purified AD169 virions immobilized in wells of a 96-well microtiter plate. Sera at various dilutions were added, and antibody binding was detected by 125 I-labeled protein A. Individual wells were then counted in a gamma counter, and the binding index was calculated by dividing counts of experimental sera bound by counts of sera from unimmunized littermate mice bound (2). Immune precipitation of 125 I-radiolabeled, gradient-purified AD169 virions by

sera from immunized mice was carried out as described previously (4). The virus-neutralizing activity of various sera as measured by the percent plaque reduction was determined in the presence or absence of complement (5% guinea pig sera) as described previously (2).

RESULTS

Expression of HCMV gp55-116 in *E. coli*. The genomic sequence encoding HCMV gp55-116 was initially identified by screening of a λ gt11 expression library with murine monoclonal antibodies specific for HCMV gp55-116. This library was constructed from cDNA prepared from poly(A)-selected RNA isolated from HCMV strain AD169-infected human fibroblast cells. In agreement with results published by other investigators, the sequence encoding this glycoprotein complex was located within the right-hand end of the *Hind*III F fragment of HCMV AD169 (15). Restriction endonuclease mapping of the *Hind*III F fragment followed by Southern transfer and hybridization with a cDNA clone representing some 60 to 70% of the complete HCMV gp55-116 gene indicated that the coding sequence was contained within a 3.6-kilobase-pair *Sac*I fragment (data not shown). Limited sequence analysis of the 5' end of the fragment demonstrated that the beginning of the glycoprotein sequence was within the *Sac*I fragment and most likely represented the complete gene encoding the gp55-116 complex. These results were consistent with recently published analysis of this HCMV gene (6).

Expression of HCMV gp55-116 in *E. coli* was obtained by insertion of a 3.0-kilobase-pair *Sca*I/*Sma*I fragment into the Klenow-repaired *Nco*I site of expression plasmid pkk233-2 (Fig. 1). This unique *Sca*I site was located some 80 base pairs downstream from the translation initiation site of the HCMV gp55-116 gene and therefore allowed the construction of a vector, pCMVS-1, containing nearly the complete gene. Transformation of *E. coli* JM105 followed by induction with IPTG resulted in production of a protein of estimated M_r 100,000 to 105,000, whereas transformation of *E. coli* with plasmid pCMVs in which the *Sca*I/*Sma*I fragment was inserted into pkk233-2 in the opposite orientation failed to produce a protein of this size (Fig. 2.). When *E. coli* cells containing pCMVS-1 were induced with IPTG and the products were analyzed by Western immunoblotting, a protein of approximate M_r 100,000 was detected, using murine monoclonal antibodies specific for gp55 of the HCMV gp55-116 complex (Fig. 3). These results demonstrated expression of the nonglycosylated equivalent of HCMV gp55-116 in *E. coli* and indicated that the epitopes recognized by these monoclonal antibodies were conserved in the procaryote-derived protein.

Expression of HCMV gp55-116 by recombinant vaccinia virus. To directly compare the immunologic properties of the procaryote-derived gp55-116 with those of eucaryote-derived protein, gp55-116 was expressed in eucaryotic cells, utilizing a recombinant vaccinia virus (Fig. 1). Recombinant vaccinia virus-infected CV-1 cells were screened for expression of HCMV gp55-116, using monoclonal antibody 7-17 in a cell-binding assay. One recombinant virus, VVCMV, was used for further characterization of eucaryote-derived gp55-116. Evidence of the expression of HCMV gp55-116 in cells infected with the recombinant vaccinia virus was obtained by analyzing HEP-2 cells infected with VVCMV by immunoblotting. Two forms of estimated M_r 150,000 to 160,000 and 55,000 were detected by monoclonal antibody 7-17 (Fig. 4). These proteins were similar in M_r to two proteins

FIG. 2. SDS-PAGE of HCMV gp55-116 expressed in *E. coli*. Expression was obtained by induction with IPTG in *E. coli* JM105 (pCMVS-1). As a control, a plasmid containing HCMV gp55-116 in the opposite orientation (pCMVS) was used to transform *E. coli* JM105 followed by induction with IPTG. Bacterial lysates were prepared and analyzed by SDS-PAGE followed by staining with Coomassie brilliant blue. Molecular weights (10^3) were estimated by comparison of migration with known standards (Sigma Chemical Co., St. Louis, Mo.) and are shown on the left.

detected by antibody 7-17 in HCMV-infected HF cells, suggesting that HCMV gp55-116 was processed authentically in VVCMV-infected HEp-2 cells. Because the *E. coli*-derived form of HCMV gp55-116 was of M_r 100,000 to

FIG. 3. Immunoblotting of *E. coli*-produced HCMV gp55-116. Bacterial lysates were prepared and separated by SDS-PAGE as described in Materials and Methods. After transfer to a nitrocellulose membrane, the membrane was cut into strips and probed with monoclonal antibody 7-17, which is specific for HCMV gp55-116, or a negative control monoclonal antibody, 48, which is reactive with Friend murine leukemia virus gp70 (2). Antibody binding was detected by 125 I-labeled protein A as described in Materials and Methods. Molecular weights (10^3) were estimated as described in the legend to Fig. 2.

FIG. 4. Expression of HCMV gp55-116 in VVCMV-infected HEp-2 cells. HEp-2 cells were infected with VVCMV or wild-type vaccinia virus (VV), and cell lysates were prepared 8 h postinfection. Polypeptides were separated by SDS-PAGE and analyzed with monoclonal antibody 7-17 by immunoblotting as described in Materials and Methods.

105,000, this result suggested that the mature protein complex expressed in VVCMV-infected HEp-2 cells contained approximately 50,000 to 60,000 daltons of co- and posttranslational modifications. The most likely form of these modifications was N linked and possibly O linked glycosylation (23; W. J. Britt, unpublished observations).

The cell surface localization of recombinant vaccinia virus-expressed HCMV gp55-116 was examined with monoclonal antibody 7-17 and a cell-binding assay. CV-1 cells grown on glass cover slips were infected with VVCMV or wild-type vaccinia virus at an MOI of 5. One set of cover slips was fixed in acetone to allow detection of intracellular proteins, while the second set of cover slips containing viable cells was left untreated to detect surface antigens. Both sets of cover slips were reacted with monoclonal antibody 7-17 followed by incubation with 125 I-labeled protein A to detect antibody binding. Recombinant vaccinia virus-derived gp55-116 was efficiently transported to the cell surface, although its expression on the cell surface was less than that found intracellularly (Fig. 5). gp55-116 could also be detected on the surface of infected cells by indirect immunofluorescence (data not shown). These results indicated that vaccinia virus-derived gp55-116 was adequately processed to allow transport to the cell surface.

The immunologic reactivity of vaccinia virus-derived gp55-116 in humans was studied with serum samples obtained from children lacking previous exposure to vaccinia virus. Radiolabeled infected cell proteins from either VVCMV- or wild-type vaccinia virus-infected CV-1 cells were precipitated with individual serum samples, and the precipitated proteins were analyzed by SDS-PAGE. The infected cell proteins were also precipitated with monoclonal antibody 7-17 to allow identification of the VVCMV-derived protein. Serum specimens from two children with congenital HCMV infection specifically precipitated a protein of esti-

FIG. 5. Cell surface transport of HCMV gp55-116 in VVCMV-infected CV-1 cells. CV-1 cells grown on cover slips were infected with a 10^{-1} or 10^{-2} dilution of VVCMV (MOI, 10 or 1, respectively) and either fixed in acetone (intracellular) or left unfixed (cell surface). The cover slips were incubated with monoclonal antibody 7-17 and reacted with ^{125}I -labeled protein A to detect antibody binding. Wild-type vaccinia virus-infected CV-1 cells (MOI, 10) served as controls for nonspecific reactivity.

mated M_r 150,000 which comigrated with a protein precipitated by antibody 7-17, whereas the serum from an age-matched child without a previous history of HCMV infection failed to precipitate this protein (Fig. 6). In this experiment, the mature forms of the gp55-116 complex could not be resolved due to the large number of proteins nonspecifically precipitated with these sera and the monoclonal antibody. This nonspecific precipitation was thought to be secondary to the use of whole *Staphylococcus aureus* bacteria as an immunosorbent because an irrelevant monoclonal antibody, 48, also precipitated the same proteins (Fig. 6). Nevertheless, these results indicated that the recombinant virus-derived protein produced in VVCMV-infected CV-1 cells expressed antigenic determinants that were recognized by antibodies in the sera of patients following naturally acquired HCMV infections.

Immunogenicity of recombinant HCMV gp55-116 in experimental animals. The availability of recombinant virus-derived HCMV gp55-116 allowed a preliminary characterization of the immunogenicity of this protein complex in experimental animals. In addition, production of this protein complex in both procaryotic and eucaryotic systems allowed us to directly compare the relative immunogenicity of both preparations. Adult mice were given 10^7 PFU of VVCMV by intradermal tail scratch and bled at days 7, 14, and 21 postimmunization. Immunization of mice with *E. coli*-derived gp55-116 was carried out by subcutaneous injection of 100 μg of crude lysate of *E. coli* cells containing pCMVS-1 which were first induced with IPTG. This group of mice was then given a booster injection 14 days later with the same amount of *E. coli* lysate, and mice were bled 7 days following the booster injection. Both immunizations induced a specific antibody response which could be detected by an immunofluorescence assay, using HCMV strain AD169-infected cells (data not shown). To quantitate this response, we used a previously described virus-binding assay which utilizes intact virions immobilized on microdilution plates (2). As expected, both immunization protocols induced virus-binding antibodies, although immunization with VVCMV resulted in a three- to fourfold-higher level of virus-binding antibodies (Table 1). The specificity of the antibody response was measured in a representative serum sample from each group of mice. Gradient-purified HCMV AD169 virions were radioiodinated and immune precipitated with monoclonal

FIG. 6. Reactivity of human sera against recombinant VVCMV gp55-116. CV-1 cells were infected with VVCMV or wild-type vaccinia virus (VV), radiolabeled with $[^{35}\text{S}]$ methionine, and lysed as described in Materials and Methods. Infected cell proteins were precipitated with control monoclonal antibody 48 (lane 1), monoclonal antibody 7-17 (lane 2), serum from a child without serologic evidence of HCMV infection (lane 3), and sera from two children with congenital HCMV infections (lanes 4 and 5). The precipitated proteins were analyzed by SDS-PAGE as described in Materials and Methods.

antibody 7-17 or sera from mice given either VVCMV- or *E. coli*-derived gp55-116. Analysis of the precipitated proteins by SDS-PAGE revealed the two predominant forms of the gp55-116 complex found in extracellular virions, gp116 and gp55 (Fig. 7). A lesser amount of gp160, the pentultimate form of this complex, was also detected (Fig. 7). A coprecipitating but unrelated protein, p68 (5), was also detected, but its precipitation was thought to be nonspecific as it was also weakly precipitated by normal mouse serum (Fig. 7). In agreement with the results obtained in the virus-binding assay, serum from the animal immunized with VVCMV generated higher levels of precipitating antibodies compared with that from the animal given the *E. coli*-derived protein (Fig. 7).

Serum specimens from the two groups of mice were then assayed for neutralizing antibodies. Again, the group immunized with VVCMV produced significantly higher levels of complement-dependent neutralizing antibodies than the group of mice given *E. coli*-derived HCMV gp55-116 (Table 2). Furthermore, we could detect significant titers of complement-dependent neutralizing antibodies in mice immunized with VVCMV as early as 7 days following inoculation (Fig. 8). The complement-dependent neutralizing response continued to increase, reaching near-maximal levels 3 weeks following VVCMV inoculation (Fig. 8). In addition, analysis

TABLE 1. Capacity of antibodies derived from pCMVS-1- and VVCMV-immunized mice to bind intact virions

Antibodies derived from:	Mouse	Binding index at given serum dilution ^a			
		50	250	500	1,000
pCMVS-1 ^b	1	4.8	2.0	1.7	1.4
	2	3.4	2.2	1.4	1.1
	3	4.6	2.1	1.6	1.0
	4	5.2	3.2	1.9	1.4
VVCMV ^c	1	12.5	6.4	4.1	2.4
	2	12.1	6.6	3.6	2.0
	3	16.0	8.5	4.1	2.2
	4	18.5	11.8	5.7	3.2
	5	12.5	6.5	3.2	1.8
Control ^d		1.0	1.0	1.0	1.0

^a Binding index at the reciprocal of the serum dilution. The binding index was calculated by dividing counts bound of experimental serum divided by those of serum from an unimmunized control littermate. A value of >2.0 was considered significantly above background.

^b Four mice were immunized subcutaneously with 100 µg of crude lysate from *E. coli* containing plasmid pCMVS-1 emulsified in complete Freund adjuvant followed 14 days later by an identical immunization with *E. coli* lysate in incomplete Freund adjuvant. Mice were bled 7 days later and assayed for virus-binding antibodies.

^c Five were inoculated with 10⁷ PFU of recombinant vaccinia virus VVCMV by tail scratch. The mice were bled sequentially, and the values shown represent the serum collection at 21 days following inoculation.

^d Pooled serum from four unimmunized, control littermate mice.

of the kinetics of the response in mice immunized with VVCMV showed preferential induction of complement-dependent neutralizing antibodies in those animals. Both immunization schedules induced comparable levels of complement-independent neutralizing antibodies at lower serum

FIG. 7. Specificity of the serologic response of mice immunized with either vaccinia virus- or *E. coli*-derived HCMV gp55-116. ¹²⁵I-labeled extracellular HCMV virions were immune precipitated with monoclonal antibody 7-17 (lane A), normal mouse serum (lane B), serum from a representative member of mice immunized with VVCMV (lane C), or *E. coli* HCMV gp55-116 (lane D) and analyzed by SDS-PAGE. The *M_r* was determined by comparison of migration with known molecular weight standards.

TABLE 2. Virus neutralization with and without complement, using sera collected from pCMVS-1- and VVCMV-immunized mice

Immunogen	Mean plaque reduction ± SD at given serum dilution ^a		
	50	250	500
– Complement			
pCMVS-1 ^b	57 ± 11	50 ± 6.8	35 ± 11
VVCMV ^b	46 ± 15	23 ± 15	5 ± 8
840 ^c	8 ± 4	0	0
None ^c	15	0	0
+ Complement			
pCMVS-1	59 ± 5.8	36 ± 10	35 ± 11
VVCMV	92 ± 3.7	87 ± 9.4	83 ± 11
840	15 ± 5	8 ± 5	0
None	25	10	0

^a Neutralizing activity at the reciprocal serum dilution shown was determined in the absence (–) or presence (+) of guinea pig serum as a source of complement. Sera from neither VVCMV- nor pCMVS-1-immunized mice neutralized HSV type 1 at a dilution of 1/100 in a complement-amplified neutralization assay (2).

^b Mice were immunized with crude *E. coli* lysate containing expression plasmid pCMVS-1 or recombinant vaccinia VVCMV as described in footnotes b and c, Table 1.

^c Control immunization groups included (i) three littermate mice immunized with 100 µg of crude cell lysate prepared from *E. coli* y1089 lysogenized with 840, a λ bacteriophage containing an intact cDNA encoding the HCMV DNA-binding protein pp49 (5) and (ii) pooled serum from four unimmunized, control littermate mice.

dilutions. However, mice immunized with *E. coli*-produced gp55-116 had significantly higher levels of complement-independent neutralizing antibodies at higher serum dilutions (Table 2). Nearly equivalent titers of complement-dependent and -independent neutralizing antibodies were produced in mice given *E. coli*-derived gp55-116 (Table 2). These results were markedly different from the response measured in animals immunized with VVCMV (Table 2) and, although the methods of immunization were not strictly comparable, suggested that, in the absence of processing events such as glycosylation, only complement-independent

FIG. 8. Kinetics of neutralizing antibodies in mice immunized with VVCMV. Mice were immunized with VVCMV as described in Materials and Methods. Sera were obtained at 7, 14, and 21 days postinoculation and assayed for neutralizing antibodies in the presence (---) and absence (—) of guinea pig sera as a source of complement. The virus-neutralizing activity of the serum at dilutions of 1/50 (○, ●), 1/250 (△, ▲), and 1/500 (□, ■) is expressed as percent plaque reduction.

neutralizing antibodies were induced. Alternatively, this result could suggest that conformational or so-called discontinuous epitopes expressed on gp55-116 required the interaction of antibody and complement to neutralize virus infectivity. Finally, to ensure that the low titers of neutralizing antibodies present in the sera of mice immunized with gp55-116 produced in *E. coli* were nonartifactual, we included several control sera in the neutralization experiments, including sera from unimmunized control littermate mice and sera from mice immunized with a crude lysate of *E. coli* which expressed an abundant nonstructural protein of HCMV, pp49 (5). None of these sera significantly reduced the number of input PFU (Table 2). The specificity of the response was further documented by demonstrating that the HCMV-neutralizing activity of sera from mice given either pCMVS-1 or VVCMV failed to neutralize HSV (Table 2).

We next examined the capacity of the two different immunization protocols to induce a boostable response, that is, an increased titer of anti-HCMV antibodies following virus challenge. Because HCMV is species specific, we could not directly challenge the immunized animals with live virus. Two different boosting schedules were used in an attempt to approximate a challenge by replicating HCMV. Mice initially given VVCMV were boosted intravenously with VVCMV-infected mouse fibroblasts. This method of boosting was utilized because it was thought that preexisting vaccinia virus immunity would significantly inhibit replication of the recombinant VVCMV virus if virus was given by tail scratch. Injection of viable cells containing replicating VVCMV was used to circumvent this potential problem. Mice originally immunized with *E. coli*-derived HCMV gp55-116 days previously were inoculated with 10^7 PFU of VVCMV by intradermal tail scratch. This method of boosting was chosen as it most closely approximated challenge of the animals with a replicating virus expressing gp55-116 which could not be directly explored because of the species specificity of HCMV. Both groups of mice were bled immediately prior to and 7 days following the booster immunization. The boost in biologically active antibodies was measured by determining the increase in neutralizing antibodies produced by these two immunization schedules. Boosting VVCMV-immunized mice with VVCMV-infected cells resulted in a significant increase in both complement-dependent and complement-independent neutralizing antibodies in four of five mice (Fig. 9). Likewise, boosting of mice initially immunized with *E. coli*-derived gp55-116 with VVCMV caused an increased neutralizing antibody response in three of the four mice (Fig. 9). In this group of mice both complement-dependent and -independent responses increased, but the increase in complement-dependent neutralizing antibodies was more dramatic (Fig. 9). This result was most likely explained by the kinetics of the primary response to VVCMV in which significant titers of neutralizing antibodies were present by 7 days postimmunization (Fig. 8). Finally, it was of interest to note the increased titers of complement-independent neutralizing antibodies following boosting of mice initially given VVCMV (Fig. 9). Although several mechanisms could account for this finding, a likely explanation would be the priming of the complement-independent neutralizing antibody response by the initial inoculation followed by expansion of this response by boosting with VVCMV-infected cells. Another explanation of this finding could be the expression of epitopes on the surface of infected cells which preferentially elicit complement-independent neutralizing antibody responses. Future experiments will address the mechanism(s) responsible for this

FIG. 9. Boosting of the neutralizing antibody response in mice immunized with recombinant HCMV gp55-116. Animals initially immunized with *E. coli*-derived HCMV gp55-116 were bled 60 days following the primary immunization and boosted with live recombinant HCMV vaccinia virus as described in the text. Animals initially immunized with VVCMV were similarly bled and boosted intravenously with 10^6 VVCMV-infected mouse 3T3 cells. Serum was obtained from both groups of animals 7 days later, and the neutralizing activity at various dilutions was determined as described in Materials and Methods. Virus-neutralizing activity was measured in the absence of complement in mice initially immunized with (A) vaccinia virus- or (C) *E. coli*-derived HCMV gp55-116 or in the presence of complement in (B) VVCMV or (D) *E. coli* HCMV gp55-116-immunized mice.

boost in the complement-independent neutralizing antibody response.

DISCUSSION

Several laboratories have detailed the genomic location and nucleotide sequence of the gene encoding the major envelope glycoprotein of HCMV variously termed gA (23), gp55-116 (4), or gB (6). Cranage et al. (6) have published the sequence of this gene and suggested that the sequence of HCMV gp55-116 (gB) was distantly related to the envelope glycoprotein B of HSV. However, major differences exist between the gB of HSV and gp55-116 (gB) of HCMV, including the following: (i) the gp55-116 precursor of HCMV has been cleaved into a heterodimer (whereas the gB of HSV has not); and (ii) HSV gB has been reported to have three regions of hydrophobicity that could act as membrane-spanning domains (21), whereas only two regions of hydrophobicity exist at the carboxyl terminus for HCMV gp55-116 (gB) (E. B. Stephens and W. J. Britt, unpublished observation). Limited sequence analysis of the 5' end of the gp55-116 gene described in this communication indicated that this gene is identical to that described by Cranage et al. (6). Furthermore, Mach and co-workers have utilized monoclonal antibody 7-17 to study the expression of this HCMV envelope gene which they originally described (15). Thus, there seems to be little doubt that all three laboratories are describing the same gene and gene product. It is also quite likely that previous descriptions of the gA complex of HCMV (23) and the glycoprotein dimer described by Rasmussen et al. (27) are equivalent to gB. Because there is

considerable confusion surrounding the nomenclature of HCMV-encoded proteins, we will continue to refer to this complex of glycoproteins as the gp55-116 complex until a consensus is reached on the nomenclature of the proteins encoded by HCMV.

The expression of the major envelope glycoprotein of HCMV in both eucaryotic and procaryotic systems has allowed us to begin characterization of its antigenic properties and its role in the pathogenesis of HCMV infections. Although the nucleotide sequence encoding this glycoprotein is known, expression of this protein in *E. coli* was difficult since the nearly full-length protein was apparently toxic to the host bacteria. Transformation of *E. coli* with plasmid pCMVS-1 in the presence of IPTG resulted in a drastic reduction in frequency of viable bacterial colonies compared with transformation in the absence of IPTG (data not shown). Other investigators have also noted this phenomenon and in at least one report suggested that the toxicity of eucaryotic viral glycoprotein gene expressed in bacteria resulted from expression of the signal peptide (29). Interestingly, these investigators also described frequent deletions of sequences within the plasmid containing the viral glycoprotein gene, including loss of regulatory regions within the vector as well as deletions in the viral gene (29). Our experience was quite similar in that plasmid pCMVS-1 was unstable during mass culture in *E. coli* and in many cases analysis of surviving *E. coli* following mass culture contained a plasmid(s) with deletions within the inserted HCMV gene (data not shown). Workers in other laboratories have also noted difficulty in expressing gp55-116 (gB) in *E. coli*, especially constructions containing the 5' terminus of the gene (M. Mach, personal communication). In contrast to these results, HCMV gp55-116 was readily expressed in mammalian cells infected with the vaccinia virus recombinant. Because the initiation codon of this gene was nearly 400 base pairs downstream of the 5' end of the *SacI* fragment inserted into the pSC11 vector, the level of expression of HCMV gp55-116 in the vaccinia virus recombinant was somewhat reduced compared with that seen in HCMV-infected human fibroblast cells. We could detect cell surface transport of this molecule as well as an M_r -160,000 form of similar M_r as the fully glycosylated pentultimate form of the gp55-116 complex (4). Thus, it appeared from these limited data that the vaccinia virus-derived gp55-116 was authentically processed. Only three intracellular forms of the complex were detected within VVCMV-infected cells, gp160, gp150, and gp55. Our inability to detect the other cleavage product, gp116, by immunoblotting was not unexpected as monoclonal antibody 7-17 reacted only with gp55 and the polypeptide precursors gp150 and gp160 (4). However, it should be noted that a previous report utilizing a similar vaccinia recombinant virus containing the HCMV gB gene also failed to detect gp116 (6). This finding is not easily reconciled with previous findings from our laboratory, and the explanation for this discrepancy is not known. Our preliminary result of the pulse-chase analyses of the synthesis of this protein complex suggested that gp116 was indeed expressed in cells infected with VVCMV recombinant (data not shown). Finally, the result of the experiments with *E. coli*-derived gp55-116 indicated that the nonglycosylated precursor of HCMV gp55-116 was of M_r 100,000 to 105,000, a finding consistent with results obtained using tunicamycin to inhibit N-linked glycosylation of the polypeptide precursor (Britt, unpublished data). This result suggested that the pentultimate form of HCMV gp55-116, gp160, contained some 50,000 to 60,000 daltons of carbohydrate modifica-

tions. Comparison of HCMV gp55-116 (gB) with that of other distantly related herpesvirus glycoproteins such as HSV gB and Epstein-Barr virus gp110 revealed that HCMV contained the highest percentage of carbohydrate modifications (38%) even though the nonglycosylated translational product of the related gene was nearly the same size in all three viruses (11, 20). Furthermore, HCMV is the only one of the three viruses in which cleavage of the precursor occurs (4). Thus, all three viruses have evolved with differing requirements for the level of glycosylation and processing of their major envelope glycoproteins. Whether this diversity resulted from their cell tropism or their mode of spread in vivo or both remains an interesting area of future study.

Recombinant-derived gp55-116 retained its immunogenicity when expressed in VVCMV-infected cells of both human and rodent origin. This finding was demonstrated not only by reactivity of previously described monoclonal antibodies, but also by serum from children with naturally acquired HCMV infections. This finding was not unexpected because other investigators have clearly demonstrated antigenic authenticity of viral glycoproteins produced by recombinant vaccinia virus-infected cells (6). In contrast to these results, the antigenicity of *E. coli*-derived HCMV gp55-116 was more restricted. Only monoclonal antibodies reactive with SDS-denatured HCMV gp55-116 recognized this procaryote-derived protein (data not shown). Furthermore, many of the monoclonal antibodies which neutralized infectious HCMV failed to recognize the *E. coli*-derived gp55-116. This result was consistent with previous reports which suggested that neutralizing antibodies reactive with this HCMV glycoprotein complex required complement for optimal activity (10, 26, 27). Because other investigators have documented the recognition of conformationally dependent or discontinuous epitopes by complement-dependent neutralizing antibodies in the study of other herpesviruses (31), it was likely that many of neutralizing antibodies reactive with HCMV gp55-116 expressed by VVCMV but not *E. coli* were directed against conformational epitopes. However, it should be noted that a subset of monoclonal antibodies with neutralizing activity reacted with *E. coli* produced gp55-116, indicating that HCMV gp55-116 contained at least two distinct sites recognized by neutralizing antibodies, one of which is independent of glycosylation of the molecule.

Immunization of mice with either *E. coli* gp55-116 or the vaccinia virus recombinant VVCMV resulted in the production of neutralizing antibodies. However, insofar as our results can be interpreted because of differing immunization schedules, this response was quantitatively and qualitatively different depending on the source of the immunogen. Immunization of *E. coli*-derived gp55-116 resulted in the production of lesser quantities of virus-binding antibodies as well as significantly lower titers of complement-dependent neutralizing antibodies than inoculation of mice with the vaccinia recombinant VVCMV. Mice given *E. coli* gp55-116 only produced complement-independent neutralizing antibodies, whereas mice given VVCMV generated large quantities of complement-dependent neutralizing antibodies but only low levels of complement-independent neutralizing antibodies. This result is in contrast to previous reports which suggested that the neutralizing antibody to HCMV gp55-116 (gB) was exclusively complement dependent (27). Our findings indicated that indeed the response to the fully glycosylated protein was biased towards production of complement-dependent neutralizing antibodies, but in the absence of native glycosylation significant titers of complement-inde-

pendent neutralizing antibodies were produced. Furthermore, boosting of animals initially inoculated with VVCMV-infected cells resulted in the production of significant titers of complement-independent neutralizing antibodies. Consistent with these findings, we have also found that neutralizing monoclonal antibodies reactive with *E. coli* gp55-116 exhibit only minimal amplification of neutralizing activity in the presence of complement (data not shown). Other workers have also noted that denatured antigens, in the form of either synthetic peptides or recombinant-derived proteins, can stimulate neutralizing antibodies against eucaryotic viruses, including herpesviruses (34) and, more recently, human immunodeficiency virus (24). Interestingly, these antibody activities were independent of complement, suggesting that at least some epitopes expressed on envelope of virions can interact with antibody directly to limit infectivity. In the case of HCMV, little is known about the functional role of the various envelope components in the process of infectivity, but our findings suggested that HCMV gp55-116 may have multiple functions and that antibodies directed against different epitopes may inhibit infectivity by different mechanisms. Delineating such functional activities may help in elucidating the pathogenesis of HCMV infections and provide directed approaches for preventing HCMV infection and associated disease.

Immunization of mice with the vaccinia recombinant virus and the *E. coli*-derived gp55-116 primed these animals for an augmented secondary response when challenged with HCMV gp55-116. We attempted to boost these animals in a manner which would simulate natural acquisition of HCMV. Because HCMV does not replicate in cells of nonhuman origin, we could not directly challenge the experimental animals and measure protection from disease. Therefore, instead of boosting in the presence of an adjuvant as other investigators did (6), we utilized VVCMV-infected cells or VVCMV virus or both. Our results were consistent with a previous report in which investigators utilized recombinant vaccinia virus-expressed HCMV gB emulsified in incomplete Freund adjuvant to demonstrate a booster phenomenon. Perhaps of greater interest was the finding that a weak, almost insignificant, complement-independent neutralizing antibody response detected following the initial inoculation with the recombinant VVCMV could be significantly boosted by exposure to VVCMV-infected cells. Overall, it appeared that primary immunization with the HCMV vaccinia virus recombinant produced higher initial levels of antibody, a more lasting immunity, and a readily boostable response compared with mice given *E. coli*-derived gp55-116. Previous reports have stressed the levels of immunity produced by recombinant vaccinia virus as well as the protection from disease afforded by this immunity (8, 9, 16). We were unable to directly determine the influence of immunization of mice with HCMV gp55-116 on resistance to disease, but if future investigations indicate that protective responses in humans are directed towards this protein, immunization with HCMV gp55-116 may be an effective means to limit the sequelae of HCMV infection.

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