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Human Cytomegalovirus Neutralizing Antibody-Resistant Phenotype Is Associated with Reduced Expression of Glycoprotein H

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We have characterized a neutralizing antibody-resistant mutant human cytomegalovirus (HCMV) obtained from a patient treated with a human monoclonal antiglycoprotein H (gH; unique long region 75) antibody. This virus exhibited resistance to several different neutralizing anti-gH murine monoclonal antibodies (MAbs), as well as to a polyvalent anti-gH serum. The resistant phenotype was unstable and could be maintained only by passage of plaque-purified virus under neutralizing MAb selection. In the absence of a MAb, the resistant phenotype reverted to a neutralizing antibody-sensitive phenotype within one passage. The predicted amino acid sequences of gH from the MAb-resistant and -susceptible parent viruses were identical. Biochemical analysis of the MAb-resistant and -susceptible parent viruses revealed a marked decrease of gH expression in the envelope of the MAb-resistant virus. Furthermore, propagation of the virus in various MAb concentrations resulted in the production of extracellular virions with various levels of resistance to the neutralizing activity of the MAb. These results suggest a mechanism for the generation of neutralizing antibody-resistant viruses which could evade host-derived antiviral antibody responses. In addition, our findings indicate that the stoichiometry of gH in the envelope of infectious HCMV virions is not rigidly fixed and therefore offer a simple explanation for production of phenotypic variants of HCMV through an assembly process in which the content of gH in the envelope of progeny virions varies randomly.

Human cytomegalovirus (HCMV) is the largest and structurally most complex of the human herpesviruses. It is a common cause of disease in immunocompromised individuals, such as allograft recipients and patients with AIDS (17, 24, 28). In addition, HCMV is the most common cause of congenital viral infection in humans (18, 40, 41). Since symptomatic infections occur almost exclusively in immunocompromised populations, resistance to HCMV infection appears to depend on an intact host immune response. Several studies have suggested that the humoral immune response may play a protective role during HCMV infection (21, 42, 45). Antibodies to two HCMV surface proteins, glycoproteins B (gB) and H (gH; unique long region 75 [U_L75]), can be detected in immune human serum, and antibodies against these proteins have been shown to neutralize HCMV infectivity in vitro (5, 9, 10, 32, 37).

gH is conserved in all three subgroups of the herpesvirus family. In studies of herpes simplex virus, gH has been shown to be essential for viral infectivity (20). Similar to the findings reported for herpes simplex virus neutralizing antibodies against gH of Epstein-Barr virus and HCMV have been shown to inhibit virus penetration but not attachment (23, 27, 29). Thus, it has been proposed that gH plays a role in membrane fusion during infection (23, 27, 29). Recent studies have also suggested that the gH homologs of varicella-zoster virus may function in virion egress and cell-cell spread of infectivity (33).

HCMV gH (gp86) is a type I glycoprotein with an estimated mass of 86 kDa (15, 31). gH has been detected in the virion envelope of HCMV and on the surface of HCMV-infected cells (15). It has been recently reported that gH is associated with another viral protein, gL, which facilitates gH transport to the cell surface (26, 39).

In this study, we characterized a clinical HCMV isolate following propagation under the selective pressure of an anti-gH neutralizing monoclonal antibody (MAb). Progeny virus highly resistant to the neutralizing activity of this MAb was isolated. Interestingly, the MAb resistance phenotype extended to other anti-gH MAbs and polyvalent anti-gH sera. Additional evidence suggests that the MAb-resistant phenotype resulted from decreased amounts of gH in the virion envelope and not from a genetic change in the gH coding sequence. These findings provide a mechanism for antibody resistance and viral persistence. Moreover, these results suggest that the stoichiometry of glycoproteins within the envelope of infectious HCMV virions can be highly variable, leading to production of phenotypic mixtures of progeny virions.

MATERIALS AND METHODS

Cells and virus. The propagation of human foreskin fibroblast (HFF) cells has been described previously (5). HCMV clinical strain FL-2 was isolated from the urine of an AIDS patient with HCMV retinitis during therapy with anti-gH human MAb MSL109 as part of a phase I study of this human antibody (30) (provided by F. Lakeman, University of Alabama at Birmingham). This virus isolate was initially plaque purified in HFF cells. Prior to serial dilutions for plaque purification, the virus-containing supernatant was filtered through a 0.8-μm-pore-size filter to remove viral aggregates. The extracellular virus was then plaque purified two additional times in media containing 10 to 15 μg of anti-gH MAb 14-4b per ml (4). MAb 14-4b was used for selection of resistant virus because the virus resistant to human MAb MSL109 was also resistant to the neutralizing activity of MAb 14-4b. Following plaque purification, supernatant from infected cells was incubated with 15 μg of MAb 14-4b per ml for 60 min at 37°C. This mixture was then inoculated onto HFF cells in media containing 10 to 15 μg of MAb 14-4b per ml. Virus passaged in this manner was designated FL-2r and was passaged in this fashion more than 10 times under MAb 14-4b selection prior to antigenic or biochemical analysis. Parent virus FL-2 was passed similarly but without exposure to an antibody. Viruses isolated from extracellular supernatant were gradient purified as described previously (5).

MAbs. gH-specific MAbs 14-4b and AP86 were produced in our laboratory, and gH-specific MAbs 5, 442, and 115 were produced as described previously (4, 37, 43). MAb 7-17 against gB has been described previously (44). Antiserum specific for gH was generated from BALB/c mice immunized with recombinant gH protein expressed in insect cells.

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Neutralization assay. The microneutralization assay used in this study has been described previously (1). Briefly, 0.3 ml of infectious virus (approximately 100 PFU) was mixed with 0.3 ml of diluted antisera or murine MAbs (100 to 0.25 μg/ml). After 60 min of incubation at 37°C, 0.2 ml of a virus-antibody mixture was added to replicate wells of a 96-well tissue culture plate containing HFF cells. Three hours later, the inoculum was removed and the monolayer was fed with fresh medium. The following day, the medium was removed and the monolayer was washed once with Dulbecco's phosphate-buffered saline, pH 7.4, and then fixed in absolute ethanol. Following fixation, the monolayer was stained with MAb p63-27, which is reactive against immediate-early gene 1 product pp72, and the number of antigen-positive cells was quantitated by immunofluorescence. Results are expressed as the mean percent reduction in fluorescent nuclei compared with control cultures containing no antibody or control nonimmune sera.

Direct DNA sequence of PCR products. DNA sequencing was performed by standard dideoxy-chain termination reactions with sequenase (U.S. Biochemical, Cleveland, Ohio) and ^{35}S-dATP (ICN, Irvine, Calif.). The region containing the gene for gH or gL was amplified by PCR from FL-2- or FL-2r-infected cells. PCR products were resolved by electrophoresis in 1% agarose gels. The band corresponding to the size of the gene for gH or gL was excised, and 3 volumes of 6 M NaI was added. The mixture was heated at 55°C for 10 min and processed by using the protocol described in the USBioclean kit (U.S. Biochemical). The DNA was resuspended in 7 μl of H_2O and subjected to sequencing reactions in accordance with the sequence protocol (U.S. Biochemical). The reaction products were resolved in 6% acrylamide gels, and sequence comparisons were performed with the BESTFIT program of the University of Wisconsin Genetics Computer Group package.

Radiolabeling of infected-cell proteins and virion surface proteins. Confluent monolayers of HFF cells in 35-mm-diameter tissue culture dishes were infected with virus FL-2 or FL-2r. When >90% of infected cells showed an early cytopathic effect, they were labeled overnight with ^{35}S-methionine as described previously (7). The cells were lysed by addition of 1 ml of wash buffer (0.1% sodium dodecyl sulfate, 1.0% Nonidet P-40, 1.0% deoxycholate in Tris-buffered saline [0.05 M Tris, 0.15 M NaCl, pH 7.4]). Gradient-purified extracellular virions were radiolabeled with ^{125}I as previously described (8).

Immunoprecipitation and immunoblotting. Immunoprecipitation and immunoblotting were performed as described previously (7). Molecular weights were estimated by comparison with molecular weight standards (Sigma Chemical Co., St. Louis, Mo.).

Nucleotide sequence accession number. The EMBL accession numbers for HCMV (AD169) gH and gL are M19882 and X17403.

RESULTS

Selection and isolation of a gH neutralizing antibody-resistant virus. A clinical strain of HCMV, designated FL-2, was isolated from an AIDS patient with HCMV retinitis during treatment with human anti-gH MAb MSL109 (30). This human antibody has been shown to be reactive with HCMV gH and exhibits potent in vitro virus-neutralizing activity (30). Because the original isolate exhibited unstable MAb resistance when assayed with both human and murine anti-gH MAbs, the virus was plaque purified twice and propagated in media containing murine anti-gH MAb 14-4b (4). MAb 14-4b was utilized for the remainder of the study because preliminary studies suggested that it competes with MAb MSL109 for binding to gH. Although the amount of the virus produced in the presence of MAb 14-4b was reduced, sufficient extracellular virus was collected for two additional cycles of plaque purification under MAb 14-4b selection. Following the second round of plaque purification, the extracellular virus was propagated for an additional 10 passages under MAb 14-4b selection. The plaque-purified virus which was serially passaged under MAb 14-4b selection was designated virus FL-2r.

Extracellular virus FL-2 grown in the absence of MAb 14-4b reached a maximum titer (10^5 PFU/ml) on day 7 postinfection, whereas production of extracellular virus FL-2r from infected cells cultured in the presence of MAb 14-4b was undetectable in the first 5 days and reached the highest concentration of 10^4 PFU/ml on day 12 (data not shown). Additional experiments suggested that in the presence of MAb 14-4b, resistant virus FL-2r spread more slowly in susceptible monolayers than did parent virus FL-2 propagated in the absence of antibody (data not shown). The size of virus-infected plaques 6 day postinfection was consistent with this result. Plaques in HFF cell monolayers infected with virus FL-2r in the presence of MAb 14-4b were estimated to be 50% smaller than the plaques induced by virus FL-2 (data not shown). Together, these experiments suggest that the growth parameters of resistant virus FL-2r and parent virus FL-2 are similar, although the level of virus production and rate of spread of parent virus FL-2 in the absence of a MAb were increased compared with those of resistant virus FL-2r passaged in the presence of a MAb.

The susceptibility of virus FL-2r to the neutralizing activity of MAb 14-4b was quantitated after multiple passages under MAb selection. Extracellular virus from these cultures was found to be highly resistant to MAb 14-4b (Fig. 1A). Extracellular virus FL-2r retained more than 92% of its input infectivity following incubation with MAb 14-4b over a broad concentration range, whereas the infectivity of parent virus FL-2 was reduced significantly over the same range of antibody concentrations. Approximately 0.25 μg of MAb 14-4b per ml neutralized 50% of parent virus FL-2 but had no effect on the infectivity of virus FL-2r. In fact, we achieved only a 5 to 10% reduction in the infectivity of virus FL-2r following incubation with the highest concentration of MAb 14-4b (100 μg/ml; Fig. 1A). In contrast, both virus FL-2r and parent virus FL-2 were neutralized to similar extents by a polyvalent anti-gB serum (Fig. 1F). By using a panel of gH-specific MAbs which recognize different antigenic sites and a polyclonal anti-gH serum, we further characterized the antibody resistance phenotype of virus FL-2r. Neutralizing gH-specific murine MAbs 5, 109, and 442 exhibited nearly identical patterns of neutralizing activity, as seen previously with MAb 14-4b (Fig. 1B to D). In addition, a similar result was observed when an anti-gH polyclonal serum was used in the neutralization assay. These results suggest the FL-2r-resistant phenotype extends to multiple MAbs, as well as polyclonal antisera, making it unlikely that an alteration of a single antigenic site is responsible for its MAb-resistant phenotype. Furthermore, because all of the antibodies used in this study recognized gH in the absence of other HCMV-encoded proteins, it is also unlikely that a mutation in a gH-associated protein, such as gL, could explain this resistant phenotype. In marked contrast to these results, the intracellular virus derived from cells infected with MAb-resistant virus FL-2r was susceptible to neutralizing MAb 14-4b, exhibiting a phenotype which was clearly different from that of the extracellular virus. The residual levels of infectivity of the intracellular virus obtained from FL-2r-infected cells following incubation with MAb 14-4b at 0, 1, 10, and 100 μg/ml were 100, 77.8, 51.4, and 17.1%, respectively.

DNA sequence comparison of genes for gH and gL in virus strains FL-2r and FL-2. To determine if a mutation within the gH coding sequence accounts for the antibody resistance of virus FL-2r, the region encoding the gH (U_L75) open reading frame was sequenced directly from PCR products of DNA amplified from virus FL-2- or FL-2r-infected cells. A series of primers were used to sequence both the coding and noncoding strands. The DNA and deduced amino acid sequences of gH from both viruses FL-2 and FL-2r were compared and aligned with the gH of HCMV laboratory strain AD169 (Fig. 2A). Several amino acid differences between viruses AD169 and FL-2 were noted, but there was no difference in the predicted amino acid sequences of gH from viruses FL-2 and FL-2r. These findings are consistent with the findings described above and indicate that the MAb-resistant phenotype is not associated with a genetic change leading to loss of MAb binding sites.

It has been reported that gL (U_L115) of HCMV forms a complex with gH and facilitates intracellular gH transport (26, 38). To exclude the possibility that a mutation in gL leads to altered expression of gH on the viral or infected-cell surface, we sequenced the U_L115 open reading frame in both viruses

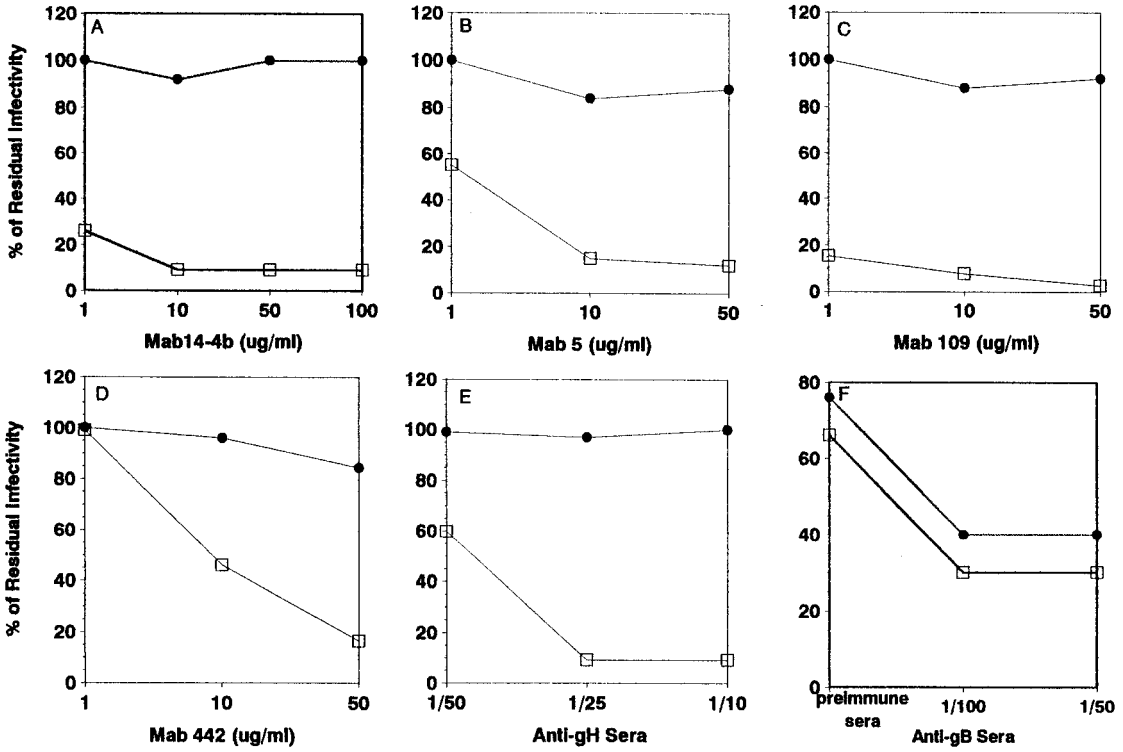

FIG. 1. Resistance of virus FL-2r to virus-neutralizing anti-gH MAbs and polyvalent anti-gH and anti-gB sera. Extracellular virus FL-2r (●) or FL-2 (□) was incubated with increasing concentrations of anti-gH MAbs (A to D) or diluted anti-gH (E) or anti-gB (F) serum, and the residual infectivity of the inoculum was determined as described in Materials and Methods.

FL-2r and FL-2. Two amino acid differences between the gL genes of viruses FL-2r and FL-2 were observed (Fig. 2B).

Reversibility of the antibody-resistant phenotype of virus FL-2r: dependence on antibody concentration. Our results indicated that the MAb-resistant phenotype of virus FL-2r is not associated with a genetic change and therefore should be reversible. Progeny viruses from FL-2r-infected cells that were passed once or twice without MAb 14-4b exhibited susceptibility to the neutralizing MAb which was similar to that of antibody-sensitive parent virus FL-2 (Fig. 3A). The concentrations of MAb 14-4b required for 50% reduction in infectivity for both viruses did not exceed 1 μg/ml (Fig. 3A). The finding that virus FL-2r altered its resistant phenotype very rapidly is consistent with our earlier findings and argues against a mutation in the amino acid sequence of either gH or gL as an explanation for its MAb-resistant phenotype. This phenotypic variation occurred within one passage without MAb 14-4b, and no intermediate phenotype was observed. Furthermore, sequence analysis of the U_L115 open reading frame amplified from infected cells following reappearance of the virus FL-2r MAb-susceptible phenotype revealed no changes in the nucleotide sequence in the gene for gL, i.e., V-17→I and V-30→A were present in both phenotypes (data not shown). This finding provides compelling evidence that the phenotypic reversion is not secondary to overgrowth of contaminating parental virus FL-2 (data not shown). The neutralization-sensitive virus population derived from virus FL-2r was then passaged in media containing MAb 14-4b. After two passages, the extracellular virus derived from these cultures was highly resistant to the neutralizing activity of MAb 14-4b, providing further evidence for reversibility of the resistant and susceptible phenotypes (Fig. 3B). This observation argues against a second-site mutation as an explanation for the reversibility of the antibody-resistant phenotype.

We next examined the quantitative relationship between the neutralizing antibody concentration in the culture media and the resistant phenotype of the extracellular virus. Cells infected with virus FL-2r were propagated in media containing various concentrations of MAb 14-4b (50, 10, 1, and 0 μg/ml). Extracellular virus was then collected and incubated with 50 μg of MAb 14-4b per ml, and the residual infectivity was determined. The virus propagated in 50 μg of MAb 14-4b per ml remained

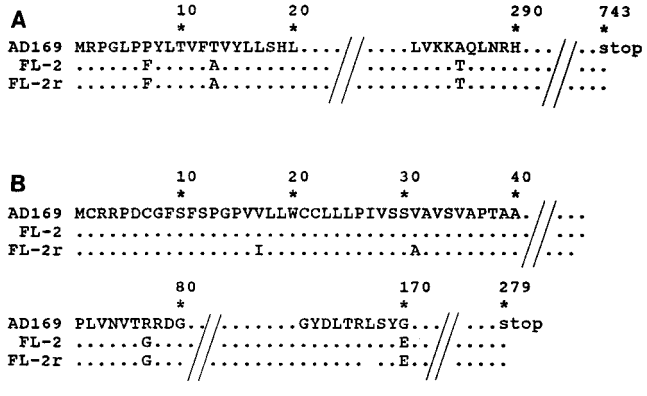

FIG. 2. Comparison of predicted amino acid sequences encoded by the gH (A) and gL (B) genes from viruses FL-2, FL-2r, and AD169. DNA encoding gH (U_L75) or gL (U_L115) was amplified from virus FL-2- or FL-2r-infected cells by PCR and sequenced directly as described in Materials and Methods. Alignment of the predicted amino acid sequences was accomplished by using the BESTFIT program of the University of Wisconsin Genetics Computer Group package. Only areas in which the predicted amino acid sequence differed from that of AD169 are shown. The gH sequences were identical for viruses FL-2 and FL-2r (A), whereas the gL sequences of the viruses differed by two amino acids (V-17→I and V-30→A) (B).

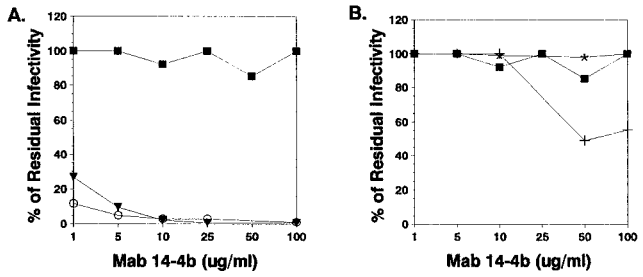

FIG. 3. Reversion of the FL-2r MAb-resistant phenotype following passage in the absence of an antibody. (A) Extracellular virus FL-2r was passaged in the presence of 15 μg of MAb 14-4b per ml (■) or passaged once (▼) or twice (○) in the absence of the MAb, and the susceptibility of progeny extracellular virus to the neutralizing activity of MAb 14-4b was determined as described in Materials and Methods. (B) The neutralizing MAb-susceptible virus obtained following passage of virus FL-2r in the absence of a MAb (A) was then subjected to one (+) or two (*) passages in the presence of 15 μg of MAb 14-4b per ml. Progeny extracellular virus from these cultures was then assayed for susceptibility to neutralization by MAb 14-4b. Extracellular virus FL-2r (■) passaged in 15 μg of MAb 14-4b per ml was included as a control.

highly resistant to the neutralizing activity of MAb 14-4b, maintaining 97% of its input infectivity (Fig. 4A). In contrast, virus FL-2r grown in media without MAb 14-4b was readily neutralized by MAb 14-4b (Fig. 4A). The virus grown in 1 and 10 μg of antibody per ml exhibited intermediate resistance phenotypes (Fig. 4A). This result suggested that the MAb neutralization-resistant phenotypes of virus FL-2r could be selected by the quantity of MAb in the culture media. In contrast, intracellular virus from these same cultures remained sensitive to neutralizing antibody regardless of the concentration of MAb 14-4b present in the culture media (Fig. 4B).

Expression of gH protein in extracellular viruses FL-2 and FL-2r and in infected cells. We characterized the expression of gH in cells infected with parent virus FL-2 and cells infected with MAb-resistant virus FL-2r to determine whether the phenotypic differences between these viruses could be explained by differences in gH expression. To define the level of gH expression, virus FL-2- or FL-2r-infected cells were radiolabeled with ^{35}S-methionine and infected-cell proteins were precipitated with gH-specific MAbs 14-4b and AP86 and gB-specific MAb 7-17 (43, 44). MAb 7-17 precipitated components of the gB complex, including the gp150 precursor and gp55 and gp116 cleavage products (7). The precipitates were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). MAb 14-4b precipitated the 86-kDa gH protein from both virus FL-2-infected and virus FL-2r-infected cells (Fig. 5A). The level of gH expression relative to that of gB expression indicated that cells infected with virus FL-2r produced quantities of gH similar to those produced by cells infected with virus FL-2. This finding suggests that phenotypic alteration of virus FL-2r cannot be ascribed to decreased synthesis of gH by virus FL-2r-infected cells.

Although our finding failed to demonstrate decreased synthesis of gH in cells infected with virus FL-2r, these studies did not quantitate the level of gH expression in extracellular virus produced by these infected cells. A potential explanation for our results is that extracellular virus FL-2r which survived MAb 14-4b selection expresses a reduced amount of gH in the virion envelope. We examined this possibility by surface radioiodination of extracellular virions obtained from virus FL-2- and FL-2r-infected cells. The latter virion preparation was obtained from infected cells cultured in the presence of MAb 14-4b. The labeled envelope proteins were immunoprecipitated with a panel of MAbs, and the precipitated proteins were analyzed by SDS-PAGE. We attempted to normalize the quantity of radiolabeled envelope components in the immunoprecipitation reactions by using gB as an internal standard for each virion preparation. As a result, lanes containing precipitated gB appeared to be overloaded because of the relative abundance of gB compared with other envelope components, including gH. The anti-gB MAb precipitated approximately equal quantities of the gB complex, including both the gp116 and gp55 components, from both FL-2 and FL-2r virions (Fig. 5B). MAb 28-4, reactive with the major capsid protein (MCP; U_L86) was also included to control for the presence of infected-cell proteins which may have contaminated the virion preparation. The anti-MCP MAb failed to precipitate a protein of the estimated 150,000-Da mass of the MCP from either virus FL-2 or FL-2r, suggesting that the initial virion preparation contained predominantly intact virions; however, several nonspecific bands were detected in lanes containing precipitates generated with the anti-MCP antibody (Fig. 5B). Anti-gH MAb AP86 precipitated gH and a previously detected but uncharacterized protein of approximately 130 kDa (p130) from FL-2 viral lysates (Fig. 5B) (4). This protein was detected inconsistently in virion preparations and appeared to be antigenically unrelated to gH, as evidenced by our inability to detect this species in an immunoblot of purified virions (data not shown). Precipitation of FL-2r virion lysates with MAb AP86 revealed much decreased amounts of gH and p130, which could be detected only after extended exposure of the gels (Fig. 5B). Because these virion preparations were purified and radiolabeled at the same time for use in the same experiments, these results suggest that extracellular virions from FL-2r-infected cultures contained less gH than the virions from FL-2-infected cultures when the envelope amount was standardized relative to gB content. Moreover, this result suggests that the MAb-resistant phenotype of virus FL-2r could be secondary to the diminished gH content.

We next analyzed the gH content in the envelope of the virions from virus FL-2r-infected cells cultured in the absence of MAb 14-4b. This population had undergone phenotypic reversion and was as susceptible to the neutralizing activity of MAb 14-4b as was parent virus FL-2. Extracellular virus from virus FL-2-infected cells was collected as part of the same experiment and served as a control. Virions from FL-2r-infected cells propagated in the absence of MAb 14-4b appeared to contain a greater amount of gH in the envelope than did

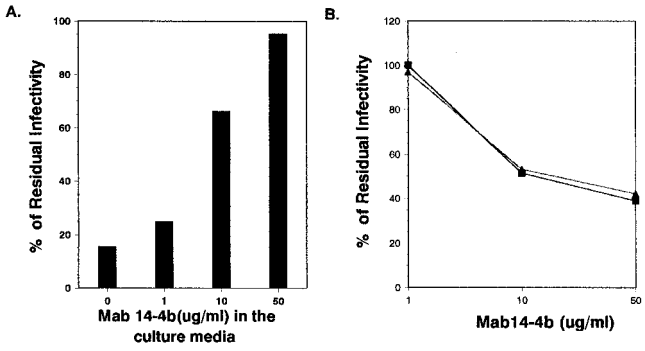

FIG. 4. Relationship between the MAb-resistant phenotype of virus FL-2r and the MAb concentrations in culture media. (A) Extracellular virus FL-2r-infected cells were passaged in media containing different concentrations of MAb 14-4b (0, 1, 10, and 50 μg/ml). Extracellular virus was collected 12 days later and used in a neutralization assay in which a single concentration of 50 μg of MAb 14-4b per ml was used. Results are presented as residual infectivity of the inoculum plotted versus the MAb concentration in the original culture medium. (B) Intracellular virus isolated from virus FL-2r-infected cells cultured in the presence of 50 μg of MAb 14-4b per ml (■) or in the absence of a MAb (▲) were tested in a neutralization assay with differing MAb concentrations.

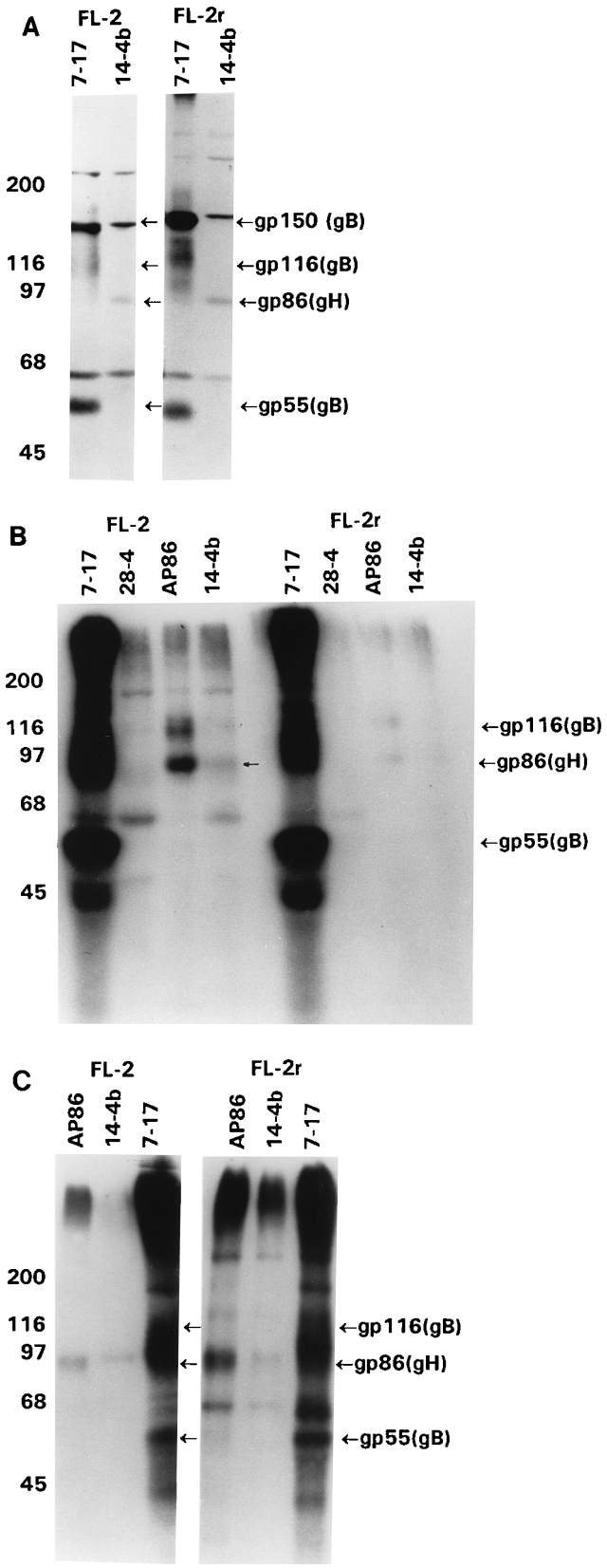

FIG. 5. Immunoprecipitation of gH protein from virus-infected cells and purified extracellular virus. (A) Human fibroblast cells infected with virus FL-2 or FL-2r (cultured in the presence of 15 μg of MAb 14-4b per ml) were pulse-labeled with ^{35}S-methionine, and infected-cell proteins were precipitated with an anti-gH (14-4b) or anti-gB (7-17) MAb and analyzed by SDS-PAGE as described in Materials and Methods. Migration was determined by comparison to molecular weight standards (Sigma Chemical Co.). The intracellular forms of gB (gp150, gp116, and gp55) precipitated by MAb 7-17 and gH (gp86) precipitated by MAb 14-4b are indicated on the right. The prominent bands migrating at an estimated 150 kDa in the MAb 14-4b precipitation were nonspecific and not antigenically related to gH. (B) Extracellular virus was obtained from virus FL-2- and FL-2r-infected cells (cultured in the presence of 15 μg of MAb 14-4b per ml) by sucrose gradient centrifugation and iodinated with ^{125}I as previously described (5, 8). Virion proteins were solubilized, precipitated with an anti-gH (AP86; 14-4b), anti-gB (7-17), or anti-MCP (U_L86; 28-4) MAb and analyzed by SDS-PAGE as described above. The components of the gB complex in virions and gH are indicated on the right. Note the difference in gH content between FL-2 and FL2r virions. The prominent protein migrating with an estimated mass of 130 kDa in the AP86 precipitates represents a coprecipitating protein which was not consistently detected in immunoprecipitations with MAb AP86 and did not appear to be antigenically related to gH (data not shown). (C) Extracellular virions were obtained from virus FL-2-infected cells and virus FL-2r-infected cells (cultured in the absence of a MAb) and subjected to iodine labeling and analysis as described for panel B. In the latter case, the virus FL-2r MAb-resistant phenotype had reverted to the MAb-susceptible phenotype. The numbers to the left are molecular sizes in kilodaltons.

neutralizing antibody-sensitive parent virus FL-2 (Fig. 5C). Together, these results and the findings described above suggest that the gH content in the envelope of extracellular virus FL-2r could be related to the susceptibility of the virus to the neutralizing activity of anti-gH MAbs such that reduced gH content in the envelop of HCMV renders these particles resistant to neutralization by anti-gH antibodies.

DISCUSSION

Our studies revealed that neutralizing antibody-resistant phenotypes of HCMV can develop independently of genetic change. The finding that neutralizing antibody-resistant extracellular virus contained quantitatively less gH than the neutralizing antibody-susceptible parent virus suggested that the mode of antibody resistance was likely a result of unfavorable binding kinetics between this population of viruses and neutralizing anti-gH antibodies. Consistent with this proposed mechanism of antibody resistance was the observation that the growth of virus FL-2r-infected cells in the presence of various concentrations of a neutralizing MAb resulted in a gradient of antibody-resistant viral phenotypes. This result suggested that viruses containing the largest amount of gH in their envelope were readily neutralized, whereas those with lesser amounts survived antibody neutralization and were scored as infectious in our assay. Although we have not definitively shown that this mechanism of antibody resistance was operative in vivo, the isolation of virus with the resistant phenotype from a patient treated with a human anti-gH MAb suggested that similar phenotypes could develop in vivo. Furthermore, the binding kinetics in vivo would likely be even less favorable than those observed in vitro for a virus containing limiting amounts of gH. Such a mechanism of virus resistance to neutralizing antibodies could provide a partial explanation for the observed persistent replication of HCMV in the presence of significant quantities of a systemic neutralizing antibody (21, 38). It should also be noted that such a mechanism of resistance to an anti-gH MAb would be consistent with the large variability in susceptibility of clinical HCMV isolates to the neutralizing activities of different murine anti-gH MAbs, even though all of the viruses used in these studies exhibited reactivity with the MAbs in binding assays (2, 37). Finally, we have preliminary evidence that this resistance phenomenon is not limited to this single clinical isolate. An additional viral isolate from a different patient enrolled in this clinical trial also exhibited the same pattern of reversible neutralizing MAb resistance, even following plaque

purification under MAb selection (data not shown). We did not study viral envelope gH expression in this isolate to determine if, indeed, the level of MAb resistance correlates with gH expression.

Alternative interpretations of our results include decreased virus release secondary to a mechanism previously described as antigenic modulation. Studies of measles virus-infected cells have detailed a mechanism by which cell surface-reactive, virus-specific antibodies can reduce the yield of infectious virus (22). Similar findings were noted with antibody-treated, murine leukemia virus-infected cells (14). In both experimental systems, antiviral antibodies reduced the expression of intracellular viral structural proteins, suggesting a mechanism for decreased virus yield (14, 22). In our studies, we found little evidence for an alteration in viral protein expression in antibody-treated infected cells. A recent study of antibody-treated varicella-zoster virus-infected cells demonstrated a redistribution of progeny virus on the cell surface, such that virus-induced syncytia were no longer observed and virus spread was inhibited (35). Our results do not exclude such a redistribution of cell surface virions, but we feel that this explanation is unlikely because HCMV does not readily form syncytia, and although contiguous cell spread is likely an important mode of virus transmission, our findings were restricted to cell-free virus. Together, our data are most consistent with neutralizing antibody selection of a phenotypically mixed population of extracellular viruses.

The findings of this study suggest a potentially unique feature of the envelope morphogenesis of HCMV. The complexity of the envelope of human herpesviruses can be appreciated by considering the numbers of individual proteins which must be inserted into the envelope to generate an infectious particle. The envelope of HCMV has been shown to contain at least six glycoproteins, and analysis of the genome has suggested that an additional 50 or more open reading frames may encode glycoproteins (13). Although several herpesvirus envelope glycoproteins were originally classified as dispensable, recent evidence has suggested that the virus may utilize different glycoproteins for different cellular receptors and, therefore, require all envelope components for the tropism observed in vivo (3, 11, 16, 19, 36). A fundamental question surrounding the study of HCMV envelope morphogenesis is the stoichiometry of the glycoprotein components of the envelope, specifically, the amount of each glycoprotein required for production of an infectious particle. Deletion of genes such as those for gH, gL, gB, and gD in herpes simplex virus has provided evidence of essential functions; however, there is little information which has related the quantity of individual glycoproteins in the envelope and function, which in most cases has been measured as infectivity (12, 20, 25, 33, 34). Our results suggest that during HCMV morphogenesis, phenotypic mixtures of viruses which vary in envelope glycoprotein composition are produced. Although a minimal amount of gH in the envelope of HCMV was likely required for recognition by anti-gH antibodies, our experiments did not define a threshold of gH expression at which HCMV was rendered noninfectious. This assembly pathway would provide several advantages for persistence of HCMV in the natural host. It would reduce the stringency of the stoichiometry of various glycoproteins within the envelope, thus reducing the structural constraints for assembly of an infectious, enveloped particle. Secondly, by generating particles with reduced glycoprotein content, progeny virus could escape host-derived antiviral antibody recognition. The latter mechanism of antibody resistance could provide a simple mechanism of phenotypic variation in a virus which has exhibited very little change in the structure of large blocks of genomic DNA, even after prolonged in vitro or in vivo passage. Finally, as noted earlier, we have observed viral phenotypes with similar reversible resistance phenotypes following MAb selection and plaque purification under MAb selection of high-passage laboratory strains such as AD169 (6a). Thus, it appears that this proposed assembly pathway is not limited to early-passage clinical isolates and may be a universal aspect of HCMV envelope morphogenesis.

Several lines of evidence suggest that the neutralizing anti-gH antibody-resistant phenotype of virus FL-2r did not arise secondary to a mutation in the gene encoding either gH or gL. In addition to the identity of the sequences of the gH genes from both parent and MAb-resistant viruses, the finding of global resistance to the neutralizing activity of multiple MAbs which recognize different epitopes on gH and polyvalent anti-gH sera argue against a single mutation following selection with one MAb. Recent investigations have suggested that the product of the gL gene may contribute to intracellular transport of gH in human fibroblasts, suggesting that the decreased expression of gH in extracellular virions could be explained by a failure of a mutant gL to efficiently facilitate transport gH to sites of envelope assembly (26, 38). We found no evidence of decreased intracellular or cell surface gH expression in parent virus FL-2- or virus FL-2r-infected cells, suggesting that the observed changes in the gL sequence of virus FL-2r did not affect gH transport (data not shown). Sequence analysis of the gL genes from the resistant and susceptible viruses revealed two amino acid changes in a hydrophobic region of the amino terminus of the molecule, potentially within an area that may serve as a signal peptide. In addition, the changes from valine to isoleucine and valine to alanine were conservative and appeared to be random mutations, as the same mutation were not detected in the gL sequence of a second clinical isolate which exhibited the same reversible antibody-resistant phenotype (data not shown). Finally, the intracellular virus isolated from virus FL-2r-infected cells exhibited a MAb-sensitive phenotype, indicating that the observed mutation in gL did not alter gH transport or virus assembly. Together, these findings suggest that the observed phenotypic changes in virus FL-2r were unlikely due to sequence differences in gL. More importantly, the current understanding of the relationship between gH and gL does not support a mechanism in which a mutation(s) in gL would produce a reversible phenotypic change in the expression of gH in the envelope of extracellular virus. In contrast to results obtained with alphaherpesvirus, an association between gH and gL has not been detected in extracellular virions. In fact, HCMV gH can induce potent virus-neutralizing antibodies independently of other virus-encoded proteins, including gL.

A trivial explanation of our findings is that we analyzed a mixture of viruses contaminated with rapidly growing wild type and a more slowly replicating, antibody-resistant viral mutant. We feel that this interpretation is unlikely for several reasons. The first is that virus FL-2r was isolated by numerous plaque purifications of extracellular virus and the progeny were passaged under antibody selection. Perhaps the most convincing evidence is the finding that the amino acid changes noted in the gL gene of virus FL-2r were conserved in the MAb-susceptible phenotype of the virus generated by propagation of FL-2r-infected cells in the absence of MAb 14-4b. This genetic marker further verified that the reversion to MAb susceptibility was not the result of outgrowth of parent virus FL-2. Lastly, we have only limited evidence which suggests that antibody-resistant virus FL-2r spread less rapidly in culture than the susceptible parent. An alternative interpretation of the decreased level of virus production from virus FL-2r-infected cells in the presence of MAb 14-4b is that the antibody-resistant FL-2r viral phenotype represents a minority of the re-

leased virus, perhaps less than 10%. Thus, the peak titer produced would be significantly less than that of the virus produced from infected cells cultured in the absence of a MAb. Once an extracellular MAb-resistant virus was produced, however, it would be expected to spread nearly as rapidly to susceptible cells as a virus produced in the absence of an antibody. This was, in fact, the case, as we documented similar intervals between initial detection of infectious, cell-free virus to peak titers in both untreated and antibody-treated cultures.

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