A Role for Human Cytomegalovirus Glycoprotein O (gO) in Cell Fusion and a New Hypervariable Locus

David A. Paterson,*,† Angela P. Dyer,* Richard S. B. Milne,*,†,1 Edgar Sevilla-Reyes,* and Ursula A. Gompels*,2

*Pathogen Molecular Biology and Biochemistry Unit, Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, University of London, Keppel Street, London WC1E 7HT, United Kingdom; and †Department of Medical Microbiology, St. George's Hospital Medical School, University of London, Cranmer Terrace, London SW17 ORE, United Kingdom

A cell fusion assay using fusion-from-without (FFWO) recombinant adenoviruses (RAds) and specific antibody showed a role in fusion modulation for glycoprotein gO, the recently identified third component of the gH/gL gCIII complex of human cytomegalovirus (HCMV). As in HCMV, RAd gO expressed multiple glycosylated species with a mature product of 125 kDa. Coexpression with gH/gL RAds showed gCIII reconstitution in the absence of other HCMV products and stabilisation by intermolecular disulfide bonds. Properties of HCMV clinical isolate, Pt, also implicated gO in cell spread. Compared to laboratory strain AD169, Pt was resistant to gH antibody plaque inhibition, but mature gH was identical. However, the gO sequences were highly divergent (20%), with further variation in laboratory strain Towne gO (34%). Thus, gO forms gCIII with gH/gL, performs in cell fusion, and is a newly identified HCMV hypervariable locus which may influence gCIII's function in mediating infection.   © 2002 Elsevier Science (USA)

Key Words: human cytomegalovirus; cell fusion; glycoprotein complex; infection; vaccine; gene variation; recombinant adenovirus; neutralising antibody.

INTRODUCTION

Human cytomegalovirus (HCMV) is a clinically important betaherpesvirus, and efforts to produce a vaccine, primarily to prevent congenital infection, are reliant on a better understanding of the virus surface glycoproteins involved in host cell infection. Conserved glycoproteins have been used in antibody-based diagnosis of primary infection during pregnancy and have also showed success as vaccines against congenital infection in animal models (Bourne *et al.,* 2001; Chatterjee *et al.,* 2001; Eggers *et al.,* 2001). However, evidence has been presented that variation in at least one of these glycoproteins can affect infection with new strains in intrauterine transmission in women with prior immunity to HCMV (Boppana *et al.,* 2001). In HCMV and other herpesviruses, together with some other enveloped viruses such as HIV, the mechanism of entry relies on the fusion of membranes, as does subsequent cell-to-cell spread, and is mediated by viral envelope glycoproteins. Although cell fusion is apparent from the cytopathic effect observed *in vivo*, it

has rarely been reported *in vitro* for HCMV, is cell-type dependent, and may differ between laboratory strains and clinical isolates (Booth *et al.,* 1978; Jarvis *et al.,* 1999; Gerna *et al.,* 2001).

To investigate further cell fusion mediated by HCMV, an assay was developed which uses "fusion-from-without" (FFWO). How does this compare to other fusion assays developed for herpes simplex virus (HSV), for example, such as neutralisation of syncytial strains in a plaque assay or transient expression of glycoprotein genes in a cell-based assay (Gompels and Minson, 1986; Gompels *et al.,* 1991; Turner *et al.,* 1998)? FFWO is inoculum-derived cell fusion by HCMV, which is mediated by virion envelope glycoproteins in the absence of virus gene expression, as demonstrated by addition of actinomycin D in the assay (Milne *et al.,* 1998). This distinguishes entry effects from factors involved in virus assembly, exit, or other effects from over 200 genome-encoded products which would be active in a plaque-based assay. Moreover, a neutralisation assay based on plaque development takes up to 2 weeks for HCMV, whereas FFWO occurs rapidly, by 1 and 8 h for HSV and HCMV, respectively (Falke *et al.,* 1985; Milne *et al.,* 1998). In HCMV, it was first demonstrated in fibroblasts, while best results are shown using virus prepared on primary human embryonic lung (HEL) cells and assayed on astrocytoma (U373MG) cell lines. Similar to other fusion assays, it correlates with effects of neutralising antibody against virus glycoproteins, thereby linking the relevance

---

[1] Present address: University of Pennsylvania, School of Dental Medicine, Department of Microbiology, 218, Levy Building, 4010 Locust Street, Philadelphia, PA 19104.

[2] To whom correspondence and reprint requests should be addressed at Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, University of London, Keppel Street, London WC1E 7HT, U.K. Fax: +44 (0)20 7637 4314. E-mail: ugompels@hgmp.mrc.ac.uk.

to the infectious process (Milne *et al.,* 1998). FFWO, similar to the other assays, does not distinguish between the linked functions in binding or penetration, but it does allow for further investigation of roles of individual glycoprotein components and their relationship to virus-induced cell fusion.

Recombinant adenoviruses (RAds), expressing glycoprotein gH/gL on the surface of infected cells, were used to examine effects on HCMV FFWO. These glycoproteins are the major components of the gCIII complex (Gretch *et al.,* 1988; Kaye *et al.,* 1992) and were shown to enhance fusion caused by HCMV viral membrane components in the FFWO assay (Milne *et al.,* 1998). This directly showed a role for gH/gL in cell fusion. It also confirmed previous indirect evidence, including inhibition of cell-to-cell spread by neutralising antibodies and inhibition of the transfer of a fluorescent probe from viral membranes to cell membranes by either an anti-gH antibody or its anti-idiotype (Rasmussen *et al.,* 1984, 1988; Cranage *et al.,* 1988; Baboonian *et al.,* 1989; Keay and Baldwin, 1991; Urban *et al.,* 1992, 1996; Simpson *et al.,* 1993; Baldwin *et al.,* 2000). Moreover, this role in cell fusion is analogous to the function of gH/gL conserved in other herpesviruses as shown in transient cell–cell fusion assays with alphaherpesvirus glycoproteins (Turner *et al.,* 1998; Klupp *et al.,* 2000; Muggeridge, 2000; Pertel *et al.,* 2001).

Analyses of the gH/gL complex in betaherpesviruses, HCMV and HHV-6, show roles for gL in cell surface transport and identify betaherpesvirus-conserved domains for gL binding to gH and fusion domains conserved across herpesvirus subgroups. Similar to other herpesviruses, gH complex formation with gL is required for cell surface and virion location of the gH/gL complex (Cranage *et al.,* 1988; Kaye *et al.,* 1992; Spaete *et al.,* 1993; Milne *et al.,* 1998). Of particular note is the observation that HHV-6 gH and HCMV gL can form heterodimers. This highlights the importance of the gH/gL complex conservation in all mammalian and avian herpesviruses studied to date with implications for effects on pathology in the betaherpesviruses (Anderson *et al.,* 1996). A betaherpesvirus-specific N-terminal domain in gH was identified which bound gL. This was established in experiments involving coexpression of HCMV gL and HHV-6 gH and immunoprecipitation of heterologous complexes (Anderson *et al.,* 1996). Further work identified a fusogenic-associated conformation in the herpesvirus-conserved C-terminal domain (NGS/TV motif) of HHV-6 gH which was mediated by N-terminal binding of gL (Anderson and Gompels, 1999). This study also showed that an N-terminal domain containing a betaherpesvirus-conserved FNFY motif, while not required for interaction with gL, was required for the fusogenic conformation in the mature form. This was based on results using an FNFY deletion mutant gH, which could only react with fusion conformation recognising antibodies while in immature glycoforms (Anderson and Gompels,

1999). This shows that complex interactions between N- and C-terminal domains are influenced by gL binding to gH and can also affect folding and presentation of the fusion-associated conformation on gH during maturation and presentation on the cell surface and virion.

Although the fusion assays and antibody results show a role for the gH/gL complex in cell fusion, gH/gL RAds do not mediate fusion when expressed on their own. They only enhance fusion in the presence of HCMV virions in the FFWO assay (Milne *et al.,* 1998). This implies that the gH/gL complex expressed from the RAds is interacting with other HCMV membrane components. A candidate for this component has recently been identified and is characterised further here. This is a third member of the HCMV gCIII complex (Huber and Compton, 1997; Li *et al.,* 1997). It is encoded by UL74 and has been subsequently designated gO (Huber and Compton, 1998). This glycoprotein therefore has the potential to affect gCIII complex assembly, maturation, and function. Sequence analyses have also shown a positional homolog of gO in other betaherpesviruses such as HHV-6. These analyses show a betaherpesvirus-specific glycoprotein with a high level of oligosaccharide modifications at both N- and O-linked sites (Gompels *et al.,* 1992; Huber and Compton, 1998). The gO genes encode a glycoprotein with an N-terminal signal sequence but no further transmembrane anchor, suggesting either a secreted glycoprotein or anchorage via a noncleavable signal sequence. Using HCMV gO-specific sera, studies of the expression of the HCMV gO revealed that it was initially processed to a 100-kDa form prior to final modification to its 125-kDa form after 3 to 6 h (Huber and Compton, 1999). The use of endoglycosidase H in HCMV-infected cells gave a pre-gO of approximately 54 kDa, correlating with the theoretical molecular weight of the polypeptide backbone of the UL74-encoded product. Heavy glycosylation of gO gives rise to a 125-kDa form (Huber and Compton, 1999).

Glycoprotein gO was identified by direct protein purification of the gCIII complex from virus (Huber and Compton, 1998). It is possible, therefore, that additional products are required to mediate formation of this gH/gL/gO complex, but no studies have yet been performed to show reconstitution of the trimolecular complex in the absence of virus. Furthermore, little is known about the function of gO. A role for gO in the fusion and entry processes has been suggested on the basis of properties of a gO deletion mutant virus (Hobom *et al.,* 2000). While the mutant is viable showing that gO is not essential for infection *in vitro*, the virus was severely impaired in growth kinetics as well as the process of cell-to-cell spread. This indicated a role in cellular spread, but the exact function of gO in the infection process has not been defined.

In this study, we use RAds and the FFWO assay to examine the role of gO during infection. We also dem-
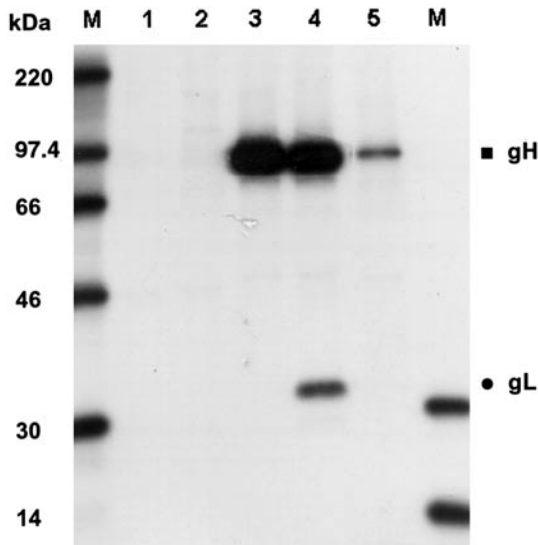
FIG. 1. Immunoprecipitation of the HCMV gH/gL complex expressed using replication-deficient recombinant adenoviruses. U373MG cells were infected with HCMV AD169, RAd gH, RAds gH and gL, and control RAd35, at 50 TCID$_{50}$ of each RAd per cell, and immunoprecipitation analysis performed. Three days postinfection, ^{35}S-labeled lysates were prepared and immunoprecipitated with the gH-specific MAb HCMV16. Lane 1, mock infection; lane 2, RAd 35; lane 3 RAd gH; lane 4, RAd gH and RAd gL. Lane 5 shows HCMV gH. The marks indicate gH and gL. In lane M are the molecular weight markers. UL115 (gL) specific expression in the infected cell preparations was confirmed by RT-PCR.

onstrate using RAds that the gH/gL/gO complex can be reconstituted without any other HCMV products. Finally, we examine properties of a clinical isolate, a natural gH MAb escape mutant, and show that while it has a conserved gH/gL, it has high variation in gO which may affect gCIII conformation and function. Further variation is shown between laboratory strains AD169 and Towne, thus defining a new hypervariable locus in HCMV which can serve as a new strain marker, influence infection, and affect vaccine formulation or efficacy.

RESULTS

Construction and characterisation of recombinant adenovirus expressing HCMV AD169 gO

Recombinant adenovirus expressing gH and gL were used to enhance fusion of HCMV in a FFWO assay (Milne *et al.,* 1998). Here we show that this RAd-expressed gH/gL interacts to form a complex (Fig. 1) similar to that shown previously using vaccinia recombinants (Kaye *et al.,* 1992). A RAd-encoding gO was constructed to test interactions with the RAd-expressed gH/gL complex and to directly show that gH, gL, and gO are necessary and sufficient to form the gCIII complex. A RAd comprising the HCMV AD169 UL74 gene was constructed and characterised. Two cloned RAds were produced, one in the coding orientation (RAd gO) and for a negative control, the noncoding inverse orientation, "inv"

(RAd gOinv). These were used to infect MRC-5 cells and gO expression examined by radioimmunoprecipitation analyses.

As gO is highly modified with multiple sites for both N-linked and O-linked glycosylation, a time-course experiment was first performed to establish optimum conditions for gO expression and maturation (Fig. 2). For this, cells were infected at different multiplicity of infection (m.o.i.'s) and radiolabelled at either 1, 2, 3, or 4 days postinfection. Cell lysates were then immunoprecipitated with the anti-gO antibody BI5, which has been previously described (Huber and Compton, 1998, 1999). Huber and Compton identified several species of gO using the BI5 antibody in pulse-chase experiments of HCMV-infected cells. These forms included the 125-kDa mature form of gO, which is present in the mature 240-kDa gCIII complex; the 100-kDa immature form of gO (gO-100), which complexes with gH-gL to form a 220-kDa precursor complex (pgCIII); and the 54-kDa nonglycosylated immature form of gO. We also detected these forms of gO in our RAd expression system. The nonglycosylated 54-kDa precursor form, pgO-54, was evident throughout the time-course assay, while the levels of both the 100-kDa and the mature 125-kDa form (gO-125) gradually increased over a 3-day period (lanes 2, 3, and 4). Maximum expression of gO was observed when cells were infected at an m.o.i. of 200 and labeled 2 days postinfection (p.i.) (lane 5). For all three m.o.i.'s tested, gO expression levels declined 4 days p.i. (data not shown). One possible explanation for this is that the high levels of gO expressed may have been toxic to the cells, resulting in cell death, leading to an overall reduction in protein expression. In addition to the gO species previously reported by Huber and Compton (1998, 1999), we also
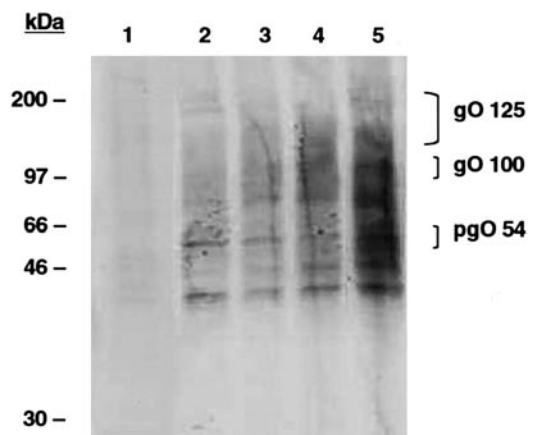
FIG. 2. Time course of RAd-expressed HCMV gO. MRC-5 cells were infected with RAd-gO at varying m.o.i.'s and radiolabelled at different times p.i. Cell lysates were immunoprecipitated with the anti-gO antibody and subjected to reducing SDS–PAGE. Lane 1, RAd-35 infected cell lysate control; lanes 2, 3, and 4, RAd-gO-infected lysates of cells infected at an m.o.i. of 100 and labeled 1, 2, and 3 days p.i., respectively; lane 5, lysate of cells infected at an m.o.i. of 200 and labeled 2 days p.i.

FIG. 3. Reconstitution of the gCIII complex. Cells were coinfected with RAd-gH, RAd-gL, RAd-gO, and RAd-gOinv at an m.o.i. of 100, radiolabelled 2 days p.i., and subjected to immunoprecipitation using either the anti-gO or anti-gH antibodies. (A) Immunoprecipitation using the anti-gH antibody and reducing SDS–PAGE. Lane 1, RAd-35-infected negative control; lane 2, RAd-gH and RAd-gL coinfected lysates; lane 3, RAd-gH, RAd-gL, and RAd-gO coinfected lysates; and lane 4, RAd-gH, RAd-gL, and RAd-gOinv coinfected cells lysates. (B) Immunoprecipitation using the anti-gO antibody and reducing SDS–PAGE. Lane 5, RAd-35-infected negative control; lane 6, RAd-gO-infected lysates; lane 7, RAd-gH, RAd-gL, and RAd-gO coinfected cell lysates; and lane 8, RAd-gH, RAd-gL, and RAd-gOinv coi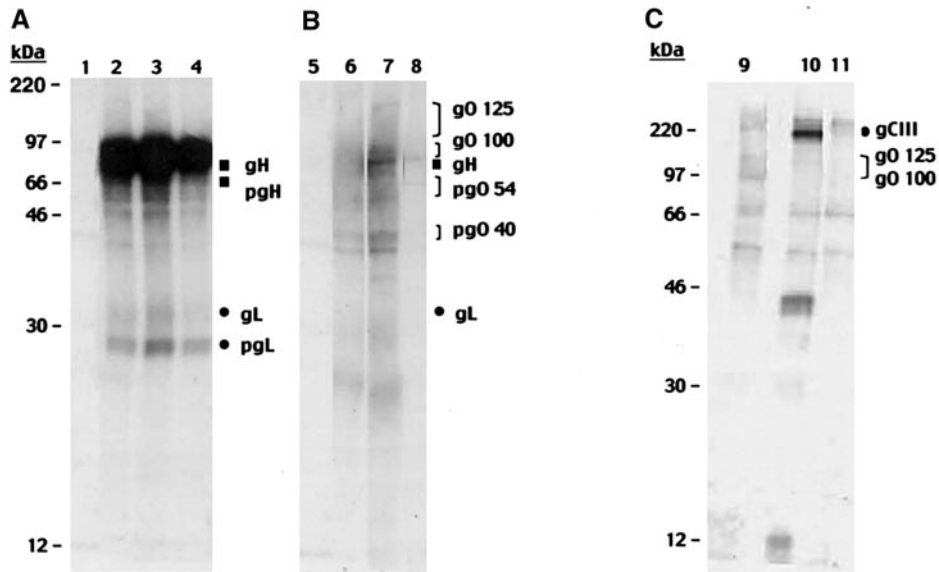nfected lysates. (C) Immunoprecipitation using the anti-gO antibody and nonreducing SDS–PAGE. Lane 9, RAd-gO-infected cell lysates; lane 10, RAd-gO, RAd-gH, and RAd-gL coinfected cell lysates; and lane 11, Rad-gOinv, RAd-gH, and RAd-gL coinfected cell lysates.

observed several other species of gO (i.e., bands between 54- and 100-kDa forms in addition to multiple forms surrounding gO-100 and gO-125). Glycoprotein O is a heavily glycosylated protein with up to 23 potential N-linked glycosylation sites and 18 potential O-glycosylation sites (Gompels et al., 1992; Huber and Compton, 1998). Thus, the additional forms that we observe may represent differentially glycosylated species of gO produced during maturation. Some bands around 40 kDa are also observed which may represent breakdown products. Overall the results show that gO can be produced in this system and receives extensive posttranslational modifications similar to those observed in the infected cell.

Reconstitution of the gCIII complex

To test whether the RAd-expressed gO formed a complex with gH and gL, MRC-5 cells were coinfected with RAds gH, gL, and gO and cell lysates were immunoprecipitated using either the anti-gH (MAb HCMV16) or anti-gO (BI5) antibody (Fig. 3). As shown in Fig. 3A (lanes 2, 3, and 4), the anti-gH antibodies coprecipitated gH (90 kDa), pgH (84 kDa), gL (34 kDa), and pgL from lysates coinfected with RAd-gH, RAd-gL, and RAd-gO (coding orientation) or RAd-gOinv (noncoding orientation). In lane 3, in which cells were infected with RAd-gH, RAd-gL, and RAd-gO, the darker bands underneath gH may represent a species of gO. As shown in Fig. 3B, the anti-gO antibody precipitated multiple forms of gO: gO-125, gO-100,

pgO-54, and pgO-40 from cells infected with gO alone (lane 6) or in combination with RAd-gH and RAd-gL (lane 7). The predicted polypeptide backbone mass of gO is 54 kDa (Gompels et al., 1992; Huber and Compton, 1998), consistent with the pgO-54 observed while pgO-40 may represent a breakdown product. The anti-gO antibody also coprecipitated gH and a very faint band representing gL from cells coinfected with RAd-gH, -gL, and -gO (Fig. 3B, lane 7). By contrast, bands representing gL and any of the gO species were absent when cells were coinfected with RAd-gOinv, -gH, and -gL (lane 8), although a faint band corresponding to gH was observed. This band appears to be background in this system, since gH levels are substantially greater when RAd-gO is used in the coinfection and gO is present (Lane 7). Thus, the results of studies performed with reducing conditions indicate the formation of a trimolecular complex similar to results observed in virus infected cells (Huber and Compton, 1998, 1999).

To determine directly if the gCIII complex could be reconstituted in our assay, anti-gO immunoprecipitated lysates were also subjected to SDS–PAGE under nonreducing conditions (Fig. 3C). The mature 240-kDa gCIII complex was observed when cells were coinfected with RAd-gO, RAd-gH, and RAd-gL (lane 10) but not when RAd-gOinv was used in the coinfection instead (lane 11). Significantly, when RAd-gO-infected lysates were immunoprecipitated with the BI5 antibody and subjected to nonreducing SDS–PAGE (lane 9), we observed the 100-

to 125-kDa gO mature forms but no other higher molecular weight forms indicative of gO multimers. The monomeric form of gO was only removed when expressed with gH/gL, as shown in lane 10, indicating disulfide bonds in the heterooligomer, but little evidence for gO homodimer. Background bands were indicated by lane 11 control of gO in the wrong coding orientation. Thus, the 240-kDa species under nonreducing conditions is consistent with intermolecular disulfide bonds mediating a covalent interaction of gH, gL, and gO to form the gCIII complex. Using a vaccinia expression system the HCMV gH/gL complex has been previously shown to form a heterodimer stabilised by disulfide bonds, although gH on its own does not form a homodimer (Kaye *et al.,* 1992) and similar results were found using the RAd system (data not shown). The lack of a prominent homodimer for either HCMV gO or gH contrasts with HHV-6 gH which clearly forms a homodimer under similar conditions (Anderson *et al.,* 1996).Taken together, these results indicate that whereas gO, similar to HCMV gH, does not form disulfide bonds with itself, it does form disulfide bonds with gH/gL to form a trimolecular gCIII complex. Formation of the mature gCIII HCMV gH/gL/gO complex formation has been demonstrated previously in HCMV-infected cells (Huber and Compton, 1998, 1999). However, by using a recombinant adenovirus expression system, we demonstrate for the first time gCIII reconstitution in the absence of the HCMV virion particle. This suggests that additional HCMV virion products are not required for these interactions. Thus, HCMV gH/gL/gO are necessary and sufficient to form this tripartite gCIII complex.

Inhibition of fusion by anti-gO antibodies

As gH, gL, and gO were shown competent at complex formation using the RAd system, the FFWO assay was used to analyse a role for gO in fusion. Anti-gO antibodies (Huber and Compton, 1998, 1999) were incorporated into the fusion assay to examine possible roles as previous results using specific antisera had shown roles for gH as well as gB (Milne *et al.,* 1998). Specific gO rabbit serum was added at dilutions of 1:30, 1:100, and 1:300 to fusogenic AD169 (titers of between 10^5 and 10^6 TCID$_{50}$/ml). The positive control was HCMV16 (HCMV gH-specific), which has been shown previously to inhibit fusion of both the virus and the FFWO assay (Cranage *et al.,* 1988; Milne *et al.,* 1998). The negative controls included normal rabbit sera and C13 (HCMV pp65-specific), which is nonneutralising and does not inhibit fusion of the virus or the FFWO assay (Cranage *et al.,* 1988; Milne *et al.,* 1998). Using methods shown previously in the FFWO assay, fusogenic AD169 virus (prepared on HEL cells) was used to superinfect U373MG cells previously infected with various combinations of gH, gL, or control RAds. These were then treated with the antibod-

ies in the overlaying media together with actinomycin D to prevent gene expression. After 16 h incubation, the plates were fixed and the cells were examined. The results show specific inhibition of fusion of the U373MG cells by the anti-gO sera (Fig. 4). Quantification of the results showed specific fusion with an endpoint beyond a 1:100 dilution (Fig. 5). The fusion inhibition achieved using the polyclonal anti-gO antibodies at a 1:30 dilution was similar to that observed with the gH-specific HCMV16 antibodies as ascites fluid diluted 1:100. The result was specific as no inhibition was observed with nonimmune sera or with nonneutralising HCMV pp65 antibodies. This demonstrates that gO has a role in cell fusion as assayed by FFWO.

A clinical isolate resistant to plaque inhibition by gH antibody and a divergent gO

In vivo evidence for a function for gO in cellular spread was explored. A clinical HCMV isolate, strain Pt, was further investigated which had unusual properties in cell spread and reactions with neutralising gH-specific antibodies. Strain Pt had identical single-cycle growth kinetics to laboratory strain AD169, but over multiple cycles (i.e., when the m.o.i. was very low, < 0.02/cell) was impaired, and grew to titers 100-fold less than AD169. This indicated an alteration in the cell-to-cell spread pathway, but not the virus entry pathway. Consistent with this result, this clinical isolate was resistant to gH-specific antibody, MAb C2 (Baboonian *et al.,* 1989), in plaque inhibition assays designed to measure cell-to-cell spread (Fig. 6A). Similar results were shown for gH MAb 14.4b. The resistance to antibody-mediated plaque inhibition was specific for these gH MAbs, while neutralising human immune sera was still active against strain Pt in the plaque inhibition assays.

However, infection with cell-free virus, as opposed to infected cell-to-cell spread, was neutralised efficiently by MAb C2 (Fig. 6B) with similar results using other gH neutralising MAbs 14.4b, 1G6 and HCMV16. Moreover the virus encoded a gH with a mature amino acid sequence identical to AD169 (Accession No. Q68571). There were no detectable differences in surface or intracellular immunoprecipitated gH and kinetics of entry were indistinguishable between Pt and AD169 (not shown). Thus, this clinical isolate may have an alternative, gH-independent mechanism for cell-to-cell spread in the presence of MAb C2. Alternatively, since this is a conformational sensitive MAb, changes in the other components of the gCIII complex, such as gL or gO, may affect conformation and function in cell spread by fusion and may indicate some selection with antibodies to gCIII. Analyses of the Pt gL sequence only showed limited differences. These included six conservative amino acid substitutions (Accession No. Q68666). Moreover there were no significant differences between gL of AD169 or
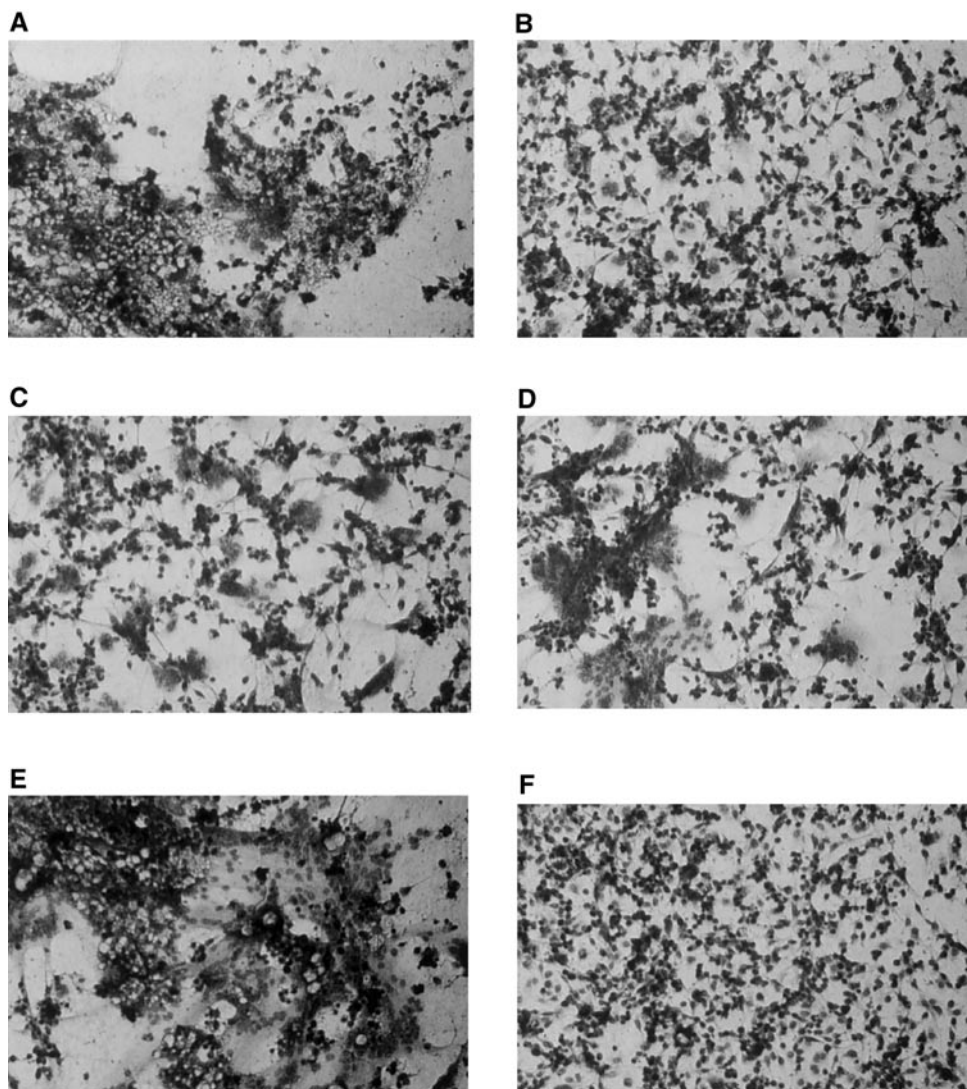
FIG. 4. Enhanced HCMV induced fusion of U373MG cells using RAds gH/gL and fusion inhibition with gO antisera. Anti-gO antibodies (Huber and Compton, 1998) were incorporated into the FFWO assay to examine any inhibitory property they may have. U373MG were infected with RAds gH and gL then superinfected with fusogenic AD169 as described under Materials and Methods. Rabbit serum specific for gO was added at dilutions of 1:30, 1:100, and 1:300. Controls included HCMV16 ascites (gH-specific; 1:100), which were shown previously to inhibit fusion, and negative control C13 (pp65-specific), which is nonneutralising and does not inhibit fusion, and nonimmune rabbit sera were employed as controls. Examples of fusion observed are shown as follows: (A) no antibody; (B) gH neutralising antibody, (C) gO antibody 1:100, (D) gO antibody 1:300, (E) nonimmune rabbit sera, (F) gO antibody 1:30. Complete inhibition of fusion are shown with gH 1:100 and gO 1:30 antibodies (B, F).

Pt in the fusion assay. When Pt or AD169 gL were used to achieve surface expression of gH, the extents of enhanced fusion in the FFWO assay performed previously (Milne *et al.,* 1998) were similar with AD169 gL = 714 (SD = 131) compared here to Pt gL = 808 (SD = 98); $P = 0.0656$. Furthermore, there were no differences in mediating cell surface expression of gH as assayed by immunofluorescence. The phenotypic changes may be due to differences in the third gCIII component, gO.

The sequence of the UL74, gO, gene from strain Pt was determined and compared to that of laboratory strain AD169. The results showed a remarkably high degree of variability (20%). This was pronounced at the N-terminal end, while the C-terminal region showed higher conser-

vation (40% N-, 6% C-terminal divergence) (Fig. 7). There were 1–3 nucleotide deletions and insertions together with mismatches in the N-terminal region, and while the C-terminal region was conserved there was a prominent 9 nucleotide deletion in strain Pt compared to AD169, resulting in loss of an encoded N-linked glycosylation site. The overall divergence observed supported the interpretation that the gO product was most likely to account for differences in the phenotype of the gCIII complex in reaction to the C2 antibody. This was further investigated by analyses of amino acid sequences and gO of another reference laboratory strain.

Previous studies of gH variation in HCMV had shown 5% divergence with AD169-like viruses most distinct from
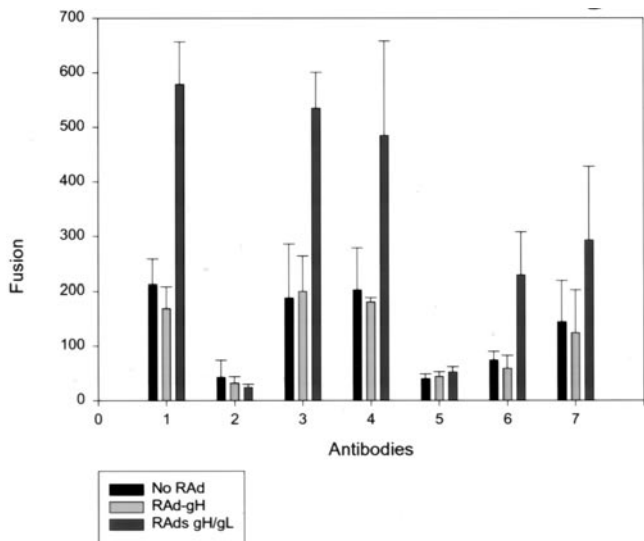
FIG. 5. Quantification of fusion enhancement with RAds gH/gL and inhibition by gO antibodies. Extent of fusion was measured as described under Materials and Methods and results are presented from triplicate experiments. The fusion axis shows the number of nuclei within syncytia per well as measured from four random views (two fields each from two duplicate wells) of 50× magnification, photographed, screen projected, and counted blind from coded images. The fusion results are shown for each antibody condition indicated in sets of no RAd (black shading), RAd-gH (no shading), or RAds gH/gL (gray shading). Set 1 has no antibody treatment; set 2, gH neutralising antibody; set 3, pp65 antibody; set 4, nonimmune rabbit sera 1:30; set 5, gO antibody 1:30; set 6, gO antibody 1:100; and set 7, gO antibody 1:300.

Towne-like viruses (Chou, 1992). As the gH and gL genes from strain Pt were both closely related to strain AD169, it was possible that the divergence in gO represented a recombinant with a Towne-like strain. This was examined by deriving the nucleotide sequence of gO from laboratory strain Towne. Surprisingly, the results showed

that the strain Pt gO remained most closely related to laboratory strain AD169, while strain Towne gO was even further divergent. Towne gO showed only 66% amino acid sequence identity in multiple alignment and dendrogram analyses and included greater nonsynonymous changes which are indicative of selection (Fig. 8). Thus, the sequence comparisons demonstrate that gO is a new hypervariable site in HCMV and indicate that changes in this gene may affect infectious spread.

DISCUSSION

We have investigated properties of HCMV gO and show directly using RAds that coexpression of gH, gL, and gO is necessary and sufficient to form a trimolecular complex consistent with the gCIII complex produced by the virus. Therefore, complex formation can occur in the absence of any other HCMV products. We also show that gO has a role in virus-mediated cell fusion as shown by fusion inhibition using gO-specific antibodies in the FFWO assay.

A role for gO in cell fusion is supported by the behavior of the clinical isolate, strain Pt, which is a naturally occurring antibody-escape mutant, resistant to inhibition of cell-to-cell spread by gH-specific neutralising MAbs. Sequence analyses of the gCIII genes suggests this phenotype is most likely due to a highly divergent gO (20% coding changes overall and 40% divergence in the N-terminal region), as Pt gH is identical to that of AD169. Although Pt gL had minor conservative substitutions, it remained functionally equivalent in gH complex formation, gH cell surface chaperone capacity, and the gH/gL fusion enhancement in the FFWO assay. Other major changes in the Pt gO sequence include a 9-nucleotide deletion in the C-terminal conserved domain which results in the lack of an encoded site for N-linked glyco-
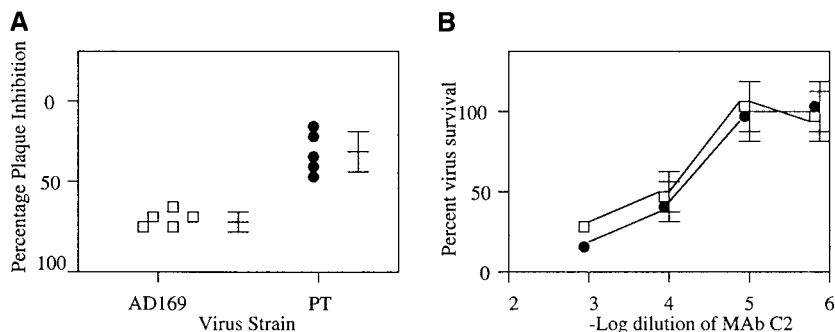
FIG. 6. HCMV clinical isolate, strain Pt, is resistant to antifusion gH antibodies but remains sensitive to gH cell-free virus neutralising antibodies. (A) Plaque inhibition assay. This assay measures inhibition of cell-to-cell spread (plaque inhibition) by gH antibody in the overlaying media; the results are from 2 weeks postinfection. Mean plaque size in the presence of gH MAb C2 was expressed as a percentage of mean plaque inhibition calculated by comparing plaque size in the presence of nonneutralising structural pp65 MAb C13. Each point on the graph represents a single experiment, and is shown in quintuplicate. The mean percentage value for each strain is shown with an error bar indicating ± 1 SD. The two means were significantly different by the Student's t test ($P = <0.0001$). (B) Cell-free virus neutralisation assay. This assay measures inhibition of virus survival after cell-free virus is incubated with gH antibody and then titrated in the absence of antibody. The mean percentage of survival for each strain of virus at each dilution of gH MAb C2 is shown ($n = 4$). Error bars represent ± SE.

```
                                                                                                                                                        120
AD169-UL74  ATGGGGAGAAAAGAGATGATGGTGAGAGACGTCCC--TAAGATGGTGTTT-CTAATATCTATATCTTTCTTGCTTGTTTCTTTCATAAACTGTAAAGTTATGTCAAAAGCGCTTTATAAT
PT-UL74     ATGGGGAGAAAAGGAGAGATG---AGAG--GTGTTTTTAATTTA-TTTTTCCTTATGACGCTAACTTTCCTGCTATTCTCTTTCATAAACTGTAGGGCTGCGGTAAGATTATCCGTAGGA
            ****************** ****  ****  **  **   * **  **  ** * ** ******** **  * ****************** *  *  ***  *

                                                                                                                                                        240
AD169-UL74  CGTCCTTGGAGGGGCTTGGTACTGTCTAAGATAGGCAAATATAAATTAGATCAGCTTAAGTTAGAAATTTTGAGACAACTAGAAACGACTATTTCTACAAAATAC---AATGTAAGTAAA
PT-UL74     CGTTACTGGTCAGGTAAAGTACTGTCTACGATAGGCAAGCAAAGACTAGACAAGTTCAAATTAGAGATTTTGAAACAACTAGAAAAGGATATCTATACGAAATACTTTAATATGACCAGA
            *** ***  **   ********* ******   * * ****  *  *   **** ** **  ****** ****** *********** *  *** *** ****** *** *  *

                                                                                                                                                        360
AD169-UL74  CAACCGGTTAAAAATCTCACTATGAACATGACAGAGTTTCCACAATACTACATTTTAGCGGGCCCCATTCAGAATTATAGTATAACCTATCTGTGGTTTGATTTTTATAGTACCCAGCTT
PT-UL74     CAACACATTAAAAATCTGACTATGAACATGACCGAGTTTCCACGGTATTACATTTTAGCGGGACCCATTCAAAATAACAGTGGTTACCTATCTTTGGTTCGATTTTTATAGCACTCAGCTT
            ****  ********** ***************** ********  * * ************* ***** ***  ** *** *** **** ****** ***** ********* ** *****

                                                                                                                                                        480
AD169-UL74  AGAAAACCCGCAAAATACGTTTACTCACAGTACAATCATACGGCTAAAACGATAACATTCAGACCCCCACCTTGTGGTACTGTGCCTTCCATGACTTGTCTTTCCGAAATGCTAAACGTT
PT-UL74     CGAAAACCGGCTAAGTACGTTTCTCAGAATACAATCATACGGCTAAAACAATAACATTCAGACCCCCATCTTGCGGTACGGTGCCTTCCATGACTTGTCTTTCCGAAATGCTAAACGTT
             ******* **  ** ****** *  **** ****************** ****** *************** **  *** ** ******************** ********* **

                                                                                                                                                        600
AD169-UL74  TCCAAACGTAATGATACTGGCGAACAAGGTTGCGGTAATTTCACCACGTTCAACCCCATGTTTTTCAATGTACCGCGTTGGAACACCAAATTGTACGTGGGTCCGACTAAGGTTAACGTA
PT-UL74     TCCAAACGTAATGATACTGGCGAACAAGGCTGCGGCAATTTCACCACGTTCAACCCCATGTTTTTCAATGTGCCACGTTGGAACACGAAGTTATATGTAGGTTCCAAGAAAGTAAACGTG
            ***************************** ***** ************************* ** ** ******* ** **   * ** ** *** ** **  **   ** ** *****

                                                                                                                                                        720
AD169-UL74  GATAGTCAAACGATTTATTTTCTAGGTTTAACCGCCCTGCTTTTACGTTACGCACAACGCAACTGTACACACAGTTTCTACCTGGTTAACGCCATGAGCCGGAATCTATTTCGCGTCCCC
PT-UL74     GATAGTCAAACGATCTCACTTTTTAGGCCTAACCGCCTTGCTTCTACGTTACGCGCAACGCAACTGTACGCACAGTTTCTACCTGGTTAACGCCATGAGCCGAAATTTATTTCGCGTCCCT
            ************** *  ***** ****  ******* ***** ********** ************** ************************************  *** **********

                                                                                                                                                        840
AD169-UL74  AAGTATATTAACGGCACCAAGTTAAAAAAACACTATGCGAAAACTAAAACGTAAACAAGCGCCCGTTAAGGAACAATTCGAAAAAAAAGCTAAGAAAACTCAGAGTACTACTACGCCATAC
PT-UL74     AAGTATATTAACGGTACTAAGTTGAAAAACACTATGCGAAAACTCAAGCGTAAACAAGCTCCAGTCAAAGAACAATCAGAAAAAAAGAGTAAGAAATCTCAAAGTACTACTACGCCATAT
            ************** ** ***** ****  ************** **  *********** ** ** ** ****** ** ****** **  ******* **** **** ***********

                                                                                                                                                        960
AD169-UL74  TTTTCCTATACAACGTCTGCCGCTCTCAACGTCACTACTAACGTGACTTATAGTATTACTACCGCCGCAAGGCGGGTTTCCACGTCTACAATTGCTTATCGTCCTGATAGCAGCTTTATG
PT-UL74     TCTCCCTATACAACGTCAACCGCTCTGAACGTCACTACTAACGCGACTTATAGTGTTACTACCACCGCAAGGCGGGTTTCCACGTCTACAATTGCTTATCGTCCTGATAGTAGCTTTATG
            * *  ********** *  ******* ************ ************* ******* *********************************************** ********

                                                                                                                                                        1080
AD169-UL74  AAGTCCATTATGGCCACACAGTTAAGGGACCTAGCAACGTGGGTGTATACCACTCTACGTTACCGGCAAAATCCTTTTTGTGAACCAAGCCGCAACCGAACCGCCGTGTCAGAATTTATG
PT-UL74     AAGTCCATTATGACCACGCAATTAAGGGACCTAGCGACGTGGGTGTATACCACTCTACGTTACCGGCAAAACCCTTTTTGTGAATCAAGCCGCAACCGAACCGCCGTATCAGAATTTATG
            ************ **** ** *************** ************************************  ********** *************************** ********

                                                                                                                                                        1200
AD169-UL74  AAAAACACGCACGTACTAATCCGTAACGAAACGCCGTACACTATTTACGGTACTCTCGACATGAGCTCCTTATATTACAACGAAACCATGTTCGTGGAAAACAAAACAGCTTCCGATAGT
PT-UL74     AAAAACACGCACGTACTAATCCGTAATGAAACACCGTACACTATTTACGGTACTCTTGATATGAGCTCCTTATATTACAACGAAACCATGTTCGTGGAAAACAAAACGGCTTCCGA----
            ************************* ***** ********************** ** ** *********************************************** ****** *

                                                                                                                                                        1320
AD169-UL74  AACAAAACTACACCTACGTCACCATCAATGGGGTTTCAGAGAACATTTATAGATCCCCTGTGGGACTATCTAGACTCGCTGCTGTTTCTAGATGAGATTCGTAACTTTAGCCTCCGGTCA
PT-UL74     -----AACTACACCTACGTCTCCATCAACGGGATTTCAGAGAACGTTTATAGATCCCCTGTGGGACTATCTAGACTCGCTGCTATTTCTAGATGAGATCCGTAACTTTAGTCTCCAGTCA
                 *************** ******* *** ***********  ******************************** ********** *********** *********** **** ****

                                                                                                                                                        1407
AD169-UL74  CCCACGTATGTAAACCTTACCCCGCCGGAACACCGCCGGGCTGTAAATCTGTCCACCCTCAATAGCCTTTGGTGGTGGTTGCAGTAA
PT-UL74     CCCACTTATGGAAACCTTACCCCGCCGGAACACCGTCGAGCTGTAAATCTGTCCACCCTTAATAGCCTTTGGTGGTGGTTGCAGTAA
            ***** **** ************************ ** ************************ ****************************
```

FIG. 7. Strain Pt has a divergent gO. Nucleotide sequence of strain Pt gO compared to that of laboratory strain AD169. The vertical lines indicate two main regions of divergence, the N-terminal region which has 40% differences containing small deletions and insertions, and a larger 9 nucleotide deletion within the conserved C-terminal domain.

sylation (at positions 400–403 of AD169), which could also affect conformation or function.

Interestingly, strain Pt also had a defect in cell-to-cell spread, giving rise to a small plaque phenotype. Although there may be other factors than the Pt gO allele, these results are consistent with properties of a recently described HCMV gO deletion mutant. These viruses have reduced plaque size and poor growth kinetics, yielding titers reduced by about 1000-fold (Hobom *et al.,* 2000). Both this result, and the fact that anti-gO antibodies inhibit HCMV-induced cell fusion as shown here, indicate that gO plays a role in cellular spread mediated by membrane fusion.

Hobom *et al.* also showed that gH was essential for infection (Hobom *et al.,* 2000), consistent with the results of a number of previous studies using gH-specific neutralising antibodies (Rasmussen *et al.,* 1984, 1988; Cranage *et al.,* 1988; Keay and Baldwin, 1991; Simpson *et al.,* 1993) and by analogy to other herpesviruses (Gompels and Minson, 1986; Gompels *et al.,* 1991; Forrester *et al.,* 1992; Roop *et al.,* 1993). It is likely, therefore, that gH is the primary functional component of gCIII in terms of modulation of fusion as also demonstrated by the fusion enhancement with gH/gL in the FFWO assay (Milne *et al.,* 1998). The results shown here together with those of Hobom *et al.* indicate that gO plays an important synergistic role in cell-to-cell spread or can direct an alternative pathway. Preliminary results on using RAds to express the gO/gH/gL and probing further the relationship to fusion suggest other membrane components are required for complete fusion, possibly a binding role such as from gB as described for homologues in the HSV fusion assay (Turner *et al.,* 1998). As noted previously, HCMV gB sera does inhibit the FFWO fusion assay (Milne *et al.,* 1998).

HCMV may use gO for infection of only certain cell types. Different gH/gL complexes mediate infection of different cell types in Epstein–Barr virus (EBV), the only other herpesvirus that has been shown to include a third component in its gH/gL complex. EBV uses the BZLF2 gene product (unrelated to HCMV UL74 gO) as the third component of the EBV gH/gL complex. Recent evidence

A

```
                    10        20        30 ^      40        50
PT gO        1 MGRKGEM-RG VFNLFFLMTL TFLLFSFINC RAAVRLSVGR YWSGKVLSTI
AD169 gO     1 MGRKEMMVRD VPKMVFLISI SFLLVSFINC KVMSKALYNR PWRGLVLSKI
Towne gO     1 MGRKGEM-RG VFNLFFLMSL TFLLFSFINC KIAV----AR FR---VKSQK
CONSENSUS      MGRK..M.R. V....FL... .FLL.SFINC .........R .....V.S..

                    60        70       *80        90       100
PT gO       51 GKQRLDKFKL EILKQLEKDI --YTKYFNMT RQH-IKNLTM NMTEFPRYYI
AD169 gO    51 GKYKLDQLKL EILRQLETTI --STKY-NVS KQP-VKNLTM NMTEFPQYYI
Towne gO    51 AKEEERQLKL RILQELASKT GDYYKHTFTP SQQKLYNITV EMKQFPPNSI
CONSENSUS      .K......KL .IL..L.... ...K...... ......N.T. .....P....

                   110       120       130       140       150
PT gO      101 LAGPIQNNSV TYLWFDFYST QLRKPAKYVF SEYNHTAKTI TFRPPSCGTV
AD169 gO   101 LAGPIQNYSI TYLWFDFYST QLRKPAKYVV SQYNHTAKTI TFRPPPCGTV
Towne gO   101 LAGPIRNHSI THLWFDFHTT QLRKPAKYVV SEYNHTGQKI TFRPPSCGTI
CONSENSUS      LAGPI.N.S. T.LWFDF..T QLRKPAKYV. S.YNHT...I TFRPP.CGT.

                   160       170       180       190       200
PT gO      151 PSMTCLSEML NVSKRNDTGC QGCGNFTTFN PMFFNVPRWN TKLYVGSKKV
AD169 gO   151 PSMTCLSEML NVSKRNDTGE QGCGNFTTFN PMFFNVPRWN TKLYVGPTKV
Towne gO   151 PSMTCLSEML NVSRRNNTGE ENCGNFTTFN PMFFNVPRWN TKLYVGPSKV
CONSENSUS      PSMTCLSEML NVS.RN.TGE ..CGNFTTFN PMFFNVPRWN TKLYVG..KV

                   210       220       230       240       250
PT gO      201 NVDSQTIYFL GLTALLLRYA QRNCTHSFYL VNAMSRNLFR VPKYINGTKL
AD169 gO   201 NVDSQTIYFL GLTALLLRYA QRNCTHSFYL VNAMSRNLFR VPKYINGTKL
Towne gO   201 NVDSQTIYFL GLAALLLRYA QRNCTRSFYL VNAMSRNIFR VPKYINSTKL
CONSENSUS      NVDSQTIYFL GL..LLLRYA QRNCT..FYL VNAMSRNIFR VPKYIN.TKL

                   260       270       280       290       300
PT gO      251 KNTMRKLKRK QAPVKEQSEK KSKKSQSTTT PYSPYTTSTA LNVTTNATYS
AD169 gO   251 KNTMRKLKRK QAPVKEQFEK KAKKTQSTTT PYFSYTTSAA LNVTTNVTYS
Towne gO   251 KNTMRKLKRK QAPVKS-ISK KSRV--STTT PYSSYT-STI FNVSTNVTYS
CONSENSUS      KNTMRKLKRK QAPVK....K K.....STTT PY..YT.S.. .NV.TN.TYS

                   310       320       330       340       350
PT gO      301 VTTTARRVST STIAYRPDSS FMKSIMTTQL RDLATWVYTT LRYRQNPFCE
AD169 gO   301 ITTAARRVST STIAYRPDSS FMKSIMATQL RDLATWVYTT LRYRQNPFCE
Towne gO   301 PIVPTR-IPT STIGYRPDEN FMKSILTTQL KDLATWVYTT LRYRDEPFCK
CONSENSUS      .....R...T STI.YRPD.. FMKSI..TQL .DLATWVYTT LRYRD.PFC.

                   360       370       380       390       400
PT gO      351 SSRNRTAVSE FMKNTHVLIR NETPYTIYGT LDMSSLYYNE TMFVENKTAS
AD169 gO   351 PSRNRTAVSE FMKNTHVLIR NETPYTIYGT LDMSSLYYNE TMFVENKTAS
Towne gO   351 PNRNRTAVSE FMKNTHVLIR NETPYTIYGT LDMSSLYYND TMPVENETAS
CONSENSUS      ..RNRTAVSE FMKNTHVLIR NETPYTIYGT LDMSSLYYN. TM.VEN.TAS

                    *  410       420       430       440       450
PT gO      401 E---TTPTSP STGFQRTFID PLWDYLDSLL FLDEIRNFSL QSPTYGNLTP
AD169 gO   401 DSNKTTPTSP SMGFQRTFID PLWDYLDSLL FLDEIRNFSL RSPTYVNLTP
Towne gO   401 DNNKTTPTSP STRFQRTFID PMWDYLDSLL FLSEIRNFSL QSSTYGNLTP
CONSENSUS      ....TTPTSP S..FQRTFID P.WDYLDSLL FL.EIRNFSL .S.T..NLTP

                   460       470
PT gO      451 PEHRRAVNLS TLNSLWWWLQ
AD169 gO   451 PEHRRAVNLS TLNSLWWWLQ
Towne gO   451 PEHRRAVNLS TLNSLWWWLQ
CONSENSUS      PEHRRAVNLS TLNSLWWWLQ
```

B

```
PT UL74     ───────────83.5%
AD169 UL74  ──────────         71.5%
Towne UL74  ────────────────────

PT gO       ───────────81.0%
AD169 gO    ──────────         66.1%
Towne gO    ────────────────────
```
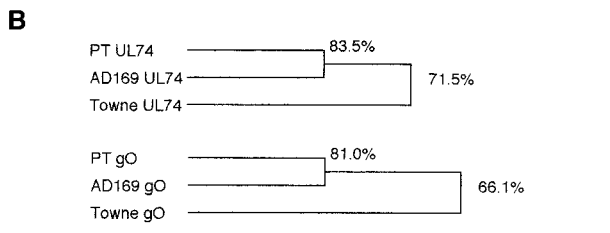
FIG. 8. Sequence of Towne gO shows a hypervariable locus. (A) Multiple sequence alignment of gO amino acid sequences from laboratory strain Towne compared to strain AD169 and clinical isolate strain Pt. Hydrophobic sequences at the N-terminal end of gO are underlined and are predicted to form part of the signal sequence/transmembrane domain. N-linked glycosylation sites are also underlined. (B) Dendrograms showing relationship between nucleotide (UL74) and encoded amino acid sequences of gO from Towne, AD169, and Pt strains.

shows that BZLF2 mediates binding to the MHC Class II molecule, and consistent with this result, the BZLF2 containing gH/gL complex is essential for EBV infection of B lymphocytes. However, BZLF2 is not required for infection of epithelial cells, where the bimolecular gH/gL complex is sufficient for virus entry and may utilise a distinct cellular receptor (Li et al., 1995; Wang et al., 1998; Molesworth et al., 2000). HCMV infects many cell types; it may therefore only require gO for cell-to-cell spread by fusion with some of these. Studies, as performed by

Hobom et al. in MRC-5 cells (Hobom et al., 2000), would be required in many different cell types to examine this further.

From the above results, it is possible that gO mediates a role in cellular spread which may be distinct from processes during initial virion infection. There is evidence that individual roles of glycoproteins in these mechanisms may differ potentially due to different presentation in the cell-free virion vs infected cell membranes. In HSV-1 for example, MAb gH escape mutants have different sensitivities to gH neutralising antibody in the presence of wild-type gH provided in the virion or the infected cells. This was done using either infections or plaque assays on wild-type gH producing cell lines (Gompels et al., 1991). Furthermore, some HSV-1 gD MAbs inhibit only cell-to-cell spread (Minson et al., 1986). Similarly, EHV-1 gp300-specific antibodies reduced plaque size and prevented fusion of infected cells but did not affect infectivity (Whittacker et al., 1992; Wellington et al., 1996). Moreover, HHV-6 gH specific MAbs show distinct properties in either neutralising infection alone or combined with inhibition of cell-to-cell spread in syncytia formation (Foa-Tomasi et al., 1991; Liu et al., 1993; Anderson and Gompels, 1999). Furthermore, Wilson et al. (1994) showed a V831-A mutation in HSV-1 gH alters only the cell surface (as opposed to virion) gH phenotype, and no longer permits syncytium formation, while still allowing virus entry. Cell fusion defects were also shown for several linker scanning mutations in the C-terminal domain of HSV-1 gH (Galdiero et al., 1997). In studies on HCMV, Li et al. characterised another natural human gH MAb escape mutant, which similar to strain Pt, also did not have changes in the coding region of the gH gene (Li et al., 1995). In this case, the level of gH expressed on the virion was reduced. In light of the results with the HCMV clinical isolate Pt, it would also be of interest to characterise the gO gene of this virus and the possible role in maturation of gH.

Since gO is either secreted or anchored by its N-terminal signal sequence, it does not have an internal cytoplasmic domain, thus differing markedly from gH. Recent data from transient expression analyses show that this signal sequence can be cleaved, and thus, similar to gL, the mature gO molecule may be secreted or membrane bound in association with gH (Theiler and Compton, 2001). The cytoplasmic domain in gB has been shown to have a nuclear localisation signal that permits nuclear envelope localisation, a site for virion envelopment (Meyer and Radsak, 2000). This mechanism for nuclear envelope localisation is not possible for gO; furthermore, the gH/gL complex has been shown to form prior to the addition of gO (Huber and Compton, 1999). Thus, presentation and function in the different membranes of the infected cell and the cell-free virus are likely to differ and can affect their roles in fusion, infection, and cellular spread. There may be many more

glycoproteins which modify the infectious process in HCMV, with over 50 encoded glycoprotein genes and additional variation observed in passages of laboratory strains, for example, the large deletions recently observed (Cha *et al.,* 1996; Dargan *et al.,* 1997). This study does highlight the importance of the gCIII complex and show that both gH and gO have a role in infection by cell fusion but exact contributions may differ, possibly in different membranes or cell types and within different strain backgrounds.

Our results also show that the gO, UL74, gene defines a new hypervariable locus in HCMV with over 34% divergence. Similarly, the related betaherpesvirus HHV-6 also encodes a gO homolog which is a site of genomic variation. In contrast the other members of the HCMV gCIII complex, gH and gL (UL75 and UL115, respectively), are relatively conserved genes, with only up to 5% divergence, with similar results also for the HHV-6 gH/gL homologues (Chou, 1992; Gompels *et al.,* 1993; Spaete *et al.,* 1993; Kasolo *et al.,* 1997; Dominguez *et al.,* 1999). The divergent gO gene is located adjacent to the conserved gH gene and is also a site of recombination and divergence between herpesvirus species (Gompels *et al.,* 1992, 1995; Huber and Compton, 1998). Similarly, the conserved gL gene is located adjacent to a divergent region encoding hypervariable chemokine genes (French *et al.,* 1999), which is also within a genomic region for recombination and divergence between herpesviruses. In the HCMV genome, this region is adjacent to a large deletion in laboratory strains, with clinical strains encoding additional potential virulence genes, including the variable UL144 open reading frame, which encodes a tumor necrosis factor receptor homolog (21% divergence between the encoded proteins of different isolates) (Lurain *et al.,* 1999). Recent results have also identified gN as another betaherpesvirus variable loci (Dal Monte *et al.,* 2001). This glycoprotein forms a complex with the conserved gM, which in other herpesviruses is a fusion modulator (Klupp *et al.,* 2000; Mach *et al.,* 2000) and is encoded by UL73 just upstream of the UL74 gO gene and at a breakpoint for rearrangement between herpesvirus species (Gompels *et al.,* 1995). Thus, these regions of divergence add accessory functions specific for individual herpesvirus species and can contribute to their virulence.

In conclusion, these studies show that gO is necessary and sufficient for the interaction with gH/gL to form the gCIII complex. It is a fusion-related gene product involved in cellular spread and is a hypervariable site which may contribute to virus virulence. It will be of interest in future work to further determine contact sites between the gH/gL/gO gCIII complex and their relationship to cell fusion, variation, and influence on infection and vaccine strategies. Recent results on intrauterine transmission show that variation in gH correlates with infection with additional HCMV strains in mothers with prior immunity (Boppana *et al.,* 2001). Our results suggest that gO warrants investigation in these or related studies and may be a potent determinant of transmission given its interaction with gH/gL, high variation, and role in cell fusion.

In previous work, we have shown heterologous gH/gL complex formation between the betaherpesviruses HCMV and HHV-6 (Anderson *et al.,* 1996). Preliminary data also suggest this complex is functionally competent in an HHV-6-based FFWO assay using HCMV gH/gL RAds, implicating heterologous interactions with HHV-6 gO. Thus, the relationship between complex formation, cell fusion, and infection can now be further investigated. This has biological implications for each betaherpesvirus, as well as in cases where these viruses frequently co-reactivate, and can co-react, such as in immunosuppressed transplant and HIV/AIDS patients (Fillet *et al.,* 1996; Ratnamohan *et al.,* 1998; Emery *et al.,* 1999; Humar *et al.,* 2000; Takemoto *et al.,* 2000; Mendez *et al.,* 2001).

MATERIALS AND METHODS

Cells, viruses, and antibodies

U373MG astrocytes, human foreskin fibroblast cells (HFFF, CB1945), human embryonic kidney 293 cells, and MRC-5 cells were obtained from the European Cell and Culture Collection (ECACC; Porton Down, Wilts, U.K.). HEL12469 fibroblasts were prepared from fetal lung tissue and shown to be free from mycoplasma infection at the Clinical Virology Laboratory, St. George's Hospital Medical School and deposited with ECACC. Cells were cultured in Eagle's minimum essential medium (EMEM; ICN Biomedicals Inc.; Cat. No. 12-102-54, supplied lacking L-glutamine), and supplemented with 10% foetal bovine serum (FBS; Gibco BRL; Cat. No. 10081-164), 2 mM L-glutamine (ICN Biomedicals; Cat. No. 16-801-49, supplied as a 200-mM stock solution), 50 IU/ml penicillin, and 50 μg/ml streptomycin (ICN Biomedicals; Cat. No. 16-700-49, supplied as a 100× concentrated stock solution), nonessential amino acids (NEAA; Sigma, supplied as a 100× concentrated stock solution; 293 cells and U373MG only), and 1 mM sodium pyruvate (Gibco BRL; Cat. No. 043-01360H, supplied as a 100-mM stock solution; U373MG only).

HCMV laboratory strain AD169 was a subculture of a former candidate live vaccine, used below passage 5 (Elek and Stern, 1974). It was propagated in HFFF CB1945 cells and for fusion assays it was passaged once in HEL12469 fibroblasts. Titrations were as described previously (Steel *et al.,* 1988; Milne *et al.,* 1998). Clinical HCMV strain Pt was isolated from a routine sample from the St. George's Hospital Diagnostic Virology Department and was used previously as a control for a rapid HCMV culture diagnostic test (Steel *et al.,* 1988). This virus grew with similar single-cycle kinetics to

AD169 (data not shown), unlike many clinical HCMV isolates characterised in the Diagnostic unit.

The HCMV gH-specific monoclonal antibody (MAb) HCMV16 (Cranage et al., 1988) was obtained from Cogent Diagnostics and the gO-specific polyclonal rabbit sera, Bl5 (Huber and Compton, 1998, 1999), was generously provided by Professor Teresa Compton, University of Wisconsin. The gH-(C2) and pp65-(C13) specific MAbs have been described previously (Baboonian et al., 1989). Samples of gH MAbs 14.4b and 1G6 were kind gifts of Professors Bill Britt, University of Alabama, and Lucy Rasmussen, Stanford University, respectively (Rasmussen et al., 1984; Bogner et al., 1992).

Virus antibody neutralisation and plaque inhibition assays

HFFF cells in 24-well dishes were infected with 50–100 PFU of HCMV strains AD169 or Pt, after 1 h absorption at 37°C, 1 ml of semisolid agarose was added containing 0.4% (w/v) agarose and either gH MAb C2 or the control nonneutralising pp65 MAb C13. Both MAbs (mouse ascites fluid) were incorporated at a dilution of 1/500 (this dilution of C2 can neutralise 90% of virus prior to absorption). Plates were incubated for 2 weeks at 37°C then fixed and stained using immunoalkaline phosphatase detection as described (Steel et al., 1988). Plaque size was measured by screen projection using a microfilm reader and averaged for each treatment.

Neutralisation of infectivity of cell-free virus by antibody was detected by a reduction in virus titer following exposure to antibody. Serial 10-fold dilutions of MAb C2 and MAb C13 at 10^{-3} dilution were prepared in growth medium, mixed with an equal volume (250 μl) of medium with 100 PFU of HCMV strain AD169 or Pt, and incubated at 37°C for 1 h. The virus/MAb mixtures were then plated in duplicate onto HFFF monolayers, incubated for 14 days under a semisolid overlay, fixed, and stained as above. For each virus, the mean number of PFU at each dilution of C2 was expressed as a percentage of the mean number of PFU in the C13 control wells.

Recombinant adenovirus

The HCMV RAd gH has been described previously (Milne et al., 1998). RAds AD169 gL and AD169 gO in coding and inverse noncoding orientations were constructed with similar methods using E1 deleted replication-deficient RAd, except construction of RAd AD169 gO employed the one-step vector, pCA14 (Microbix, Canada) in place of pMV100/pMV60. The UL74 gene was amplified from HCMV strain AD169 virion DNA using PFU polymerase with the following primers modified to contain BamHI restriction enzyme sites: UL74-1 5′ CGGTGCGGGGGATCCTCCTCTGTC 3′; UL74-2 5′ GACATTGCTGGATCCAGAACTTTA 3′. The amplified product was digested with BamHI, gel purified, ligated with BamHI-

digested phosphatased pCA14, and transformed into Escherichia coli strain JM109. The resulting plasmid was cotransfected with pJM17 into E1 complementing 293 cells to produce recombinant adenovirus RAd gO and cloned by limiting dilution as described previously (Milne et al., 1998). Control RAd35 (which expresses Escherichia coli lacZ) was a generous gift of Dr. Gavin Wilkinson (Department of Medicine, University of Wales, Cardiff, U.K.).

DNA sequencing and analyses

Sequencing of AD169 gH and Pt gH were as described (Milne et al., 1998). Plasmid pMV100 AD169 and Pt gL sequences were determined using a Li-cor 4000LS automated sequencer, and reactions were performed using Thermosequenase (fluorescent-labeled primer cycle sequencing kit following the manufacturer's protocol (Amersham)). UL115 sequencing primers were for both coding and complementary DNA strands as follows: UL115-3 5′AATAGAGGATCCAGACTTGATGTGCCGCCG3′, UL115-4 5′ CGGACCAAGCTTGAGCCTTTATTATCAGCA3′ UL115-7 5′GTTGGCGGCCTCCGGCGT3′; UL115-8 5′GCTATCGCAACTTATTCG3′; UL115-A 5′GGTGACGCAGGCAGA3′; UL115-B 5′AGGGCATCACACTCT3′; UL115-C 5′TCGAAGCCTAACACG3′; UL115-D 5′CAGCATCTTCACGGA3′ UL115-E 5′CGAGCCATCGCCGCA3′ UL115-F 5′GCTGACGCTGTTGAG3′; UL115-G 5′AGTGTCCAGGAAAGC3′ UL115-H 5′CCGTTGGTGAATGTT3′ UL115-I 5′CACCCAACAGGCATC3′; UL115-J 5′TGTTTCCTCAGTCGC3′.

Sequencing of plasmid DNA or PCR products (UL74) from strain Pt, AD169, and Towne were performed using a Perkin–Elmer ABI sequencer. Primers used for sequencing UL74 from both strands were as follows: UL74-1 5′-CGGTGCGGGGGATCCTCCTCTGTC-3′; UL74-2 5′-GACATTGCTGGATCCAGAACTTTA-3′; UL74-3 5′-AGCGGGCCCCATTCA-3′ UL74-4 5′-CGTAGATAGTCAAAC-3′; UL74-5 5′-TACTACCGCCGCAAG-3′ UL74-7 5′-GTTCGGTTGCGGCTT-3′; UL74–9gO2 5′-AGACAAGTCATGGAAGG-3′ UL74-10 5′-TTGCCTATCTTAGAC-3′; gO1 5′-CTCCTCTGTCATGGGGAGAAA-3′; F1A 5′-AGTTTCCCCCGAATTCCATTTTAG-3′; F2 5′-AGTTTTTACCTGGTTAACGCC-3′; F3 5′-ACCGCCGTATCAGAATTTATG-3′; R1A 5′-TTGACAGGCGCTTGTTTAC-3′; R2 5′-GGAAGCCGTTTCGTTTTCCAC-3′; R3 5′-TTACTGCAACCACCACCAAAG-3′ gO F4 5′ CCG TAC ACT ATT TAC GGT A 3′; gO R4 5′ CTC ATC GTC CAG CTT TGC G 3′ A primer specific for the major immediate early promoter contained in the plasmid vector was also used to confirm 5′-terminal sequences on inserted genes gO and gL, MIEP (HCMV) 5′CGCAAATGGGCGGTAGGCGTG3′.

Results were collected and the electrophorerograms examined to compile the final sequence. When PCR products were sequenced, three independent sequencing reactions were run from different PCR reactions, which had been performed using PFU polymerase, to

give good fidelity. Results presented are consensus se-
quences derived from such experiments. The sequences
were compiled from the results of bidirectional sequenc-
ing using Perkin–Elmer ABI Sequence Navigator. Multi-
ple alignments were produced based on CLUSTAL anal-
yses and dendrograms were generated from these re-
sults (Higgins and Sharp, 1989). The Pt and Towne gO
sequences are deposited in GenBank/EMBL databases.

Cell fusion assay

This has been described in detail elsewhere (Milne *et
al.,* 1998). Briefly, U373MG cells were seeded in 96-well
plates (Falcon) (5×10^4 per well) and infected the fol-
lowing day with approximately 50 $TCID_{50}$/cell RAd gH or
RAd gH and gL. At 72 h postinfection, cells were super-
infected with fusing AD169 (prepared in HEL12469 cells,
titer between 10^5 and 10^6). Actinomycin D, 25 μg/ml
(Sigma), and relevant antibodies were added at the ap-
propriate dilutions. Control HCMV16 gH MAb ascites
was diluted 1:100, and several dilutions of the gO anti-
body were used as noted in the text. Plates were centri-
fuged at 800 *g* for 15 min at room temperature (Heraeus
SEPATECH Labofuge 400E, rotor 8177) prior to overnight
incubation (14–16 h) at 37°C. Medium was then removed
and cells were fixed for 10 min in methanol and stained
for 1 h in Giemsa (1:20 dilution; Sigma, Cat. No. GS-500).
Following bench-top air-drying, wells were viewed and
the fusion quantified as previously described using pro-
jected cell images and counting nuclei in fused syncytia
(Milne *et al.,* 1998). This quantification is similar to that
used for a herpesvirus "fusion-from-within" cellular assay
(Turner *et al.,* 1998).

Radioimmunoprecipitation assay

MRC-5 cells grown in 25-cm² flasks were infected with
RAds at an m.o.i. of either 50, 100, or 200. At 1–4 days
postinfection, cells were labeled overnight with 50 μCi
[^{35}S]methione (>1000 Ci/mmol, Amersham) in methio-
nine-deficient media (ICN). Labeled cells were washed
two times in ice-cold PBS, lysed with cold RIPA buffer
(150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 1% Nonidet
P-40, 1% sodium deoxycholate, 1 mM EDTA) and cell
debris pelleted by centrifugation at 13,000 rpm for 30 min
at 4°C. For immunoprecipitation of the gCIII complex,
RAd-infected lysates were rotated with 1–2 μl of the
appropriate antibody for 2 h at 4°C. Immune complexes
were incubated with Protein A–Sepharose for an addi-
tional 2 h at 4°C, washed three times with 0.5 ml of RIPA
buffer with half-strength detergents as described (Ander-
son *et al.,* 1996) and once with 25 mM Tris (pH 7.4). For
the RAd-gO time course, infected cell lysates were im-
munoprecipitated using the IMMUNOcatcher Kit accord-
ing to the manufacturer's instructions (CytoSignal Re-
search Products). Immunoprecipitates were resus-
pended in sample buffer and subjected to SDS–PAGE on

precast 4–12% minipolyacrylamide gels (Novex) under
denaturing, reducing, or nonreducing conditions as de-
scribed previously (Anderson *et al.,* 1996). Gels were
fixed and treated with Amplify (Amersham) for 15 min
prior to exposure to Hyperfilm (Amersham) for autora-
diography.

REFERENCES

Anderson, R. A., and Gompels, U. A. (1999). N- and C-terminal external
domains of human herpesvirus-6 glycoprotein H affect a fusion-
associated conformation mediated by glycoprotein L binding the
N-terminus. *J. Gen. Virol.* **80,** 1485–1494.

Anderson, R. A., Liu, D. X., and Gompels, U. A. (1996). Definition of a
human herpesvirus-6 betaherpesvirus-specific domain in glycopro-
tein gH that governs interaction with glycoprotein gL: Substitution of
human cytomegalovirus glycoproteins permits group-specific com-
plex formation. *Virology* **217,** 517–526.

Baboonian, C., Blake, K., Booth, J. C., and Wiblin, J. C. (1989).
Complement-independent neutralising monoclonal antibody with
differential reactivity for strains of cytomegalovirus. *J. Med. Virol.*
29, 139–145.

Baldwin, B. R., Zhang, C. O., and Keay, S. (2000). Cloning and epitope
mapping of a functional partial fusion receptor for human cytomeg-
alovirus gH. *J. Gen. Virol.* **81,** 27–35.

Bogner, E., Reschke, M., Britt, W., and Radsak, K. (1992). Recognition of
compartmentalised intracellular analogs of glycoprotein H of human
cytomegalovirus. *Arch. Virol.* **126,** 67–80.

Booth, J. C., Beesley, J. E., and Stern, H. (1978). Syncytium formation
caused by human cytomegalovirus in human embryonic lung fibro-
blasts. *Arch. Virol.* **57,** 143–152.

Boppana, S., Rivera, L., Fowler, K., Mach, M., and Britt, W. (2001).
Intrauterine transmission of cytomegalovirus to infants of women
with preconceptional immunity. *New Engl. J. Med.* **344,** 1366–1371.

Bourne, N., Schleiss, M., Bravo, F., and Bernstein, D. (2001). Precon-
ception immunization with a cytomegalovirus (CMV) glycoprotein
vaccine improves pregnancy outcome in a guinea pig model of
congenital CMV infection. *J. Infect. Dis.* **183,** 59–64.

Cha, T. A., Tom, E., Kemble, G. W., Duke, G. M., Mocarski, E. S., and
Spaete, R. R. (1996). Human cytomegalovirus clinical isolates carry at
least 19 genes not found in laboratory strains. *J. Virol.* **70,** 78–83.

Chatterjee, A., Harrison, C. J., Britt, W. J., and Bewtra, C. (2001). Modi-
fication of maternal and congenital cytomegalovirus infection by
anti-glycoprotein B antibody transfer in guinea pigs. *J. Infect. Dis.*
183, 1547–1553.

Chou, S. (1992). Molecular epidemiology of envelope glycoprotein H of
HCMV. *J. Infect. Dis.* **166,** 604–607.

Cranage, M. P., Smith, G. L., Bell, S. E., Hart, H., Brown, C., Bankier, A. T.,
Tomlinson, P., Barrell, B. G., and Minson, T. (1988). Identification and
expression of a human cytomegalovirus glycoprotein with homology

to the Epstein-Barr virus BXLF2 product, varicella-zoster virus gpIII, and herpes simplex virus type 1 glycoprotein H. *J. Virol.* **62,** 1416–1422.

Dal Monte, P., Pignatelli, S., Mach, M., and Landini, M. (2001). The product of human cytomegalovirus UL73 is a new polymorphic structural glycoprotein (gpUL73). *J. Hum. Virol.* **4,** 26–34.

Dargan, D. J., Jamieson, F. E., Maclean, J., Dolan, A., Addison, C., and McGeoch, D. J. (1997). The published DNA sequence of human cytomegalovirus strain AD169 lacks 929 base pairs affecting genes UL42 and UL43. *J. Virol.* **71,** 9833–9836.

Dominguez, G., Dambaugh, T., Stamey, F., Dewhurst, S., Inoue, N., and Pellett, P. (1999). Human herpesvirus 6B genome sequence: Coding content and comparison with human herpesvirus 6A. *J. Virol.* **73,** 8040–8052.

Eggers, M., Radsak, K., Enders, G., and Reschke, M. (2001). Use of recombinant glycoprotein antigens gB and gH for diagnosis of primary human cytomegalovirus infection during pregnancy. *J. Med. Virol.* **54,** 135–142.

Elek, S. D., and Stern, H. (1974). Development of a vaccine against mental retardation caused by cytomegalovirus *in utero. Lancet* **1,** 171.

Emery, V., Atkins, M., Bowen, E., Clark, D., Johnson, M., Kidd, I., McLaughlin, J., Phillips, A., Strappe, P., and Griffiths, P. (1999). Interactions between beta-herpesviruses and human immunodeficiency virus in vivo: Evidence for increased human immunodeficiency viral load in the presence of human herpesvirus 6. *J. Med. Virol.* **57,** 278–282.

Falke, D., Knoblich, A., and Muller, S. (1985). Fusion from without induced by herpes simplex virus type-1. *Intervirology* **24,** 211–219.

Fillet, A., Reux, I., Joberty, C., Fournier, J., Hauw, J., Hoang, P., Bricaire, F., Huraux, J., and Agut, H. (1996). Detection of human herpes virus 6 in AIDS-associated retinitis by means of in situ hybridization, polymerase chain reaction and immunohistochemistry. *J. Med. Virol.* **49,** 289–295.

Foa-Tomasi, L., Boscaro, A., Di, G. S., and Campadelli-Fiume, G. (1991). Monoclonal antibodies to gp100 inhibit penetration of human herpesvirus 6 and polykaryocyte formation in susceptible cells. *J. Virol.* **65,** 4124–4129.

Forrester, A., Farrell, H., Wilkinson, G., Kaye, J., Davis-Poynter, N., and Minson, T. (1992). Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. *J. Virol.* **66,** 341–348.

French, C., Menegazzi, P., Nicholson, L., Macaulay, H., DiLuca, D., and Gompels, U. (1999). Novel, nonconsensus cellular splicing regulates expression of a gene encoding a chemokine-like protein that shows high variation and is specific for human herpesvirus 6. *Virology* **262,** 139–151.

Galdiero, M., Whiteley, A., Bruun, B., Bell, S., Minson, T., and Browne, H. (1997). Site-directed and linker insertion mutagenesis of herpes simplex virus type 1 glycoprotein H. *J. Virol.* **71,** 2163–2170.

Gerna, G., Percivalle, E., Baldanti, F., Sozzani, S., Lanzarini, P., Genini, E., Lilleri, D., and Revello, M. (2001). Human cytomegalovirus replicates abortively in polymorphonuclear leukocytes after transfer from infected endothelial cells via transient microfusion events. *J. Virol.* **74,** 5629–5638.

Gompels, U. A., Carrigan, D. R., Carss, A. L., and Arno, J. (1993). Two groups of human herpesvirus 6 identified by sequence analyses of laboratory strains and variants from Hodgkin's lymphoma and bone marrow transplant patients. *J. Gen. Virol.* **74,** 613–622.

Gompels, U. A., Carss, A. L., Saxby, C., Hancock, D. C., Forrester, A., and Minson, A. C. (1991). Characterisation and sequence analyses of antibody-selected antigenic variants of herpes simplex virus show a conformationally complex epitope on glycoprotein H. *J. Virol.* **65,** 2393–2401.

Gompels, U. A., Carss, A. L., Sun, N., and Arrand, J. R. (1992). Infectivity determinants encoded in a conserved gene block of human herpesvirus-6. *J. DNA Sequencing Mapping* **3,** 25–39.

Gompels, U. A., and Minson, A. (1986). The properties and sequence of glycoprotein H of herpes simplex type 1. *Virology* **153,** 230–247.

Gompels, U. A., Nicholas, N., Lawrence, G., Jones, M., Thompson, B. J., Efstathiou, S. E., Craxton, M. A., and Macaulay, H. A. (1995). The DNA sequence of human herpesvirus-6: Structure, coding content, and genome evolution. *Virology* **209,** 29–51.

Gretch, D. R., Kari, B., Rasmussen, L., Gehrz, R. C., and Stinski, M. F. (1988). Identification and characterisation of three distinct families of glycoprotein complexes in the envelopes of human cytomegalovirus. *J. Virol.* **62,** 875–881.

Higgins, D. G., and Sharp, P. M. (1989). Fast and sensitive multiple sequence alignments on a microcomputer. *Comput. Appl. Biosci.* **5,** 151–153.

Hobom, U., Brune, W., Messerle, M., Hahn, G., and Koszinowski, U. H. (2000). Fast screening procedures for random transposon libraries of cloned herpesvirus genomes: Mutational analysis of human cytomegalovirus envelope glycoprotein genes. *J. Virol.* **74,** 7720–7729.

Huber, M. T., and Compton, T. (1997). Characterisation of a novel third member of the human cytomegalovirus glycoprotein H-glycoprotein L complex. *J. Virol.* **71,** 5391–5398.

Huber, M. T., and Compton, T. (1998). The human cytomegalovirus UL74 gene encodes the third component of the glycoprotein H-glycoprotein L-containing envelope complex. *J. Virol.* **72,** 8191–8197.

Huber, M. T., and Compton, T. (1999). Intracellular formation and processing of the heterotrimeric gH-gL-go (gCIII) glycoprotein envelope complex of human cytomegalovirus. *J. Virol.* **73,** 3886–3892.

Humar, A., Malkan, G., Moussa, G., Greig, P., Levy, G., and Mazzulli, T. (2000). Human herpesvirus-6 is associated with cytomegalovirus reactivation in liver transplant recipients. *J. Infect. Dis.* **181,** 1450–1453.

Jarvis, M., Wang, C., Meyers, H., Smith, P., Corless, C., Henderson, G., Vieira, J., Britt, W., and Nelson, J. (1999). Human cytomegalovirus infection of Caco-2 cells occurs at the basolateral membrane and is differentiation state dependent. *J. Virol.* **73,** 4552–4560.

Kasolo, F. C., Mpabalwani, E., and Gompels, U. A. (1997). Infection with AIDS-related herpesviruses in human immunodeficiency virus-negative infants and endemic childhood Kaposi's sarcoma in Africa. *J. Gen. Virol.* **78,** 847–856.

Kaye, J. F., Gompels, U. A., and Minson, A. C. (1992). Glycoprotein H of human cytomegalovirus (HCMV) forms a stable complex with the HCMV UL115 gene product. *J. Gen. Virol.* **73.**

Keay, S., and Baldwin, B. (1991). Anti-idiotype antibodies that mimic gp86 of human cytomegalovirus inhibit viral fusion but not attachment. *J. Virol.* **65,** 5124–5128.

Klupp, B. G., Nixdorf, R., and Mettenleiter, T. C. (2000). Pseudorabiesvirus glycoprotein M inhibits membrane fusion. *J. Virol.* **74,** 6760–6768.

Li, L., Coelingh, K., and Britt, W. (1995). Human cytomegalovirus antibody-resistant phenotype is associated with reduced expression of glycoprotein-H. *J. Virol.* **69,** 6047–6053.

Li, L., Nelson, J. A., and Britt, W. J. (1997). Glycoprotein H-related complexes of human cytomegalovirus: Identification of a third protein in the gCIII complex. *J. Virol.* **71,** 3090–3097.

Li, Q., Turk, S. M., and Hutt-Fletcher, L. M. (1995). The Epstein-Barr virus (EBV) BZLF2 gene product associates with the gH and gL homologs of EBV and carries an epitope critical to infection of B cells but not of epithelial cells. *J. Virol.* **69,** 3987–3994.

Liu, D. X., Gompels, U. A., Foa-Tomasi, L., and Campadelli-Fiume, G. (1993). Human herpesvirus-6 glycoprotein H and L homologs are components of the gp100 complex and the gH external domain is the target for neutralizing monoclonal antibodies. *Virology* **197,** 12–22.

Lurain, N. S., Kapell, K. S., Huang, D. D., Short, J. A., Paintsil, J., Winkfield, E., Benedict, C. A., Ware, C. F., and Bremer, J. W. (1999). Human cytomegalovirus UL144 open reading frame: Sequence hypervariability in low-passage clinical isolates. *J. Virol.* **73,** 10040–10050.

Mach, M., Kropff, B., DalMonte, P., and Britt, W. J. (2000). Complex

formation of human cytomegalovirus glycoprotein M (gpUL100) and glycoprotein N (gpUL73). *J. Virol.* **74,** 11881–11892.

Mendez, J. C., Dockrell, D. H., Espy, M. J., Smith, T. F., Wilson, J. A., Harmsen, W. S., Ilstrup, D., and Paya, C. V. (2001). Human beta-herpesvirus interactions in solid organ transplant recipients. *J. Infect. Dis.* **183,** 179–184.

Meyer, G., and Radsak, K. (2000). Identification of a novel signal sequence that targets transmembrane proteins to the nuclear envelope inner membrane. *J. Biol. Chem.* **275,** 3857–3866.

Milne, R. S. B., Paterson, D. A., and Booth, J. C. (1998). The glycoprotein H/L complex of human cytomegalovirus modulates fusion from without. *J. Gen. Virol.* **79,** 855–865.

Minson, A., Hodgman, T., Digard, P., Hancock, D., Bell, S., and Buckmaster, E. (1986). An analysis of the biological properties of monoclonal antibodies against glycoprotein D of herpes simplex virus and identification of amino acid substitutions that confer resistance to neutralization. *J. Gen. Virol.* **67,** 1001–1013.

Molesworth, S., Lake, C., Borza, C., Turk, S., and Hutt-Fletcher, L. (2000). Epstein-Barr virus gH is essential for penetration of B cells but also plays a role in attachment of virus to epithelial cells. *J. Virol.* **74,** 6324–6332.

Muggeridge, M. (2000). Characterization of cell-cell fusion mediated by herpes simplex virus 2 glycoproteins gB, gD, gH, and gL in transfected cells. *J. Gen. Virol.* **81,** 2017–2027.

Pertel, P. E., Fridberg, A., Parish, M. L., and Spear, P. G. (2001). Cell fusion induced by herpes simplex virus glycoproteins gB, gD, and gH-gL requires a gD receptor but not necessarily heparan sulfate. *Virology* **279,** 313–324.

Rasmussen, L., Nelson, M., Neff, M., and Merigan, T. C. (1988). Characterisation of two different human cytomegalovirus glycoproteins which are targets for virus neutralising antibody. *Virology* **163,** 163.

Rasmussen, L. E., Nelson, R. M., Kelsall, D. C., and Merigan, T. C. (1984). Murine monoclonal antibody to a single protein that neutralizes the infectivity of human cytomegalovirus. *Proc. Natl. Acad. Sci. USA* **81,** 876–880.

Ratnamohan, V., Chapman, J., Howse, H., Bovington, K., Robertson, P., Byth, K., Allen, R., and Cunningham, A. (1998). Cytomegalovirus and human herpesvirus 6 both cause viral disease after renal transplantation. *Tranplantation* **66,** 877–882.

Roop, C., Hutchinson, L., and Johnson, D. C. (1993). A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells and its particles lack glycoprotein H. *J. Virol.* **67,** 2285–2297.

Simpson, J. A., Chow, J. C., Naker, J., Avdalovic, N., Yuan, S., Au, D., Co, M. S., Vasquez, M., Britt, W. J., and Coelingh, K. L. (1993). Neutralising monoclonal antibodies that distinguish three antigenic sites on human cytomegalovirus glycoprotein H have conformationally distinct binding sites. *J. Virol.* **67,** 489–496.

Spaete, R. R., Perot, K., Scott, P. I., Nelson, J. A., Stinski, M. F., and Pachl, C. (1993). Coexpression of truncated cytomegalovirus gH with UL115 gene product or the truncated human fibroblast growth factor receptor results in transport of gH to the cell surface. *Virology* **193,** 853–861.

Steel, H. M., Booth, J. C., Tryhorn, Y. S., and Stern, H. (1988). A simple immunoalkaline phosphatase method for the rapid diagnosis of cytomegalovirus (CMV) infection. *Serodiagnosis Immunother. Infect. Dis.* **2,** 193–200.

Takemoto, Y., Takatsuka, H., Wada, H., Mori, A., Saheki, K., Okada, M., Tamura, S., Fujimori, Y., Okamoto, T., Kakishita, E., and Kanamura, A. (2000). Evaluation of CMV/human herpesvirus-6 positivity in bronchoalveolar lavage fluids as early detection of acute GVHD following BMT: Evidence of a significant relationship. *Bone Marrow Transplant.* **26,** 77–81.

Theiler, R. N., and Compton, T. (2001). Characterization of the signal peptide processing and membrane association of human cytomegalovirus glycoprotein O. *J. Biol. Chem.* **276,** 39226–39231.

Turner, A., Bruun, B., Minson, T., and Browne, H. (1998). Glycoproteins gB, gD, and gHgL of herpes simplex virus type 1 are necessary and sufficient to mediate membrane fusion in a Cos cell transfection system. *J. Virol.* **72,** 873–875.

Urban, M., Britt, W., and Mach, M. (1992). The dominant linear neutralizing antibody-binding site of glycoprotein gp86 of human cytomegalovirus is strain specific. *J. Virol.* **66,** 1303–1311.

Urban, M., Klein, M., Britt, W., Hassfurther, E., and Mach, M. (1996). Glycoprotein H of human cytomegalovirus is a major antigen for the neutralizing humoral immune response. *J. Gen. Virol.* **77,** 1537–1547.

Wang, X., Kenyon, W., Li, Q., Mullberg, J., and Hutt-Fletcher, L. (1998). Epstein-Barr virus uses different complexes of glycoproteins gH and gL to infect B lymphocytes and epithelial cells. *J. Virol.* **72,** 5552–5558.

Wellington, J., Love, D., and Whalley, J. (1996). Evidence for involvement of equine herpesvirus 1 glycoprotein B in cell-cell fusion. *Arch. Virol.* **141,** 167–175.

Whittaker, G., Bonass, W., Elton, D., Halliburton, I., Killington, R., and Meredith, D. (1992). Glycoprotein 300 is encoded by gene 28 of equine herpesvirus type 1: a new family of herpesvirus membrane proteins? J. Gen. Virol. **73,** 2933–2940.

Wilson, D. W., Davis-Poynter, N., and Minson, A. C. (1994). Mutations in the cytoplasmic tail of herpes simplex virus glycoprotein H suppress cell fusion by a syncytial strain. *J. Virol.* **68,** 6985–6993.