Human cytomegalovirus glycoprotein H/glycoprotein L complex modulates fusion-from-without

Richard S. B. Milne,‡ David A. Paterson and James C. Booth†

Department of Medical Microbiology, St George’s Hospital Medical School, London SW17 0RE, UK

Glycoprotein H/glycoprotein L (gH/gL) complexes of herpesviruses are required for fusion of infecting virions with host cell membranes. In human cytomegalovirus (HCMV), neutralizing monoclonal antibodies (MAb) specific for gH inhibit the transfer of a fluorescent probe to the host cell from labelled virus particles. In similar fashion, in the present study, neutralizing gH-specific MAb inhibited HCMV-induced fusion-from-without in monolayers of both human embryonic fibroblasts and continuous astrocytoma cells (U373). No fusion was detected in cells co-infected with defective recombinant adenovirus vectors that elicited high-level expression of gH and gL, indicating that surface-expressed gH was not intrinsically fusogenic. However, when such cells were superinfected with HCMV that gave fusion-from-without, the resulting cell-to-cell fusion was considerably enhanced. Thus, under our experimental conditions, gH/gL on the cell surface functioned to increase membrane fusion once this was initiated by other components in the virus envelope.

Introduction

Infection with human cytomegalovirus (HCMV), of the subfamily Betaherpesvirinae, is mostly subclinical but may be associated with a mononucleosis-like illness, intrauterine transmission and a range of clinical problems in immunosuppressed patients. Efforts to develop recombinant vaccines, for the prevention of congenital abnormalities, have been focused mainly on the viral surface glycoproteins B (gB; UL55) and H (gH; UL75), both of which elicit good neutralizing antibody responses (Britt, 1996). The major HCMV envelope glycoproteins have been assigned to three families on the basis of their reactivity against particular neutralizing monoclonal antibodies (MAb; Gretch et al., 1988): the gCI glycoproteins comprise different forms of gB; gCII bind to proteoglycan receptors on the cell surface; gCIII include gH, and also glycoprotein L (gL; UL115) without which gH is not presented on the cell surface (Kaye et al., 1992; Spaete et al., 1993). By analogy with the properties of gH/gL for other herpesviruses (Forrester et al., 1992; Roop et al., 1993), both are probably essential for infectivity in HCMV.

In the virion, gB and gH/gL function after virus attachment and during virus entry by membrane fusion (Keay & Baldwin, 1991; Rasmussen et al., 1991). In the infected cell they probably participate in the generation of infectious progeny virus, as shown for herpes simplex virus (HSV; Desai et al., 1988) and in the direct spread of infection cell-to-cell, as shown by the fact that plaque size is reduced when MAb with specific neutralizing activity is present in the overlay medium (Pachl et al., 1989; Simpson et al., 1993). The involvement of gH in fusion was shown by Keay & Baldwin (1991) by the fact that the transfer of a fluorescent probe from labelled virion envelopes to the host cell membrane, which occurred within 40 min after inoculation, was inhibited by neutralizing MAb specific for gH and by its anti-idiotypic. Whether gH/gL in HCMV has intrinsic fusogenic activity, like gB (Bold et al., 1996), or is a modulator of fusion after initiation by other components of the virus, is not known.

The present study describes the production of microscopically visible cell fusion by HCMV soon after inoculation of monolayers of human embryonic fibroblasts or continuous human astrocytoma cells, U373. This resembles the fusion-from-without described for HSV by Falke et al. (1985) and Walev et al. (1991) and is inhibited by neutralizing antibodies specific for HCMV gB and gH, thereby indicating that both

Author for correspondence: David Paterson.
Fax +44 181 672 0234, e-mail dpaterso@sghms.ac.uk
† Present address: Pathogen Molecular Biology and Biochemistry Unit, Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK.
‡ This paper is dedicated to the memory of Dr Jim Booth, who died on Saturday 24 January 1998.
these glycoproteins are involved. We have used this fusogenic virus, together with defective recombinant adenoviruses capable of delivering high-level expression of HCMV gH and gL, to investigate the fusogenic properties of cell surface-expressed gH/gL.

Methods

Cells and virus. Cultures of diploid human embryonic lung (HEL) fibroblasts, prepared by the Clinical Virology Laboratory, from foetal lung tissue, and shown to be free of mycoplasma, were propagated in Eagle's MEM (Bioshuttle) containing 2 mM glutamine and 10% foetal bovine serum (Gibco BRL). Other strains of HEL, of human skin fibroblasts (HSF), the 293 cells and the line of continuous human astrocytoma cells (U373MG), if not produced in-house, were purchased from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK) and propagated in the medium as above; for the medium the 293 cells was further supplemented with 1% (v/v) non-essential amino acids (NEAA; Sigma) and, in the case of the U373 cells, with 1% NEAA and 1 mM pyruvate.

The HCMV strain AD169 was a subculture of that previously used as a candidate live vaccine (Elek & Stern, 1974) and sequenced (Chee et al., 1990); others were in-house clinical isolates, adapted to tissue culture. For the production of virus with fusogenic activity, confluent monolayers of HEL/2469 fibroblasts in 75 cm² flasks were inoculated with 1 ml undiluted virus supernate (10⁶–10⁷ TCID₉₀/ml) from a previous culture showing gross cytopathology and, after 1 h adsorption at 37 °C, the culture was re-fed with 20 ml growth medium and returned to the incubator. Daily sampling of the culture medium detected fusogenic activity by 4–5 days after inoculation, at the same time as the peak titres of infectivity (10⁶–10⁷ TCID₉₀/ml). Fusogenic activity was most reliably detected in undiluted culture supernatant but in some preparations it could be detected up to a dilution of one in eight, the inocula usually providing input multiplicities of 2–20 TCID₉₀ per cell. Nevertheless, fusogenic activity did not necessarily relate to infectivity in that some preparations with infectivity titres greater than 10⁶ TCID₉₀/ml were negative for fusion when tested undiluted and, occasionally, preparations containing 10⁵ TCID₉₀/ml were positive for fusion. Greatest success in producing fusogenic virus by active infection of infected culture supernatants.

Specific antibodies. The complement-independent neutralizing gH-specific MAb were: C2 (Baboonian et al., 1989) and HCMV-16 (Cogen Diagnostics; Cranage et al., 1988). The non-neutralizing MAb were: C13 (specific for ppUL83; Baboonian et al., 1989) and HCMV-34 (specific for gB; Cogen Diagnostics), all in the form of mouse ascitic culture supernatants.

Assay procedures. Infectivity was titrated in microplate cultures of HEL cells which were stained immunocytochemically for HCMV early protein after 2 days at 37 °C (Steel et al., 1988). Indirect immunofluorescence (IF) for viral antigens was performed on cells growing on glass coverslips in 24-well dishes after fixation and permeabilization, as required, according to Cranage et al. (1988); incubation with the primary and secondary antibodies [fluorescein-labelled goat F(ab')₂, anti-mouse immunoglobulins; Tago, TCS Microbiology] was for 1 h at 37 °C.

Fusion assay. Confluent monolayers in 96-well or 24-well culture plates were re-fed with 100 µl and 500 µl respectively, of virus with fusogenic activity (heat or diluted in fresh growth medium) then incubated at 37 °C, as required, before fixing in methanol for 10 min at room temperature and staining with Giemsa. End-point titres of virus fusion activity were read on the basis of detection of cells containing four or more nuclei.

For estimating the degree of cell-to-cell fusion quantitatively, the fixed and stained monolayers were photographed at uniform magnification and the number of nuclei per microscope field was counted. Mean values for several fields were compared for statistical significance by the unpaired Student t test.

Defective recombinant adenoviruses (RAd). The full coding sequences for HCMV gH or gL were cloned separately into the transient expression vector pMV100 (Wilkinson & Akrigg, 1992; Jacob et al., 1992) so placing them under the control of the HCMV major immediate early (MIE) promoter and upstream of a polyadenylation signal. After excision, the entire expression cassette was re-ligated into the adenovirus transfer vector pMV60, placing it into sequences flanking the adenoviral E1 region. Human epidermoid carcinoma cells (293 cells), constitutively expressing the adenoviral E1 gene function, were then cotransfected, using the calcium phosphate precipitation technique (Graham & van der Eb, 1974; Wigler et al., 1977), with the pMV60 construct and a plasmid, pM17, containing the full-length genome for adenovirus type 5 but with the plasmid pBRX inserted into the E1a region. Recombination gave rise to a defective adenovirus type 5 carrying the expression cassette for gH or gL in place of the E1a region; after cloning three times by limiting dilution in 293 cells, each recombinant virus elicited high-level expression of HCMV gH or gL in a variety of cell lines, but replicated only in 293 cells. The procedure, devised by McGrory et al. (1988) was undertaken with plasmids kindly supplied by Gavin Wilkinson (University of Wales, Department of Medicine, Cardiff, UK), with the permission of F. Graham (McMaster University, Hamilton, Ontario, Canada). For control purposes the recombinant adenovirus RAd-35 was used, which expresses the Escherichia coli lacZ gene, a generous gift of Gavin Wilkinson (Wilkinson & Akrigg, 1992).

Characterization of the recombinant RAd showed that DNA extracted from 293 cells infected with RAd-gH or RAd-gL was positive by PCR for the appropriate HCMV gene sequence. Cells infected with RAd-gH were positive by immunofluorescence against the gH-specific MAb HCMV-16; immunoprecipitation of ¹²⁵I-labelled lysates of these and HCMV-infected cells by the same MAb revealed a single band of Mr 90000–92000. MAb specific for gL was not available, but immunoprecipitation by MAb HCMV-16 of cells co-infected with both RAd-gH and RAd-gL revealed a band of Mr 31000–33000 in addition to that of Mr 90000–92000; also, immunofluorescence of non-permeabilized infected HEL fibroblasts showed strong surface fluorescence against MAb HCMV-16 which was not detected on cells infected with RAd-gH or RAd-gL alone. No proteins were precipitated nor immunofluorescence detected with MAb C13 specific for ppUL83. Details of these investigations are given in Milne (1990).

The gH (UL75) and gL (UL115) genes were amplified by PCR from DNA extracted from HSF cells infected with an HCMV clinical isolate, strain Pl (Milne, 1996). The gH coding sequence was amplified using Prm polymerase (Stratagene) with the primers Hxb-1 (5' TATCTCTCATGACCTACCTGATGACCGC) and Hxb-2 (5' TAGCTCTACACCCGATATGATACCA), which introduced an XbaI site (underlined) at either end of the amplicon to allow cloning into pMV100. The gL gene was amplified from the same template material using Taq polymerase (Boehringer) with the primers UL115-3 (5' AATAGAGTGAGACTTGATGTCGCCG) and UL115-2
U373 cells in microplates were re-fed with 100 µl of neat fusogenic virus and serial dilutions thereof, in fresh medium, which were then centrifuged for 15 min at 20 °C, at 800 g in an MSE Mistral 3000i centrifuge before fixing and staining as above.

Results

Fusogenic activity in human embryonic fibroblasts

Virus (strain AD169) with fusogenic activity was produced most reliably in HEL12469 cells but also in a line of HSF cells. Cell fusion was demonstrated in these and in other strains of HEL fibroblast cells (Fig. 1). Time course studies on unfixed cultures revealed loss of elongated fibroblastic shape and fusion by 6 h after inoculation but becoming more extensive overnight (14 h). Virus strains other than AD169 also produced fusion in HEL12469 cells but serial propagation of known fusogenic harvests of AD169 in these cells did not improve fusogenic yields nor result in the selection of virus with enhanced fusogenic attribute.

The specificity of the fusion was demonstrated in inhibition tests with acute and convalescent sera from three cases of primary HCMV infection in pregnant women: 100 µl virus suspension with fusogenic activity was mixed with 10 µl serum and incubated at 37 °C for 1 h before testing on confluent monolayers of HEL12469 cells which were fixed and stained after incubation at 37 °C for 16–24 h. The acute sera, which were negative for neutralizing antibody to HCMV, and negative by diagnostic enzyme immunoassays for HCMV IgG and IgM antibodies, did not inhibit the development of fusion, in contrast to the convalescent sera, which were positive by all three serological tests described and markedly inhibited the development of fusion (data not shown).

Fusogenic preparations of HCMV gave no fusion when inoculated (100 µl), undiluted, onto monolayers of Hep2 cells (susceptible to infection and fusion by paramyxoviruses) or B95 cells (susceptible to infection and fusion by measles virus). Moreover, HEL12469 cells, uninfectected and infected with fusogenic HCMV, were negative when tested by immunofluorescence against MAbs specific for Mycoplasma pneumoniae, influenza virus types A and B, parainfluenza viruses types 1–3, respiratory syncytial virus, HSV types 1 and 2, human herpesvirus 6 and varicella-zoster virus (data not shown).

U373 cells

Fusogenic AD169 virus, grown in HEL12469 cells, also produced fusion in monolayers of U373 cells, this being detected first by 2 h after inoculation but peaking by 7–24 h at 37 °C (Fig. 2). Centrifugation of the inoculum onto the cell monolayers increased titres of fusogenic activity by about tenfold, e.g. from undiluted, in unspun cultures, to titres of 8–16. Centrifugation enabled fusogenic activity to be detected in virus harvests that otherwise showed negative or minimal activity (Fig. 3) and, for a given dilution of virus, increased by twofold to threefold the number of nuclei per microscope field which were enclosed within syncytial cells, e.g. from a mean of 169 (SD = 21, n = 5) up to a mean of 436 (SD = 113, n = 5; P = 0.0008). Fusogenic activity was removed completely, along with infectivity, by filtration through membranes of average pore diameter 0.22 µm but not through 0.45 µm (data not shown).

Fusion from without?

To establish whether the fusion was dependent on the expression of viral gene products, actinomycin D was used to inhibit virus and cellular transcription. Monolayers of U373 cells, in microwells, were re-fed with 100 µl medium or fusogenic AD169 virus, both containing 25 µg/ml actinomycin D, and were incubated for 2 h at 37 °C. Control cultures
were processed in parallel but without the drug. After 14 h at 37 °C, the degree of cell fusion was not significantly different in the absence and presence of the drug [e.g. the mean numbers of nuclei per microscope field that were contained within syncytial cells were, respectively, 670 (SD = 153, n = 3) and 736 (SD = 79, n = 3), P = 0.48], but the fusion was more easily recognized in the latter case because of reduced cell rounding.

The effectiveness of the actinomycin D in inhibiting virus transcription was examined by nested RT–PCR with primers specific for the HCMV MIE gene and by single-round PCR with primers for the HRS housekeeping gene, in both cases carried out on total RNA extracted from U373 cells at 14 h after inoculation with fusogenic virus (Fig. 4). In the presence of actinomycin D, no HCMV MIE transcripts were detected in 2 µl undiluted total extracted RNA after conversion to cDNA (see Fig. 4, lanes 4 and 9) whereas in the absence of actinomycin D, positive results were obtained on testing the cDNA undiluted (see Fig. 4, lanes 3 and 8) and in dilutions of up to 10⁻⁴ (results not shown). In the case of the HRS gene, reactivity was detected in the neat sample from the cultures treated with actinomycin D (see Fig. 4, lanes 4 and 9), also in the neat sample in the absence of the drug (see Fig. 4, lanes 2, 3, 7 and 8) and up to a dilution of 10⁻³ (results not shown).

Ultraviolet irradiation of fusogenic AD169 virus decreased the infectivity from 10⁵.6 TCID₅₀ per 20 µl to undetectable, within 10–20 min, but with no loss in fusogenic activity (titres of 8–16 after centrifugation), albeit the fusogenic titre was reduced after longer exposure (Fig. 5). Titration, before and after inactivation of infectivity, for expression of HCMV IE and early gene products, showed that these were lost in parallel with infectivity and without significant decrease in fusogenic titre. These and the results of the experiments with actinomycin D indicated that fusion occurred independently of viral gene expression, which suggested a mechanism of fusion-from-without.
**HCMV gH/gL and membrane fusion**

**Fig. 3.** Centrifugal enhancement of fusion by HCMV, strain AD169, in monolayers of human astrocytoma (U373) cells: infected cells (a) with and (b) without centrifugation of the inoculum; uninfected cells (c) with and (d) without centrifugation. The cultures were fixed in methanol at 14 h post-infection and stained with Giemsa.

**Fig. 4.** Inhibition of HCMV gene expression by actinomycin D (25 µg/ml) in U373 cells as demonstrated by RT–PCR on extracted cell nucleic acids before (lanes 7, 8 and 9) and after (lanes 2, 3 and 4) double digestion with DNAse I, RT–PCR for histidyl tRNA synthetase mRNA (product sizes: spliced mRNA target, 110 bp; unspliced mRNA or genomic DNA targets, 361 bp), and RT–PCR for HCMV MIE mRNA (product sizes for mRNA and DNA targets: first-round PCR, 256 bp; second-round nested PCR 216 bp). Lanes 1 and 6: marker DNA (pUC19 MspI digest); lanes 2 and 7, uninfected cells without actinomycin D; lanes 3 and 8, infected cells without actinomycin D; lanes 4 and 9, infected cells with actinomycin D; lanes 5 and 10, water control.

**Fig. 5.** Inactivation, by ultraviolet irradiation, of HCMV fusion-from-without activity (D) and of infectivity as judged by titrations for specific cytopathic effect (E) and the expression of IE (☐) and early (●) antigens in inoculated cells by immunocytochemical staining with specific MAb.

**Effect of specific antibodies on virus fusion**

Confluent monolayers (HEL12469 or U373 cells) were overlaid with samples of fusogenic virus (containing 25 µg/ml actinomycin D) that had been preincubated for 1 h at 37 °C with the neutralizing gH-specific MAb C2 or HCMV16 or, as control, with the non-neutralizing MAb C13 (specific for ppUL83). The development of fusion was inhibited by both of the gH-specific MAb but not by MAb C13 (data not shown).

Similar experiments showed inhibition of fusion by post-immunization guinea-pig sera specific for gB but not by pre-immunization serum nor the non-neutralizing MAb specific for gB (HCMV-34) (data not shown). The same results were obtained when the cultures were treated with actinomycin D for 1 h at 37 °C before inoculation with fusogenic virus, this
Fig. 6. Monolayers of U373 cells, 3 days post-infection with 50 TCID$_{50}$ per cell of recombinant adenoviruses (RAd) expressing HCMV gH or gL and stained by immunofluorescence with the gH-specific MAb HCMV16 to detect internal or cell surface expression of gH: uninfected cells showing (a) internal and (b) surface staining; RAd-gL-infected cells showing (c) internal and (d) surface staining; RAd-gH infected cells showing (e) internal and (f) surface staining; cells co-infected with RAd-gH and RAd-gL showing (g) internal and (h) surface staining; cells infected with control RAd35 (expressing E. coli lacZ) showing (i) internal and (j) surface staining. Cells co-infected with RAd-gH and RAd-gL and showing (k) internal and (l) surface staining with control MAb C13 specific for HCMV pUL83.
Fig. 7. Enhanced fusion in monolayers of human astrocytoma (U373) cells co-infected with 10–100 TCID₅₀ per cell of recombinant adenoviruses (RAd) expressing HCMV-gH (RAd-gH) and HCMV-gL (RAd-gL), by superinfection, 3 days later, with HCMV, strain AD169, in the presence of 25 µg/ml actinomycin D. The cells were fixed in methanol 14 h following superinfection, and stained with Giemsa: infected initially with (a) RAd-gH and RAd-gL then superinfected; (b) RAd-gH and RAd-gL but not superinfected; (c) RAd-gH alone then superinfected; (d) RAd-gL alone, superinfected; (e) RAd-gH and RAd-35 (expressing E. coli lacZ), not superinfected; (f) RAd-gL and RAd-35, not superinfected; (g) RAd-gH and RAd-35, superinfected; (h) RAd-gL and RAd-35, superinfected.
also containing actinomycin D, followed 1 h later by washing three times with fresh medium, to remove the unadsorbed inoculum, then by the addition of the various antibodies. Thus, the neutralizing gH-specific MAbs and the post-immunization guinea-pig serum against gB, when added to the virus before or after adsorption, specifically inhibited the development of cell fusion (data not shown).

**Cell surface HCMV gH/gL as a mediator of membrane fusion**

Defective recombinant human adenoviruses, separately expressing HCMV gH (RAd-gH) and HCMV gL (RAd-gL), were prepared. HEL12469 and U373 cells which were inoculated with 10–100 TCID\textsubscript{50} per cell of RAd-gH alone showed peak expression of this glycoprotein, intracytoplasmically, after 2–3 days at 37 °C, as judged by immunofluorescence staining with MAb HCMV-16 on permeabilized fixed cells (Fig. 6). The staining was distributed within the cytoplasm and around the nuclear membrane but not within the nucleus, in keeping with the results of others (Cranage et al., 1988; Rasmussen et al., 1984). In cells infected with RAd-gH and which had been fixed but not permeabilized, no immunofluorescence was detected with the gH-specific MAb HCMV-16. Thus, although infection with the RAd-gH resulted in expression of high levels of intracellular gH, this glycoprotein was not presented on the cell surface. On the other hand, cells infected with both RAd-gH and RAd-gL, and which had been fixed but not permeabilized, showed abundant granular expression of gH on the cell surface by immunofluorescence with HCMV-16 (Fig. 6), fully consistent with established evidence (see Introduction) that an important function of gL is the presentation of gH on the cell surface.

Monolayers of U373 and HEL12469 cells infected with 10–100 TCID\textsubscript{50} per cell of RAd-gH or RAd-gL, or of both RAds together, showed no evidence of fusion during 3 days of incubation at 37 °C, indicating that gH, when presented on the cell surface, possessed no intrinsic fusogenic activity (Fig. 7).

To see if there was a role for gH/gL in modulating membrane fusion, experiments were carried out in which U373 cells infected with different combinations of the RAds were superinfected 3 days later with undiluted AD169 virus suspension from a preparation that gave low-level fusogenic activity. Monolayers in microplates were inoculated, in groups, with RAd-gH and RAd-gL, and with the control RAd-35, both singly and in pair-wise combinations, using 10–100 TCID\textsubscript{50} per cell; cultures not inoculated with RAd were processed in parallel. After 3 days at 37 °C, representative cultures from all groups were re-fed either with the preparation of fusogenic HCMV or with fresh medium. In all cases the inoculum contained 25 μg/ml actinomycin D, one purpose of which was to prevent HCMV MIE gene products complementing the replication defect in the RAd (Tevithia & Spector, 1984; Tevithia et al., 1987).

![Mean no. of nuclei per field, which are within syncytial cells](image)

**Fig. 8.** Quantitative estimates of the extent of cell-to-cell fusion in monolayers of human astrocytoma (U373) cells co-infected with 10–100 TCID\textsubscript{50} per cell of recombinant adenoviruses (RAd) expressing HCMV-gH (RAd-gH) and HCMV-gL (RAd-gL), by superinfection, 3 days later, with HCMV, strain AD169, in the presence of 25 μg/ml actinomycin D. The cells were fixed in methanol 14 h following superinfection, and stained with Giemsa. Numbers of nuclei were counted per microscope field as described in Methods: monolayers infected initially with (a) RAd-gH and RAd-gL then superinfected; (b) RAd-gH and RAd-35 (expressing E. coli lacZ) then superinfected; (c) RAd-gL and RAd-35 then superinfected; (d) RAd-gH alone then superinfected; (e) RAd-gL alone then superinfected; (f) RAd-gH and RAd-gL, not superinfected; (g) RAd-gH and RAd-35, not superinfected; (h) RAd-gL alone, and RAd-35, not superinfected. The mean value for (o) is significantly greater (P < 0.004) than for any of the other categories by univariate comparison.

After 14 h incubation at 37 °C, small numbers of syncytia were detected in the cultures superinfected with AD169 3 days after inoculation with RAd-gH or the RAd-gL alone or in combination with the RAd-35. These syncytia were similar in size and number to the syncytia that were present in the control cultures inoculated with the fusogenic AD169 preparation alone. In the cultures that were superinfected 3 days after co-inoculation with RAd-gH and RAd-gL, however, there was extensive cell fusion which involved most of the cells in the monolayer (Fig. 7).

Quantitative estimates of the degree of cell fusion confirmed that this was significantly greater in cultures co-inoculated with RAd-gH and RAd-gL followed by super-infection with fusogenic AD169 virus, than in cultures inoculated with one or other of these RAds in combination with RAd-35 before exposure to the same preparation of AD169 (Fig. 8). Pretreatment of the superinfecting AD169 virus with the neutralizing MAb specific for gH (C2 and HCMV-16) or with post-immunization guinea-pig serum specific for gB, completely inhibited the development of fusion in cultures preincubated with any pair-wise combination of the three RAds. No such inhibition was detected with the non-neutralizing MAb specific for ppUL83 (C13), with the pre-immunization guinea-pig serum nor with the non-neutralizing MAb specific for gB (HCMV-34). Thus, the recombinant gH/gL expressed on the cell surface, despite displaying no fusogenic activity of its own, significantly enhanced cell-to-cell fusion when this was initiated by fusogenic preparations of HCMV strain AD169.
Discussion

Fusogenic activity associated with HCMV has been demonstrated only rarely, either in cell cultures undergoing short-term chronic infection (Booth et al., 1978), or following exogenous exposure to the virus. Garnett (1979) described fusion in a particular line of HEL fibroblasts 3–4 days after inoculation with strain AD169. In the present study, cell fusion was demonstrated with high-titrated AD169 virus particularly, but not exclusively, when prepared in HEL12469 cells, both in these and other strains of human fibroblasts and in the cell line U373. The most important requirement for achieving fusion was probably the quantity of virus within preparations but a contributory factor might have been the lipid composition of the host cell and hence the virus envelope; however, attempts to enhance the fusogenic activity of HCMV preparations by adding cholesterol, as has been shown for rotaviruses (Falconer et al., 1995), were unsuccessful (unpublished findings).

The fusogenic activity was shown to be specific for HCMV in inhibition studies using paired acute and convalescent sera from cases of primary infection in pregnancy. Moreover, despite taking several hours to reach its maximum in HEL12469 from cases of primary infection in pregnancy. Moreover, inhibition studies using paired acute and convalescent sera confirming that both these glycoproteins are involved in the transfer of fluorescent probe from labelled virions to unlabelled cultured cells is inhibited by neutralizing MAb and was inhibited specifically by antibodies against gH and gB, confirming that both these glycoproteins are involved in the process but providing no information on the individual properties of the glycoproteins themselves. In varicella-zoster virus, gH/gL is intrinsically fusogenic in that HeLa cells transfected to express both genes have been shown to undergo extensive polykaryocytosis (Duus et al., 1995). In the present study, U373 cells expressing surface HCMV gH as a result of co-infection with defective RAds encoding this gene and the gene for gL showed no evidence of fusion. Given that these same cells readily underwent fusion-from-without when exposed to the whole virus, this argues against HCMV gH/gL being intrinsically fusogenic.

Studies on other herpesviruses have provided indirect evidence of a role for the homologous gH/gL molecules in membrane fusion. In human herpesvirus 6, gH-specific MAb inhibit virus-induced fusion in cultured J-Jhan cells (Liu et al., 1993). Likewise, in Epstein–Barr virus, MAb specific for gp85 inhibit membrane fusion but not virus attachment in cultured B-cell lines (Miller & Hutt-Fletcher, 1988). Pseudorabies virus that has gH deleted attaches to cultured cells but does not penetrate unless fusion between virus and cell is induced artificially by treatment with polyethylene glycol (Babic et al., 1996); similar findings have been reported for HSV (Forrester et al., 1992; Roop et al., 1993). Affinity-purified gH/gL for bovine herpesvirus 1 blocks virus penetration into MDBK cells but not the initial attachment (van Drunen Littel-van den Hurk et al., 1996). Mutations to the C terminus of HSV gH modulate the fusogenic activity of virus strains that are intrinsically syncytiogenic because of a mutation in gB (Wilson et al., 1994). Gompels & Minson (1989) have reported cell-to-cell fusion in COS cells transiently expressing HSV gH; Forrester et al. (1992) demonstrated that Vero cells, which had been modified to express HSV-1 gH inducibly, in response to HSV-1 infection, produced widespread syncytial cytopathology following infection with wild-type non-syncytiogenic HSV-1. These findings point to the gH homologues of herpesviruses other than of varicella-zoster virus being modulators rather than initiators of membrane fusion (Spear, 1993), at least in the in vitro cell systems in which the investigations were carried out.

In the present study, fusion of U373 cells showing high-level coexpression of HCMV gH/gL was demonstrated most extensively after superinfection with fusogenic HCMV. Under such conditions, therefore, cell surface HCMV gH/gL appears to modulate fusion that is initiated by another component of the virus envelope. Evidently, the expression of recombinant gH/gL in abundance on the cell surface did not obliterate the receptor sites for the attachment of whole HCMV to the same cell (Keay et al., 1989, 1991); presumably surface gH/gL on one expressing cell could still recognize its receptor on other contiguous expressing cells. Exposure of such expressing cells to preparations of fusogenic whole AD169 virus provided the crucial event for initiating extensive mixing of plasma membrane components through the action of cell surface gH/gL. A likely candidate for the initiator is gB, because of its...
known intrinsic fusogenic activity (Bold et al., 1996; Tugizov et al., 1994) and the inhibition of the fusion-from-without by neutralizing anti-gB sera; alternatively, one of the gCII family of HCMV glycoproteins or other proteins may be required (Huber & Compton, 1997; Li et al., 1997).

Richard Milne was supported by a Wellcome Trust Prize Research Studentship. David Paterson holds a Medical Research Council Research Studentship. We are indebted to Dr Gavin Wilkinson, University of Wales, and Dr Yuri Boriskin and Dr Phil Butcher of this Department for their valued advice.

References


Received 18 June 1997; Accepted 24 November 1997