CD13 (Human Aminopeptidase N) Mediates Human Cytomegalovirus Infection

CECILIA SÖDERBERG,¹ TERRENCE D. GIUGNI,^{2*} JOHN A. ZAIA,² SUSANNE LARSSON,¹
JOHANNA M. WAHLBERG,¹ AND ERNA MÖLLER¹

Department of Clinical Immunology and Center for BioTechnology, NOVUM, Karolinska Institute at Huddinge Hospital, Stockholm, Sweden,¹ and Division of Pediatrics, City of Hope National Medical Center, Duarte, California 91010-0269²

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Human cytomegalovirus (HCMV) infects cells by a series of processes including attachment, penetration via fusion of the envelope with the plasma membrane, and transport of the viral DNA to the nucleus. The details of the early events of HCMV infection are poorly understood. We have recently reported that CD13, human aminopeptidase N, a metalloprotease, is present on blood cells susceptible in vitro to HCMV infection (C. Söderberg, S. Larsson, S. Bergstedt-Lindqvist, and E. Möller, *J. Virol.* 67:3166–3175, 1993). Here we report that human CD13 is involved in HCMV infection. Antibodies directed against human CD13 not only inhibit infection but also block binding of HCMV virions to susceptible cells. Compounds known to inhibit aminopeptidase activity block HCMV infection. HCMV-resistant murine fibroblasts have heightened susceptibility to HCMV infection after transfection with complementary DNA encoding human CD13. A significant increase in binding of HCMV was observed in the CD13-expressing transfectants compared with neomycin-resistant control mouse cells. However, murine fibroblasts transfected with mutant CD13, lacking a portion of the aminopeptidase active site, remained susceptible to HCMV infection. Thus, human CD13 appears to mediate HCMV infection by a process that increases binding, but its enzymatic domain is not necessary for infection.

Human cytomegalovirus (HCMV), a member of the herpesvirus family, is responsible for significant morbidity and mortality in immunocompromised groups (12, 25, 39). HCMV infection of healthy individuals infrequently gives rise to clinical symptoms, whereas infection of the immunocompromised host often leads to a severe or fatal disease. Treatment of this infection is limited by the fact that the early events of HCMV infection, involving attachment to and penetration of the host cell, are largely unknown (10).

The cellular molecules involved in attachment to the cell surface have been identified for certain viruses (18) and include sialyloligosaccharides for a number of viruses, including influenza virus (23), specific cellular glycoproteins such as intercellular adhesion molecule I for human rhinovirus (9), and phospholipids such as phosphatidylserine for vesicular stomatitis virus (26). For the herpesviruses, the binding of Epstein-Barr virus to B cells is mediated by attachment to the C3d complement receptor CR2 (CD21) (8). Cell surface heparin sulfate proteoglycans have been shown to serve as the initial attachment molecule for both herpes simplex virus type 1 (HSV-1) and HSV-2 (37) and for the initial HCMV attachment to cells (5, 14). HCMV infection of human fibroblasts is thought to be mediated by specific binding to one or more membranes glycoproteins (31), and at least four different cell surface proteins have been suggested to be important in HCMV binding and fusion. A cell surface heparin-like molecule has been linked to HCMV attachment via the gCII glycoprotein complex and gB of HCMV (5, 14). HCMV has been shown to bind to a 30- to 34-kDa (1, 22, 32) membrane protein found in a wide range of cell types (22). Grundy et al. have suggested that HCMV binds to class I HLA molecules via virus-bound β 2-microglobulin (11). Finally, a 92.5-kDa protein

on human fibroblasts has been shown to bind the 86-kDa glycoprotein (gp86) of HCMV (16). Antibodies directed against the 92.5-kDa protein prevent virus fusion with the plasma membrane but not virion binding (15), suggesting that this protein plays a role in virus penetration. These data suggest that separate cellular proteins are required for HCMV binding to and fusion with the host cell plasma membrane.

Recently, we have shown that peripheral blood mononuclear cells susceptible in vitro to HCMV infection have in common the CD13 surface marker (28). CD13 is human aminopeptidase N (hAPN), an N-terminal exopeptidase with preference for neutral amino acids. It is present on the apical surfaces of human lung, renal, and intestinal epithelial cells and fibroblasts, as well as on granulocytes, monocytes, and their bone marrow progenitors (17, 20, 21, 27). hAPN plays a role in the digestion of peptides in the gut (21) and in the breakdown and inactivation of peptide neurotransmitters in the brain (17).

Since circumstantial evidence links CD13 with cells that are infected by HCMV, we considered it as a candidate cellular surface molecule that could play a role in the early events of HCMV infection. Recently, porcine CD13 has been reported to be a binding protein for transmissible gastroenteritis virus (6), and human CD13 serves the same function for certain human coronavirus (HCV) serotypes (38). Thus, the aim of this study was to investigate a possible role for human CD13 in the early events of in vitro infection of fibroblasts by HCMV. We here show that compounds that interact with CD13 inhibit HCMV infection of fibroblasts, probably by an inhibition of binding. Expression of human CD13 in mouse cells increases binding and entry of HCMV to these cells, suggesting that CD13 plays a role in cellular attachment or penetration of HCMV. Finally, the aminopeptidase activity of CD13 expressed on the cell surface is not essential for this role in HCMV infection.

* Corresponding author.

MATERIALS AND METHODS

Cells and viruses. Human embryonic lung fibroblasts (MRC-5 [American Type Culture Collection, Rockville, Md.] and HL734 [provided by V. Sundqvist, National Bacteriological Laboratory, Stockholm, Sweden]) and mouse NIH 3T3 cells (provided by S. Kane, City of Hope National Medical Center, Duarte, Calif.) were maintained in bicarbonate-free minimal essential medium with Hanks' salts (GIBCO BRL, Grand Island, N.Y.) supplemented with 25 mM HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid], 10% fetal bovine serum (HyClone, Logan, Utah), glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 µg/ml) (all from GIBCO BRL). The NIH 3T3 transfectants expressing normal human aminopeptidase N (hAPN-3T3) (20), or a truncated form of the protein lacking 39 amino acids from a region within the active site (hAPNMUT-3T3) (3), or a control transfectant carrying the neomycin-containing plasmid without a CD13 cDNA insert (NEO-3T3) (all provided by A. T. Look, St. Jude Children's Research Hospital, Memphis, Tenn.) (20) were maintained in this complete medium with the addition of 800 µg of G418 (Geneticin; GIBCO BRL) per ml.

HCMV strains RC256 (provided by E. Mocarski, Stanford University, Palo Alto, Calif.), a recombinant Towne strain with the *Escherichia coli* β-galactosidase (β-gal) gene under the control of the HCMV major early gene promoter inserted by homologous recombination (29), AD169, and Towne were propagated in human embryonic lung fibroblasts grown in complete medium with 5% fetal bovine serum, and the titer of infectious virus was determined by expression of the major immediate-early (mIE) gene following a modified shell vial assay (30) or by plaque assay (35). HCMV was radiolabeled with [³⁵S]methionine by replacing the medium in cultures of infected MRC-5 monolayers with methionine-free Dulbecco's modified Eagle's medium (ICN, Irvine, Calif.) supplemented with 2% fetal bovine serum and [³⁵S]methionine (TRAN³⁵S-LABEL; ICN) at 250 µCi/ml when 30% cytopathic effect was observed. Virus was collected 4 days later. Cell debris was removed by centrifugation at 1,500 × g for 15 min, and the virus was pelleted from the medium by centrifugation at 25,000 rpm in a Beckman SW-28 rotor for 1 h through a 5-ml layer of 20% (wt/vol) D-sorbitol (Sigma, St. Louis, Mo.). The pellet was resuspended in buffered saline. The specific activity of the ³⁵S-labeled HCMV ranged from 3.8 × 10⁴ to 1.9 × 10⁵ cpm/µg, as determined by liquid scintillation counting of trichloroacetic acid-precipitable counts normalized to total virion protein by bicinchoninic acid protein assay (Pierce, Rockford, Ill.).

Semliki Forest virus (SFV) (provided by H. Garoff, Karolinska Institute at Huddinge Hospital, Stockholm, Sweden) was propagated in baby hamster kidney (BHK-21) cells, and the titer of infectious virus was determined by plaque assay (13).

Inhibition of HCMV infection by CD13-specific antibodies. HL734 cells grown in 96-well plates were infected with HCMV in the absence or presence of various antibodies. Human CD13-specific mouse monoclonal antibodies, U71 and U81 (ascites fluids; both provided by D. Bourel, Centre Regional de Transfusion Sanguine, Rennes, France), WM47 (Dakopatts, Glostrup, Denmark), or anti-Leu M7 (Becton Dickinson, San Jose, Calif.), or various control antibodies, a mouse ascites fluid from mice bearing the NS-1 myeloma (Sigma), mouse immunoglobulin G (mIgG; Sigma), a mouse monoclonal anti-human fibroblast surface protein (Sigma), or a rabbit anti-human epidermal growth factor receptor (EGFR) serum (provided by S. Cohen, Vanderbilt University, Nashville, Tenn.),

were added during incubation of virus with cells. Characterizations of the specific interaction of the CD13-specific antibodies with human CD13 and cells expressing this molecule have been carried out by a number of investigators (2, 3, 7, 19). Briefly, the antibodies used in this study bind to the mouse cell transfectant (hAPN-3T3) expressing normal hAPN (reference 3 and unpublished results) (see also Fig. 4). Two of these antibodies, U71 and U81, do not bind to the mouse cell transfectant (hAPNMUT-3T3) that expresses a mutated form of human CD13 (3) (see Fig. 4), and they recognize a 150-kDa band on immunoblots of fractionated lysates from hAPN-3T3 and MRC-5 cells (unpublished data). The antibodies WM47 and MY7 specifically immunoprecipitate two [³⁵S]methionine-labeled polypeptides of the sizes expected for hAPN, 130 and 150 kDa, from lysates of metabolically labeled hAPN-3T3 (references 2 and 3 and unpublished data). These six antibodies recognize at least three different epitopes of hAPN (7, 19), and all mediate similar inhibitions of HCMV infection as reported in this study.

Monolayer cultures were incubated with HCMV or with HCMV plus antibody for 2 h at 4°C, washed, and incubated at 37°C for 16 h. The cells were assayed for HCMV infection by expression either of the mIE gene of HCMV or of β-gal, if cells were infected with HCMV RC256. For assaying the expression of β-gal, the cells were fixed in 1% phosphate-buffered glutaraldehyde for 15 min at room temperature, washed, and incubated with Bluo-Gal (430 µg/ml; GIBCO BRL) for 2 to 16 h at 37°C (29). Infected cells were detected by the presence of precipitated blue substrate in the cytoplasm of cells, as visualized by light microscopy. For spectrophotometric analysis, β-gal expression was assayed by incubation with *p*-nitrophenyl-β-D-galactopyranoside (1 mg/ml; GIBCO BRL) and the optical density at 410 nm was measured to detect free *p*-nitrophenol liberated by cleavage of the substrate by β-gal. The expression of mIE nuclear antigen was detected after fixation in methanol-acetone by incubation with a mouse monoclonal antibody directed against the 72-kDa HCMV mIE protein (NEN-DuPont, Boston, Mass., or BIOSOFT, Paris, France) followed by incubation with either a biotinylated goat anti-mouse antibody (Sigma) and staining with the ABC kit (Vector Laboratories, Burlingame, Calif.) according to the manufacturer's instructions with 3-amino-9-ethyl carbazol (AEC; Sigma) as the substrate or a phycoerythrin- or fluorescein isothiocyanate-labeled F(ab')₂ fragment of rabbit anti-mouse IgG (Dakopatts). Infected cells were detected by the visualization of brown precipitate in nuclei by light microscopy or by detection of fluorescence-labeled nuclei with a Nikon microscope fitted with epi-illumination optics.

Expression of HCMV-encoded antigens. Kinetic studies of expression of HCMV-encoded antigens were performed after infection of 80% confluent monolayers of HL734, NIH 3T3, hAPN-3T3, and hAPNMUT-3T3 cells with HCMV strain AD169 or RC256. Cells were inoculated at a multiplicity of infection of 1 to 10 for 2 h at 4°C, washed, and incubated in fresh media. At days 1, 3, and 7 postinfection, the cells were fixed and assayed for the expression of either HCMV mIE antigen, pp65, or β-gal, as described above. The assay for pp65 expression was identical to the mIE assay except that a monoclonal antibody directed against pp65 (NEN-Dupont) was utilized as the primary antibody. The number of HCMV-antigen-positive cells was determined and presented as a percentage of total cells.

Inhibition of virus infection by aminopeptidase inhibitors. To measure the effect of aminopeptidase inhibitors on virus infection, HL734 cells were incubated with increasing concentrations of bestatin, actinonin, 1,10-phenanthroline, or 2,2'-

dipyridyl (all from Sigma) for 1 h at 37°C, infected with HCMV strain AD169 at a multiplicity of infection of 1 or SFV at a multiplicity of infection of 30 for 1 h at 37°C, washed, and incubated in fresh media for 6 h at 37°C. To assay for infection, cells were trypsinized and fixed in ice-cold acetone-methanol (1/1) and HCMV antigens were detected with a monoclonal antibody to the mIE antigen followed by the phycoerythrin-labeled secondary antibody. Infected cells were counted by flow cytometric analysis as previously described (28). Cells infected with SFV were detected by using a rabbit polyclonal immune serum against SFV (provided by H. Garoff) followed by a rhodamine-conjugated goat anti-rabbit IgG (BioSys, Compiègne, France).

Cell surface aminopeptidase activity. APN activity of intact cells was measured by a spectrophotometric assay previously described by Ashmun and Look (2). Briefly, 1×10^4 to 5×10^4 cells were plated in 96-well plates, incubated overnight in fresh media, washed, and then incubated with alanine-*p*-nitroanilide (6 mM) (Sigma) in isotonic buffer at 37°C. At various times, the released free *p*-nitroanilide was measured spectrophotometrically at 410 nm. The APN activity was normalized to cell number as measured by bicinchoninic acid protein assay and expressed as mean values of triplicate determinations.

Virus-binding assays. Binding of HCMV was carried out by incubating increasing concentrations of ^{35}S -labeled HCMV with confluent monolayers of cells in 96-well plates for 2 h at 4°C. Incubation was carried out in the presence of a CD13-specific monoclonal antibody (anti-Leu M7) or control mIgG at a concentration of 16.5 $\mu\text{g}/\text{ml}$ or excess of unlabeled HCMV. Cells were then washed five times with HEPES-buffered saline (20 mM HEPES [pH 7.4], 150 mM NaCl) and solubilized with 0.5 M NaOH, and then ^{35}S -labeled virus bound to cells was measured quantitatively by liquid scintillation counting and normalized to the cell number determined by bicinchoninic acid protein assay.

Flow cytometric analysis of cell surface expression of CD13. HL734, NIH 3T3, and NIH 3T3 transfectants expressing the normal form or a deletion mutant form of CD13 were analyzed by flow cytometry as previously described by Ashmun et al. (3). Cells were incubated at 4°C for 30 min in a titrated excess of a CD13-specific monoclonal antibody, U71, MY7 (provided by J. D. Griffin, Dana-Farber Cancer Institute, Boston, Mass.), or WM15 (provided by E. Favaloro and K. F. Bradstock, Westmead Hospital, New South Wales, Australia), or an isotype-matched control (Sigma), washed, and incubated with fluoresceinated affinity-purified goat antiserum to mIgG (Coulter, Hialeah, Fla.) for 30 min at 4°C. The cells were washed and resuspended in staining medium containing 0.25 mM propidium iodide and analyzed with a Coulter EPICS 753 flow cytometer. Dead cells labeled with propidium iodide were excluded from the analysis. Frequency distribution profiles of the $\log_{10}$ fluorescence of each sample were plotted, and a modal value of 1.0 was assigned to the cells stained with control myeloma protein.

RNA PCR for detection of HCMV gene transcription. Detection of mIE mRNA was performed by an RNA polymerase chain reaction (PCR) assay previously described by Söderberg et al. (28). Briefly, whole cell RNA was extracted from cultured, infected and uninfected, HL734, NIH 3T3, hAPN-3T3, and hAPNMUT-3T3 cells 7 days postinfection by using the acid guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sachi (4). cDNA was synthesized with the First-Strand cDNA Synthesis Kit (Pharmacia LKB Biotechnology, Uppsala, Sweden), and 1 μl of the cDNA product was used for the subsequent PCR amplification. The presence of HCMV-specific cDNA and DNA was detected by

a nested PCR method that utilizes forward and reverse primers from exons 1 and 2 of the mIE gene (28). As a control for the presence of cDNA and DNA in each sample, primers from exons 6 and 7 of the glucose 6-phosphate dehydrogenase (G6PD) gene were used. The PCR amplification was carried out as previously described by Söderberg et al. (28) with 50 ng of total cellular DNA or cDNA as the template. The final amplification products were fractionated on a 1% agarose gel containing ethidium bromide and were visualized with UV light.

Southern blot and hybridization with oligonucleotide probes. The PCR amplification products were transferred onto a nylon membrane by vacuum blotting, and hybridization with HCMV-specific oligonucleotide probes, MIE-1 for the intron between exons 1 and 2 of the mIE gene and MIE-2 for exon 2 of the mIE gene, was performed as described previously (28). Following hybridization with oligonucleotide probes labeled at the 3' ends with ^{32}P , the membranes were exposed to X-Omat S film (Kodak) with intensifying screens for 3 h.

Infectious virus production. HL734 and NIH 3T3 cells and 3T3 transfectants were infected as described above with AD169. At 7 days postinfection, the media and cells were collected and subcultured, after clarification and sonification, respectively, on human fibroblasts by incubation at 37°C for 2 h, and then the fibroblasts were washed and incubated in complete media overnight. Fibroblasts were assayed for infection by immunocytochemistry staining for the mIE antigen of HCMV as described above.

RESULTS

Inhibition of HCMV infection by CD13-specific antibodies.

To investigate the role that CD13 plays in the early interaction of HCMV with human fibroblasts, cell strains HL734 and MRC-5 were exposed to virus in the presence of different human CD13-specific monoclonal antibodies (U71, U81, WM47, and anti-Leu M7). As shown in Fig. 1 for U71, these CD13-specific monoclonal antibodies protected HL734 from infection with HCMV RC256 (compare panels c and b). No protection was observed in cells treated with a control mouse ascites fluid obtained from BALB/c mice bearing the NS-1 myeloma (Fig. 1d) or in cells treated with antibodies directed either to the EGFR (anti-EGFR) (Fig. 1e) or to a fibroblast-specific antigen (data not shown), both known to be present on the surface of these fibroblasts. The dose-response curve of HCMV inhibition by U71 indicated that inhibition was directly proportional to the amount of CD13-specific antibody used (Fig. 2A). The dose-dependent inhibition of HCMV infection was determined by an assay that detected the cleavage of *p*-nitrophenyl- β -D-galactopyranoside, a soluble substrate for β -gal. The highest concentration of U71 used in this assay resulted in a 43% decrease in substrate cleavage, indicating a decrease in the expression of the marker gene β -gal, compared with a greater-than-90% decrease in the total number of cells expressing β -gal (Fig. 1c). Side-by-side comparisons of these different assays show that to decrease the amount of expressed β -gal to a level similar to the number of β -gal-positive cells visualized by precipitable substrate requires a higher dose of antibody. The discrepancy in the percentages of inhibition generated by these two assays could be due to the presence of a few positive cells capable of generating a relatively high level of substrate cleavage or to more efficient substrate cleavage in the presence of low-level β -gal production.

The CD13-specific monoclonal antibodies, U81, WM47, and anti-Leu M7, which recognize at least two different epitopes of the CD13 glycoprotein (7, 19), similarly inhibited HCMV

FIG. 1. Inhibition of HCMV Towne strain (RC256) infection of HL734 cells by U71, a human CD13-specific monoclonal antibody. Monolayer cultures of HL734 cells were uninfected (a) or infected with HCMV RC256 in the absence (b) or presence (c through e) of a human CD13-specific antibody (c), control mouse ascites (d), or a polyclonal antibody to the EGFR (e). Inoculation was by incubation with virus or virus plus antibody at 4°C for 2 h; cells were then washed and incubated with complete medium at 37°C overnight. Infected cells were detected by assaying for β -gal activity by incubation with Blueo-Gal as described in Materials and Methods, and they appear darkly stained (panels b, d, and e).

infection in a dose-dependent fashion, and the effect of maximal concentration is shown in Table 1. Although the ascites fluids, U71 and U81, were used at a high total protein concentration, 1.8 mg/ml, and the actual concentration of the CD13-specific antibodies in these ascites fluids is not known, a similar concentration of a control ascites had no effect on infection by HCMV (Fig. 2A). The purified monoclonal antibodies WM47 and anti-Leu M7 at a dose that resulted in 90% or greater inhibition of infection of cells by HCMV had no inhibitory effect on infection by an unrelated virus, SFV (Table 1). Anti-Leu M7 at a concentration as high as 16 μ g/ml had an inhibitory effect no greater than that of mIgG on the infection of MRC-5 cells by HSV-1 as measured by plaque formation (data not shown). U81 and U71 both are more effective in inhibiting cell-associated aminopeptidase activity than WM47 or anti-Leu M7 (Table 1) (3). Thus, we have shown that HCMV infection of fibroblasts in vitro is inhibited by CD13-specific antibodies and not HSV-1, a related enveloped virus, or SFV, an unrelated virus. All CD13-specific antibodies tested to this point are able to inhibit infection whether or not they inhibit aminopeptidase activity (Table 1).

Inhibition of HCMV infection by aminopeptidase inhibitors.

In order to investigate whether the inhibition of hAPN activity can influence HCMV infection, four different aminopeptidase inhibitors, actinonin (33), bestatin (24, 36), 2,2'-dipyridyl, and 1,10-phenanthroline (34), were evaluated for effect on both enzymatic activity and HCMV infection. Bestatin and actinonin are competitive inhibitors of aminopeptidases (24, 33, 36), and 1,10-phenanthroline and 2,2'-dipyridyl inhibit aminopeptidase activity by chelating zinc required for enzyme function (34). These compounds were used at concentrations which were demonstrated to inhibit hAPN in cells using alanine-*p*-nitroanilide as the substrate (Table 1). Toxicity of these substances, measured by trypan blue uptake in cells treated with the inhibitors, was less than 10% at the highest concentrations used (data not shown). As shown in Fig. 2B, bestatin inhibited HCMV infection, measured by the expression of the mIE gene, in a dose-dependent manner with a 50% inhibitory concentration of 2.5 mM. Similarly, actinonin, 1,10-phenanthroline, and 2,2'-dipyridyl inhibited HCMV infection with 50% inhibitory doses of 3.3, 0.25, and 2.8 mM, respectively (Table 1). The same results were obtained whether the expression of mIE or β -gal activity was used as the endpoint for infection, indicating that the results of these experiments are not merely due to inhibition of β -gal activity by the APN inhibitors.

FIG. 2. Effect of hAPN inhibitors on HCMV infection. (A) Infection of HL734 with HCMV RC256 was performed as described in the legend to Fig. 1 in the presence of serial dilutions of either U71, a CD13-specific mouse ascites fluid (■), or a control mouse ascites fluid (□). Cells were washed, fixed with glutaraldehyde, and assayed for β -gal expression by incubation with *p*-nitrophenyl- β -D-galactopyranoside, and then infection was quantitatively determined by spectrophotometric measurement of free *p*-nitrophenol produced by the cleavage of substrate by β -gal. (B) Monolayer cultures of HL734 were infected with HCMV strain AD169 (■) or with SFV (□) in the presence of increasing concentrations of bestatin. Infection was quantitatively determined by flow cytometric analysis using an antibody to the mIE protein of HCMV or immune serum to SFV and enumeration of infected cells, as described in Materials and Methods.

TABLE 1. Effect of anti-human CD13 monoclonal antibodies and aminopeptidase inhibitors on HCMV infection

Inhibitor	Inhibition of ^a :		
	HCMV infection	SFV infection	Aminopeptidase activity
Monoclonal antibodies^b			
U71	+	ND	+
U81	+	ND	+
WM47	+	—	—
Anti-Leu M7	+	—	—
Chemical inhibitors^c			
Actinonin (3.3)	+	—	+
Bestatin (2.5)	+	—	+
1,10-Phenanthroline (0.25)	+	—	+
2,2'-Dipyridyl (2.8)	+	—	+

^a Inhibition of HCMV and SFV infection was determined as described in the legend to Fig. 2. Inhibition of enzymatic activity was measured by using the substrate alanine-*p*-nitroanilide, as described in Materials and Methods. +, >50% inhibition compared with controls; —, no inhibition; ND, not done.

^b Purified antibodies (WM47 and anti-Leu M7) and ascites fluids (U71 and U81) were tested at various concentrations, and the results shown are for the dose that gave maximum effect for each antibody: U71, 1.8 mg/ml; U81, 2 mg/ml; WM47, 20 µg/ml; anti-Leu M7, 3.7 µg/ml.

^c Chemical inhibitors were tested at the following concentrations, which gave maximum inhibition without toxicity to the cells: actinonin, 5.45 mM; bestatin, 4.35 mM; 1,10-phenanthroline, 0.45 mM; 2,2'-dipyridyl, 3.75 mM. The 50% inhibitory dose (millimolar concentration) is given in parentheses.

Expression of APN in mouse cells. To further investigate the importance of human CD13 and its aminopeptidase domain during HCMV infection, murine cells (NIH 3T3) which were transfected with cDNA for hAPN (hAPN-3T3) (20) or for a truncated form of the protein lacking 39 amino acids from a region within the active site (hAPNMUT-3T3) (3) were obtained. To confirm the level of APN activity in these cells, they were tested for the ability to cleave the aminopeptidase substrate alanine-*p*-nitroanilide at 37°C (Fig. 3). At various times after incubation, the level of aminopeptidase activity was determined by spectrophotometric measurement of free *p*-nitroanilide, a cleavage product of the soluble substrate. A high level of enzymatic activity was present in the hAPN-3T3 cells compared with that in HL734. There was little detectable activity in the parent NIH 3T3 or in the hAPNMUT-3T3 cells. The presence of surface human CD13 was assessed on the

FIG. 3. APN activity on the surface of intact human embryonic lung fibroblasts and 3T3 transfectants. The APN activity on the surface of HL734 (■), NIH 3T3 (●), hAPN-3T3 (□), or hAPNMUT-3T3 (△) cells expressed as optical density of the reaction product *p*-nitroanilide was measured spectrophotometrically as described in Materials and Methods.

human cells and on the NIH 3T3 transfectants by flow cytometric analyses using human CD13-specific monoclonal antibodies, U71, MY7, and WM15. The parental NIH 3T3 cells are negative for human CD13 expression (Fig. 4B); hAPNMUT-3T3, the cell line having the truncated form of human CD13, expresses low levels (Fig. 4D); and HL734 and MRC-5 express intermediate levels (Fig. 4A and data not shown), with HL734 expressing significantly higher levels than MRC-5. The hAPN-3T3 cells express very high levels of hAPN (Fig. 4C). The flow cytometric analyses showing surface expression of human CD13 by antibody binding were identical with all three CD13-specific antibodies, except that the antibodies U71 and WM15 failed to react with the hAPNMUT-3T3 line that no longer had the epitope recognized by U71 or WM15 (24).

Role of CD13 in HCMV binding. To begin to understand in which part of the viral life cycle CD13 might play a role, we analyzed the effect of human CD13-specific antibodies on binding of ³⁵S-labeled HCMV virions to MRC-5 cells (Fig. 5a). Incubation of cells with increasing concentrations of ³⁵S-labeled virions resulted in increased binding of HCMV. If binding was performed in the presence of control mIgG, there was no effect on binding. However, binding in the presence of human CD13-specific antibodies (anti-Leu M7) resulted in a decrease in HCMV binding (Fig. 5a). The extent of binding in the presence of CD13-specific antibodies was similar to that seen in the presence of a 25-fold excess of unlabeled HCMV (data not shown), suggesting that CD13-specific antibodies block the specific binding of HCMV to fibroblasts.

To determine whether CD13 could play a direct role in binding of HCMV to cells, we compared the binding of ³⁵S-labeled HCMV to hAPN-3T3 cells that express high levels of human CD13 and to control NEO-3T3 cells. Monolayer cultures of the 3T3 transfectants were exposed to increasing concentrations of ³⁵S-labeled HCMV in the presence or absence of excess unlabeled HCMV at 4°C. Specific binding was calculated by subtracting the values obtained at each virus concentration in the presence of an approximate 25-fold excess of unlabeled HCMV from the values which occurred in the absence of excess unlabeled HCMV. Both the NEO-3T3 and the hAPN-3T3 cells showed an increase in cell-associated virus with increasing viral input (Fig. 5b). The nonspecific binding for the hAPN-3T3 cells ranged from 15 to 50% of total binding from the low to the high amount of input virus. The parental 3T3 showed slightly greater nonspecific binding, reaching 65% of total binding at the highest virus concentration. The hAPN-3T3 cells showed a marked increase in specific HCMV binding over the control NEO-3T3 cells (Fig. 5b). At the highest concentration of radiolabeled virus used, the CD13-expressing cells showed a 4.1-fold increase in specific HCMV binding compared with the controls.

CD13-specific antibodies were tested for their ability to inhibit binding of HCMV to hAPN-3T3 cells. hAPN-3T3 cells were incubated with a single concentration of ³⁵S-labeled HCMV virions in the absence or presence of a 25-fold excess of unlabeled virions, anti-Leu M7, or a control mIgG (both at 20 µg/ml), as described in the legend to Fig. 5. The specific binding of HCMV to hAPN-3T3 cells was inhibited by 85% by this concentration of anti-Leu M7, while no inhibition of binding was observed with the control mIgG.

Role of CD13 on HCMV infection in vitro. To test, directly, whether the expression of CD13 on the surface of cells would enhance the susceptibility of cells to HCMV infection in vitro, we compared the susceptibilities of the parental mouse cells and the CD13-expressing transfectants to HCMV infection. The 3T3 cells and the 3T3 transfectants were challenged with HCMV RC256 by incubation with the virus at 4°C for 2 h.

FIG. 4. Flow cytometric analysis for CD13 in human embryonic lung cells and NIH 3T3 transfectants. HL734 (A), NIH 3T3 (B), hAPN-3T3 (C), and hAPNMUT-3T3 (D) cells were stained with CD13-specific monoclonal antibody (MY7) (solid line) or with an isotype-matched control antibody (dotted line). Similar results were obtained for HL734, NIH 3T3, and hAPN-3T3 cells utilizing CD13-specific antibodies U71 and WM15; however, these antibodies do not bind to the mutant CD13 expressed in hAPNMUT-3T3 cells (data not shown).

Nuclear staining for the mIE antigen was greatly enhanced in the transfectants compared with the parental cells (Fig. 6 and Table 2). Similarly, β -gal activity was observed in a greater number of the cells expressing human CD13 than in the parental cells (Table 2).

To further characterize the kinetics of HCMV antigen expression and to determine whether infected mouse cells expressing human CD13 could produce and release infectious virus, AD169- or RC256-infected cultures of 3T3 cells, 3T3 transfectants, and HL734 fibroblasts, a positive control for infection, were harvested 1, 3, and 7 days postinfection and assayed for expression of the 72-kDa mIE antigen, β -gal, pp65, or infectious particles. Data from a representative experiment are shown in Table 2. As shown in Fig. 6, 19% of the permissive fibroblasts, HL734, had positive nuclei at day 1 postinfection, increasing to 90% by day 7. In comparison, the NEO-3T3 cells expressed less than 0.25% positive cells, with no significant increase from days 1 to 7 postinfection. In hAPN-3T3, the percentage of cells with positive nuclei reached a high of 2.5% at days 3 to 7 postinfection, a 10-fold increase over the controls, which did not express human CD13.

Our results with aminopeptidase inhibitors suggest that the enzymatic activity of CD13 is important in HCMV infection. To more directly address this question, we compared the susceptibility to HCMV infection of the transfectants expressing native CD13 to that of those expressing the mutant form, lacking enzymatic activity. Virus infection was observed in a

greater number of cells expressing the truncated form of human CD13 than in those with unmodified hAPN (Fig. 6; compare panels c and d). In a quantitative experiment, 7.6 to 13% of the hAPNMUT-3T3 cells were positive for mIE expression 1 to 3 days postinfection, a 5.2-fold increase over the value for the hAPN-3T3 cells and a 50-fold increase compared with the value for parental cells (Table 2).

Expression of HCMV gene products in hAPN-transfected murine cells. To determine the presence of active HCMV DNA transcription in the 3T3 transfectants, both DNA and RNA were prepared from in vitro-infected cells, cDNA was synthesized from RNA preparations, and nested PCR was performed with DNA or cDNA as the template and primers to the mIE gene of HCMV or to the G6PD gene as a positive control, as described in Materials and Methods (Table 2). No HCMV DNA or cDNA could be detected in uninfected cells, whereas mIE primers yielded 332- and 218-bp products for DNA and cDNA, respectively, in in vitro-infected HL734, NIH 3T3, hAPN-3T3, and hAPNMUT-3T3 cells. Although this PCR assay is not quantitative, the cDNA samples from the HL734 fibroblasts yielded the strongest amplified band when visualized on an ethidium bromide-stained agarose gel. The cDNA samples from the NIH 3T3 cells yielded a very weak band. All the samples yielded a 272- or a 95-bp product, for DNA or cDNA, respectively, and amplification using the control G6PD primers indicated that DNA and cDNA were

FIG. 5. Effect of CD13-specific antibodies and CD13 expression on binding of ³⁵S-labeled HCMV to cells. (a) Binding of HCMV to MRC-5 cells was carried out by incubating increasing concentrations of ³⁵S-labeled HCMV with MRC-5 cells for 2 h at 4°C in the presence of mIgG (○) or antibody to human CD13 (anti-Leu M7) (●) at a concentration of 16.5 µg/ml. (b) Binding of HCMV to NEO-3T3 (●) and hAPN-3T3 (○) cells was carried out by incubating increasing concentrations of ³⁵S-labeled HCMV with 3T3 transfectants for 2 h at 4°C in the presence or absence of excess unlabeled HCMV. Virus bound was determined by subtracting counts of virus that bound in the presence of excess unlabeled HCMV from total counts of virus bound and normalizing to cell number as determined by the bicinchoninic acid protein assay.

present. To test the specificity of the amplification reactions, the PCR products were transferred to nylon membranes and Southern blot hybridizations were performed with ³²P-labeled oligonucleotides specific for the intron between exons 1 and 2 (MIE-1) and for exon 2 (MIE-2) of the mIE gene, as described in Materials and Methods. The MIE-1 probe hybridized to both the 332- and 218-bp amplification products, whereas the MIE-1 probe hybridized only with the 332-bp cDNA amplification product in all the cells, confirming the presence of HCMV DNA and transcription of mIE mRNA in the in vitro-infected 3T3 cells and transfectants.

Progression of infection appears not to go beyond the immediate-early stage in the mouse cells. We were unable to detect the expression of the major matrix protein, pp65, from 1 to 7 days postinfection in the 3T3 transfectants or to show the

presence of infectious virus at 7 days postinfection in either subcultured media or cells (Table 2).

DISCUSSION

In the present study, we have shown that HCMV infection of fibroblasts, as well as binding of virions to these cells, can be inhibited by different monoclonal antibodies directed against human CD13 (Fig. 1 and Table 1). We have shown that four different monoclonal antibodies directed against at least two different epitopes of human CD13 inhibit HCMV infection. This inhibition did not appear to be due to nonspecific steric hindrance by antibody to cell surface marker, since antibodies to the EGFR (Fig. 1e) and to a human fibroblast surface antigen failed to inhibit HCMV infection. Antibodies to human CD13 do not inhibit the infection of cells by SFV, an unrelated enveloped virus, or by HSV-1, a related herpesvirus. Human CD13 expression on murine cells increases the binding of HCMV to these cells, and this binding can be inhibited by antibodies directed at CD13, suggesting that human CD13, hAPN, is an attachment protein for HCMV.

To evaluate the role of aminopeptidase activity in HCMV infection, we assessed the ability of different monoclonal antibodies and of aminopeptidase inhibitors to inhibit APN activity and HCMV infection. Of the CD13-specific monoclonal antibodies utilized in this study, U71 and U81 inhibit APN activity by binding to an epitope that is contained within or overlaps a 39-amino-acid segment that contains the putative zinc-binding domain of the active site (3), while WM47 and anti-Leu M7 do not bind to this domain or inhibit aminopeptidase activity (Table 1). All four of these antibodies inhibit HCMV infection (Fig. 2 and Table 1). Thus, HCMV infection of fibroblasts in vitro is inhibited by CD13-specific antibodies whether or not they inhibit aminopeptidase activity. Additionally, all aminopeptidase inhibitors tested inhibited HCMV infection (Fig. 2B and Table 1), and these inhibit the enzyme either by direct binding to hAPN or by chelating zinc required for the aminopeptidase activity. Furthermore, HCMV infection was enhanced in both murine cells expressing human CD13 and murine cells expressing enzymatically inactive CD13 (Fig. 6), in which 39 amino acids were deleted from the active site. In fact, the cells expressing the mutated form of CD13 showed a fivefold greater expression of HCMV-associated gene products than did the murine cells expressing native human CD13, even though the mutant cells express lower levels of surface CD13 as measured by MY7 binding. These data suggest two points: the enzyme activity of human CD13 is not critical for HCMV infection, and a nonenzyme domain of human CD13 is important for HCMV infection of fibroblasts.

Since active enzyme is not essential for HCMV infection, it is not clear how the hAPN-inhibiting compounds which block infection act; one possible explanation is that they change the conformation of the CD13 molecule and, thus, alter a molecular domain which is important for infection. However, we have been unable to show inhibition of binding of two CD13-specific antibodies, anti-Leu M7 and WM47, to normal human fibroblasts, HL734, by concentrations of the aminopeptidase inhibitors, 2,2'-dipyridyl or bestatin, that inhibit enzymatic activity and HCMV infection. The fact that different anti-CD13 monoclonal antibodies, recognizing different epitopes on the CD13 molecule, all inhibit infection is not unexpected. The size of antibodies is such that steric inhibition might occur irrespective of the fact that one set of antibodies recognize the enzymatically active domain and the other set recognize one or more epitopes unrelated to aminopeptidase activity. However, it will be of importance in future experiments to delineate the

FIG. 6. Expression of mIE in in vitro HCMV infection of human fibroblasts and mouse cells expressing human CD13. Cell lines consisting of parental murine NIH 3T3 cells (b), transfected NIH 3T3 cells expressing high levels of native human CD13 (hAPN-3T3) (c) or mutant human CD13 (hAPNMUT-3T3) lacking 39 amino acids, including the zinc-binding site, necessary for enzymatic activity (d), and human HL734 (a) were infected with HCMV RC256 and assayed for the expression of HCMV mIE. Infection was performed and assayed as described in Materials and Methods.

part of the CD13 molecule having the central role for HCMV infection.

It has recently been reported that hAPN (CD13) and porcine APN serve as attachment proteins for HCV-229E (38) and transmissible gastroenteritis virus (6), respectively. Like HCMV infection, HCV-229E infection is inhibited by CD13-specific antibodies that inhibit APN activity and APN inhibitors that act by chelating the zinc required for enzymatic activity; but unlike HCMV infection, it is not inhibited by bestatin and actinonin, which bind competitively to the active site (24). In addition, HCV-229E can infect murine cells expressing human CD13 (hAPN-3T3) but not murine cells expressing the mutated form (hAPNMUT-3T3), whereas HCMV can infect both forms of murine cells. Thus, although infection by HCMV, HCV-229E, and transmissible gastroenteritis virus is mediated by cell surface expression of hAPN, the domain important for HCMV infection appears to differ from that utilized by HCV-229E and transmissible gastroenteritis virus. The domain of CD13 which is important for HCMV infection is currently under investigation.

It is of interest to note that, even though the membrane-bound proteins utilized by viruses to gain entry into cells are quite diverse, CD13 might serve a general function that is important for the entry of certain enveloped viruses into the cell. The fact that two related enveloped RNA coronaviruses and an unrelated enveloped DNA herpesvirus would utilize

the same cellular molecule in the early events of infection focuses attention on CD13 as a route of entry utilized potentially by many viruses. Thus, it is possible that other enveloped viruses utilize APN glycoproteins or other related metalloproteases to gain entry into their respective host cells. It is possible that the special feature of hAPN and of other related molecules, utilized by enveloped viruses, is merely that they are expressed in abundance on the apical surfaces of epithelial cells in the alimentary and respiratory tracts. The physiological role of the apical expression of the CD13 molecule is not completely understood (17, 21). Studies of the importance of the CD13 cell surface structure to the physiological function of such cells are warranted.

As indicated earlier, a number of cellular proteins have been shown to interact with HCMV virions (1, 11, 14, 16, 32), and these appear to play a role in binding to or fusion with the plasma membrane. The molecular size of CD13, 130 to 150 kDa, would preclude its being any of these previously described HCMV-binding proteins. In addition, these previously described cell surface molecules with binding affinity for HCMV are also expressed on cells which are resistant to infection (22). It is possible that attachment to or fusion with the plasma membrane involves a complex of proteins that includes CD13. The data presented here and by others (22) clearly show that HCMV can bind to cells, such as NIH 3T3, that do not express human CD13 (Fig. 5). The exact role of

TABLE 2. HCMV gene expression in murine cell transfectants

HCMV product	Expression in cell line ^a :			
	HL734	NIH 3T3	hAPN-3T3	hAPNMUT-3T3
mIE antigen				
Day 1	19	<0.25	1.7	7.6
Day 3	25	<0.25	2.5	10
Day 7	90	<0.25	2.4	13
pp65				
Day 1	—	—	—	—
Day 3	4.5	—	—	—
Day 7	10	—	—	—
mIE DNA	+	+	+	+
mIE RNA	+	+	+	+
G6PD DNA	+	+	+	+
G6PD RNA	+	+	+	+
Infectious virus ^b				
IC	+	—	—	—
EC	+	—	—	—

^a Percentage of cells expressing the 72-kDa mIE antigen or pp65 of HCMV was determined 1, 3, and 7 days postinfection with AD169 as described in Materials and Methods. —, no positive cells were detected. Presence of HCMV DNA and expression of HCMV mIE RNA were detected by PCR utilizing primers specific for the mIE gene of HCMV as described in Materials and Methods. Primers specific for the G6PD gene were used as a positive control for the presence of DNA and cDNA. +, an amplified product was detected. The production of infectious virus was determined by culturing normal human fibroblasts with sonicated cells or clarified media, respectively, from cultures of cells collected 7 days postinfection with AD169 as described in Materials and Methods. +, infectious virus was detectable; —, no infectious virus was detectable.

^b IC, intercellular; EC, extracellular.

CD13 in the early events of HCMV infection are not known, and it is possible that there are other molecules with which it interacts and which are important for HCMV infection.

The distribution of CD13 and the types of cells infected by HCMV appear to correlate, and this suggests that the expression of CD13 plays a role in determining the tissue tropism of HCMV. Even murine cells expressing human CD13 show increased binding and are more susceptible to HCMV infection than cells which do not express CD13, as assayed by expression of mIE RNA and protein (Table 2 and Fig. 6). Clearly, this increased expression of HCMV mIE indicates that there is an increase in the number of cells in which virus entry occurs when hAPN is expressed on the surface of the cell. The ability of CD13-expressing murine transfectants to undergo enhanced HCMV infection indicates the potential importance of human CD13 in target cell tropism. Thus, human CD13 appears to be a host protein with a significant role in the normal biology of HCMV infection. Understanding this role could lead to improved methods for prevention and treatment of this infection.

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