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# Involvement of Aminopeptidase N (CD13) in Infection of Human Neural Cells by Human Coronavirus 229E

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**Attachment to a cell surface receptor can be a major determinant of virus tropism. Previous studies have shown that human respiratory coronavirus HCV-229E uses human aminopeptidase N (hAPN [CD13]) as its cellular receptor for infection of lung fibroblasts. Although human coronaviruses are recognized respiratory pathogens, occasional reports have suggested their possible neurotropism. We have previously shown that human neural cells, including glial cells in primary cultures, are susceptible to human coronavirus infection in vitro (A. Bonavia, N. Arbour, V. W. Yong, and P. J. Talbot, J. Virol. 71:800–806, 1997). However, the only reported expression of hAPN in the nervous system is at the level of nerve synapses. Therefore, we asked whether hAPN is utilized as a cellular receptor for infection of these human neural cell lines. Using flow cytometry, we were able to show the expression of hAPN on the surfaces of various human neuronal and glial cell lines that are susceptible to HCV-229E infection. An hAPN-specific monoclonal antibody (WM15), but not control antibody, inhibited the attachment of radiolabeled HCV-229E to astrocytic, neuronal, and oligodendrocytic cell lines. A correlation between the apparent amount of cell surface hAPN and the level of virus attachment was observed. Furthermore, the presence of WM15 inhibited virus infection of these cell lines, as detected by indirect immunofluorescence. These results indicate that hAPN (CD13) is expressed on neuronal and glial cell lines in vitro and serves as the receptor for infection by HCV-229E. This further strengthens the neurotropic potential of this human respiratory virus.**

Human coronaviruses (HCV) are large enveloped RNA viruses that contain a single-stranded message sense RNA of approximately 30 kb (19). Virions of the 229E strain of HCV are composed of a 50- to 60-kDa nucleocapsid phosphoprotein (N) associated with genomic RNA (38, 43) and three envelope proteins: the 21- to 25-kDa membrane glycoprotein (M) embedded within the lipid membrane, the 200-kDa surface glycoprotein (S) which forms the spikes protruding from the virions, and a 9- to 12-kDa small membrane glycoprotein (E) (19, 36). The S glycoproteins of coronaviruses, which can induce neutralizing antibody and mediate cell fusion, have been shown to bind to specific host cell receptor glycoproteins (18, 19).

The cellular receptor used by HCV-229E for infection of human lung fibroblasts has been described to be human aminopeptidase N (hAPN [CD13]) (51). CD13 (EC 3.4.11.2) is a zinc-dependent metalloprotease of 150 kDa composed of 967 amino acids (27) that is encoded by a gene present on the long arm (q11-qter) of chromosome 15 (48). This membrane-bound N-terminal exopeptidase has a preference for neutral amino acids (3) and is widely distributed at the surface of many cells and tissues such as the renal-proximal tubule epithelium, and lung and intestinal epithelium, as well as cells of the granulocytic and monocytic lineage (23, 27). It can also be found on blood-brain-barrier-associated pericytes and on synaptic membranes of the central nervous system (CNS) (25, 27). Some known functions of CD13 include the digestion of peptides in the gut and the cleavage and inactivation of peptide neurotransmitters in the brain (9, 28, 39). So far, there has been no report of the presence of this enzyme on human CNS glial cells, although it has been found in cultures of pig neuronal and astrocytic glial cells (4).

APN also acts as a receptor for other coronaviruses of the same antigenic group as HCV-229E. Porcine APN was found to be a major receptor for porcine transmissible gastroenteritis virus (TGEV) (17), although an additional second receptor restricted to the villous enterocytes of newborn pigs has also been reported (49). Even if porcine and human APN have more than 80% amino acid homology, TGEV can utilize porcine but not human APN, while HCV-229E can utilize human but not porcine APN (16). Feline infectious peritonitis virus (FIPV) and feline enteric virus (FeCV), which are members of the same antigenic group as HCV-229E and TGEV, use a feline APN (46). Interestingly, the latter study showed that feline APN can also serve as a functional receptor for HCV-229E, TGEV, and canine coronavirus (CCV). Unexpectedly, it has also been shown that CD13 mediates human cytomegalovirus infection (41).

While HCV are recognized respiratory pathogens that cause as much as 30% of common colds in humans (33), their possible neurotropism has been documented previously (22). For example, coronavirus-like particles were found in the brain of a patient with multiple sclerosis (MS) (45), although their size was later shown to be untypical of coronaviruses. Also, two coronaviruses (SD and SK) were isolated from the CNS of two patients with MS (10), although they are probably murine contaminants (50). Nevertheless, the neurotropic murine coronavirus JHM is able to infect the CNS of nonhuman primates after an intracerebral inoculation that can result in acute encephalomyelitis (31). Astrocytes were described as the target cells in white-matter lesions during acute infection (32). Moreover, this virus can propagate from the periphery to the CNS following an intravenous or intranasal inoculation (11). This propagation seems to follow the mode of entry of mouse hep-

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atitis virus (MHV) into the CNS of mice via the olfactory nerve (5, 26). Recent studies have shown that HCV RNA can be detected in the CNS of patients with MS (14, 30, 42) and of control subjects (2). Other studies have reported the susceptibility of continuous human neural cell lines to acute and persistent HCV infection (1, 13, 44). Also, we have recently shown that primary neural cell cultures are susceptible to infection by HCV, which appears to preferentially target astrocytes and microglial cells (7).

Since CD13 serves as a receptor for HCV-229E infection of human lung fibroblasts and since various human neural cells are susceptible to infection by HCV-229E, we wanted to investigate whether CD13 acts as a cellular receptor for infection of these cells. However, reports of the expression of CD13 in the nervous system are limited to synaptic membranes. Therefore, our first objective was to verify the expression of CD13 on astrocytes, neurons, oligodendrocytes, and microglia. Our second objective was then to verify if a monoclonal antibody against CD13 could block HCV-229E virus attachment and infection of human neural cell lines.

## MATERIALS AND METHODS

**Cells and virus.** The human embryonic lung cell line L-132 and the neural cell lines H4 (neuroglioma [brain]), SK-N-SH (neuroblastoma [metastasis to bone marrow]), U-87 MG (astrocytoma), and U-373 MG (glioblastoma) were all originally obtained from the American Type Culture Collection (ATCC; Rockville, Md.). The fetal microglial cell line CHME-5 was generously provided by Marc Tardieu (Université Paris-Sud, Paris, France) (20). The astrocytic cell line GL-15 was from V. Bocchini (University Medical School, Perugia, Italy) (6). The monocytic cell line THP-1 was a gift of Daniel Oth (Institut Armand-Frappier, Laval, Québec, Canada). MO3.13 is an immortalized human-human hybrid cell line expressing many oligodendrocyte markers, including myelin basic protein, proteolipid protein, and galactocerebroside (29). L-132 cells were grown in a humidified chamber at 37°C and 5% (vol/vol) $CO_2$ in a medium consisting of Earle's minimal essential medium (MEM) and Hanks' medium M199 (1:1 [vol/vol]), supplemented with 5% (vol/vol) fetal bovine serum (FBS), 1.7% (vol/vol) sodium bicarbonate, 2 mM L-glutamine, and 50 μg of gentamicin (Gibco Canada, Burlington, Ontario, Canada) per ml, while neural cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% (vol/vol) FBS, 2 mM L-glutamine, and 50 μg of gentamicin per ml, THP-1 cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% (vol/vol) FBS, 2 mM L-glutamine, 50 μg of gentamicin per ml, and $2 \times 10^{-5}$ M 2-mercaptoethanol (Bio-Rad, Mississauga, Ontario, Canada).

Strain 229E of HCV was originally obtained from the ATCC and was propagated as described previously (21). HCV-229E was radiolabeled by replacing the medium in cultures of infected L-132 monolayers at a multiplicity of infection (MOI) of 0.1 with methionine-free MEM (ICN, Costa Mesa, Calif.) supplemented with 1% (vol/vol) dialyzed FBS and [$^{35}$S]methionine-cysteine (Tran$^{35}$S-Label; ICN) at 200 μCi/ml, 5 h after initiation of the infection. Radiolabeled virus was collected at 43 h postinfection. Cell debris were removed by centrifugation at 10,000 × *g* for 20 min. Virus was then concentrated by the addition of 10% (wt/vol) polyethylene glycol 8000 (Sigma, Oakville, Ontario, Canada) supplemented with 0.5 M NaCl, overnight incubation at 4°C, and centrifugation at 10,000 × *g* for 30 min. The virus was subsequently purified by centrifugation at 148,000 × *g* in a Beckman SW41 rotor on a 10 to 50% (wt/vol) Nycodenz discontinuous gradient (Accurate Chemical & Scientific Corporation, Westbury, N.Y.) for 3 h. Fractions containing virus were identified by liquid scintillation counting, pooled, dialyzed against Dulbecco's phosphate-buffered saline (PBS), and stored at −70°C.

**Flow cytometric analysis of cell surface expression of CD13.** L-132, THP-1, and human neural cell lines were assayed for expression of cell surface CD13. The cells were detached from the culture flask with PBS containing 0.4 mM EDTA. The cells were then washed twice with cold PBS, and $10^6$ cells were stained with 10 μl of a phycoerythrin (PE)-conjugated monoclonal antibody (MAb) directed against CD13 (SJ1D1; Immunotech, Burlington, Ontario, Canada) or a PE-conjugated isotypic control MAb (679.1Mc7; Immunotech) or in the absence of MAb for 45 min at 4°C in a dark room. The cells were then washed twice with PBS, suspended in 1 ml of PBS, and analyzed with a Coulter EPICS XL-MCL flow cytometer. Dead cells labeled with 0.1 μg of ethidium bromide 10 min before the analysis were excluded from the analysis.

**Virus-binding assays.** Binding saturation of HCV-229E was verified by incubating increasing amounts of $^{35}$S-labeled HCV-229E ($0.3 \times 10^5$ to $1.3 \times 10^5$ dpm) with 95% confluent monolayers of L-132 cells in 24-well plates for 1 h at room temperature. Afterwards, the cells were washed four times with DMEM supplemented with 1% (vol/vol) FBS to eliminate unattached $^{35}$S-labeled HCV-229E and then solubilized with 200 μl of 1% (wt/vol) sodium dodecyl sulfate (SDS). $^{35}$S-labeled HCV-229E bound to cells was quantitated by liquid scintillation counting in a Packard liquid scintillation analyzer (model 2200CA; Packard, Downers Grove, Ill.).

**Preparation of HCV-229E-infected cell lysates.** A 50% confluent monolayer of L-132 cells was inoculated with HCV-229E at an MOI of 0.1 and incubated at 33°C with 5% (vol/vol) $CO_2$ for 2 h. Culture medium with 1% (vol/vol) FBS was then added, and the incubation was allowed to continue for 42 h. After three cycles of freezing at −70°C and thawing at 37°C, the cells and the culture medium were sonicated on ice twice for 1 min. This solution was then centrifuged at 10,000 × *g* for 30 min. Afterwards, the supernatant was collected and centrifuged at 100,000 × *g* for 1 h. The viral pellet was then resuspended with PBS in 1/50 of the initial volume and sonicated on ice twice for 1 min. This solution was then centrifuged at 1,000 × *g* for 10 min to collect the supernatant containing the HCV-229E antigen.

**ELISA.** The absence of interaction between the anti-CD13 MAb (azide-free WM15; Biodesign International, Kennebunk, Maine) and HCV-229E was determined by an enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated overnight at room temperature with HCV-229E-infected clarified cell lysates, as described above, at 3.125 or 6.25 μg/ml. Afterwards, the plates were washed and blocked with PBS containing 10% (vol/vol) FBS and 0.2% (vol/vol) Tween 20 for 90 min at room temperature. The anti-CD13 MAb WM-15 or a positive control MAb (5-11H.6 specific for the S protein of HCV-229E) was then added at a concentration of 5 μg/ml, and fivefold serial dilutions were made. After a 2-h incubation period at room temperature, the plates were washed five times with PBS containing 0.1% (vol/vol) Tween 20 and the secondary antibody, peroxidase-labeled goat anti-mouse immunoglobulin G (IgG; Kirkegaard & Perry Laboratories), was added at a dilution of 1/2,000. Following a 2-h incubation period at room temperature, the plates were washed five times with PBS containing 0.1% (vol/vol) Tween 20 and the substrate solution (0.05 M citric acid [pH 5.0], 0.1 M sodium phosphate dibasic, 2.2 M *O*-phenylene diamine, 3 mM hydrogen peroxide) was added. The reaction was stopped after 30 min in the dark with 1 N HCl. The plates were read at 492 nm in an SLT Easy Reader (model EAR400AT; SLT-Labinstruments, Salzburg, Austria).

**Inhibition of binding of radiolabeled HCV-229E to cells by CD13-specific MAb.** Monolayers of L-132, CHME-5, H4, SK-N-SH, GL-15, U-87 MG, U-373 MG, and MO3.13 cells were grown in 24-well plates to 95% confluence. The monolayers were then washed and incubated for 1 h at room temperature in the absence or presence of various antibodies or semipurified HCV-229E (positive control for inhibition of attachment). The antibodies used in this experiment were a human CD13-specific mouse MAb, which was purchased in an azide-free form to prevent any cytotoxic effects (WM15), or various control antibodies, including a human leukocyte antigen-specific antiserum (15 μg) obtained from Suzanne Lemieux (Institut Armand-Frappier), a tobacco mosaic virus (TuMV)-specific mouse MAb (6D; 15 μg) obtained from Jean-François Laliberté (Institut Armand-Frappier), or mouse MAb anti-HCV-229E (5-11H.6; 15 μg). Afterwards, 100,000 dpm of semipurified $^{35}$S-labeled HCV-229E was added to the monolayers for 1 h at room temperature. The cells were then washed four times with DMEM supplemented with 1% (vol/vol) FBS and solubilized with 200 μl of 1% (wt/vol) SDS. $^{35}$S-labeled HCV-229E bound to cells was quantitated by liquid scintillation counting in a Packard liquid scintillation analyzer.

The statistical significance of differences in attachment of $^{35}$S-labeled HCV-229E to cells after various treatments was determined by a one-way analysis of variance (ANOVA). When a difference ($P < 0.05$) was observed in the mean values between the treatment groups (groups 2 to 7), the statistically significant difference was further analyzed by an all-pairwise multiple-comparison procedure (Tukey test) to determine which treatments (groups 2 to 7) yielded results that were different from each other (53).

**Inhibition of HCV-229E infection of neural and control cell lines by a CD13-specific MAb.** Monolayers of L-132, H4, SK-N-SH, GL-15, and MO3.13 cells were grown in 12-well plates to 90% confluence. The cell monolayers were then washed with PBS, and culture medium with or without various antibodies was added. The antibodies used were as described above. After a 1-h incubation period at room temperature, cell monolayers were infected with HCV-229E at an MOI of 0.1 for 1 h in a humidified chamber at 33°C and 5% (vol/vol) $CO_2$. Cell monolayers were then washed with PBS, and the corresponding antibodies were added again as described above. Cell monolayers were then detached from the culture flask with PBS–0.4 mM EDTA and suspended in 0.6 ml of appropriate culture medium supplemented with 1% (vol/vol) FBS. Approximately 25 μl of this cell suspension was then added to each well of a 12-well glass slide (Flow; ICN Biomedical Canada Ltd., Mississauga, Ontario, Canada), and infection was allowed to continue for 22 h at 33°C and 5% (vol/vol) $CO_2$. Antibodies remained in the culture media throughout the experiment. The slides were washed once in PBS and fixed in cold acetone at −20°C for 20 min.

To detect HCV-229E viral antigen, we added a 1/2,000 dilution of guinea pig antiserum directed against HCV-229E (National Institute of Allergy and Infectious Diseases, Bethesda, Md.) or normal guinea pig antiserum. After a 45-min incubation period in a humidified chamber at 37°C with 5% (vol/vol) $CO_2$, the slides were washed three times in PBS. This was followed by the addition of a fluorescein-labeled affinity-purified goat antibody to guinea pig IgG (KPL) at a 1/500 dilution and incubation for 45 min in a humidified chamber at 37°C with 5% (vol/vol) $CO_2$. After another round of washes, the slides were mounted with

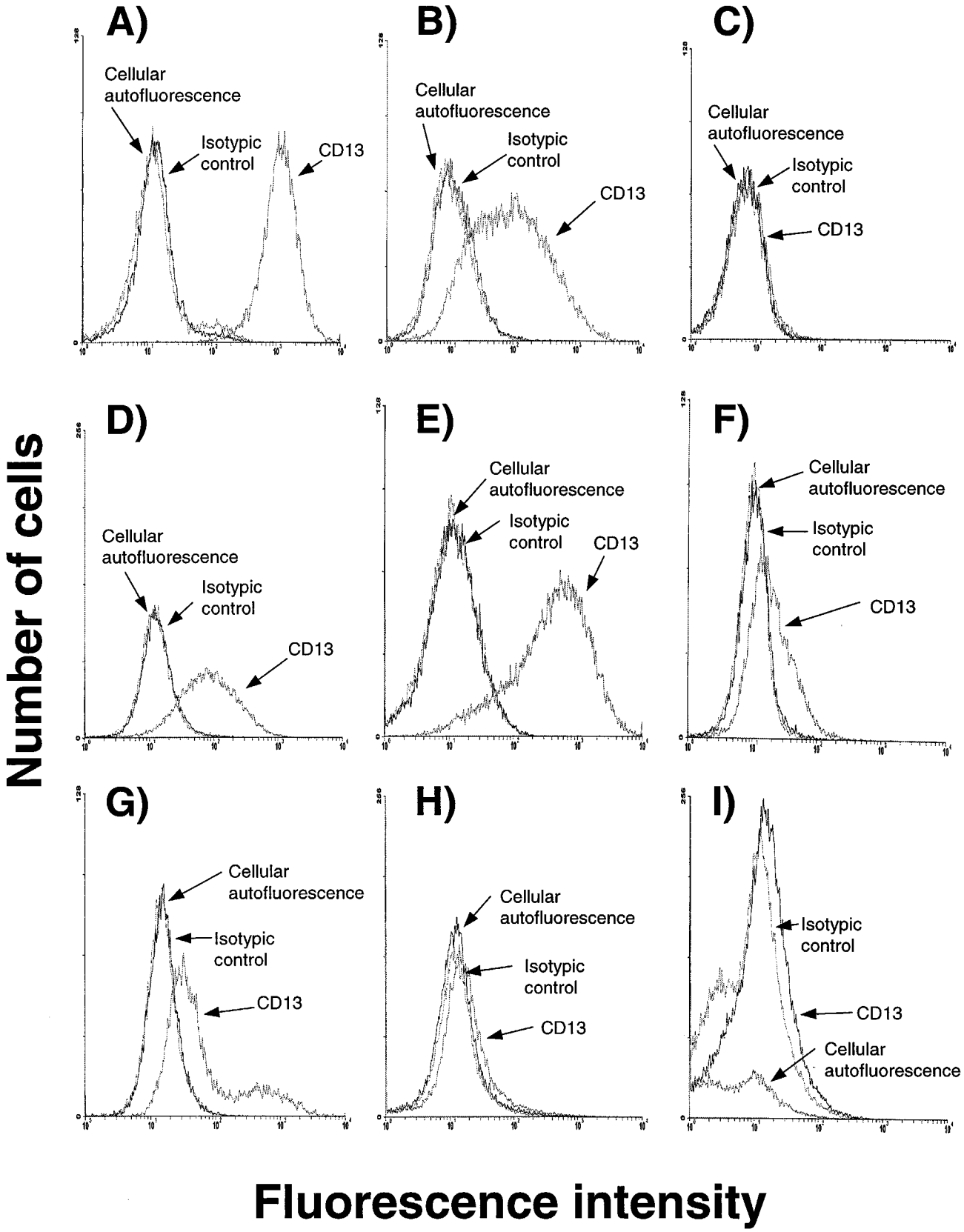



FIG. 1. Flow cytometry analysis of cell surface expression of CD13. Cells were labeled with either an anti-CD13 PE-conjugated antibody or a PE-conjugated isotypic control antibody. (A) THP-1 monocytic cell line (2-log-unit signal displacement); (B) L-132 human lung fibroblast cell line (1-log-unit signal displacement); (C) CHME-5 microglial cell line (no signal displacement); (D) H4 neuronal cell line (1-log-unit signal displacement); (E) SK-N-SH neuronal cell line (2-log-unit signal displacement); (F) MO3.13 oligodendrocytic cell line (0.2-log-unit signal displacement); (G) GL-15 astrocytic cell line (0.5-log-unit signal displacement); (H) U-87 MG astrocytic cell line (0.1-log-unit signal displacement); (I) U-373 MG astrocytic cell line (0.1-log-unit signal displacement). These profiles are representative of at least two separate experiments.

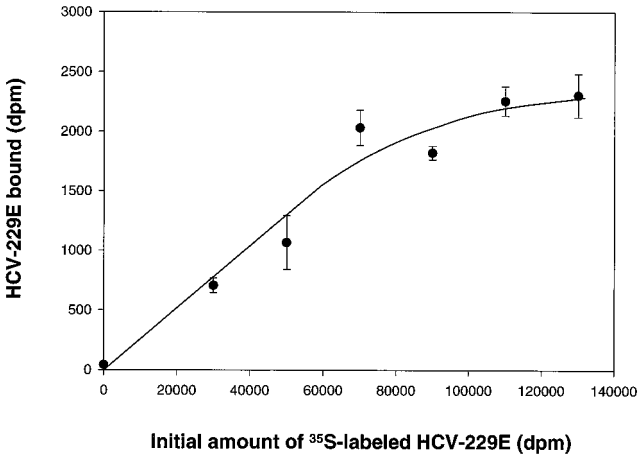



FIG. 2. Specific attachment of $^{35}$S-labeled HCV-229E onto L-132 cells. Binding of HCV-229E to L-132 cells was carried out by incubation of increasing amounts of $^{35}$S-labeled HCV-229E, in triplicate, on monolayers of L-132 cells for 1 h at room temperature. Cell monolayers were washed four times with DMEM supplemented with 1% (vol/vol) FBS to eliminate unattached virus and were solubilized with 1% (wt/vol) SDS. Cell-bound $^{35}$S-labeled HCV-229E was quantitated by liquid scintillation counting. This profile is representative of two separate experiments.

glass coverslips by using glycerol-PBS (9:1) and were observed with a Leitz fluorescence microscope (Dialux 20 model).

## RESULTS

**Expression of CD13 on human neural cell lines.** Expression of the cell surface protein CD13 on human neural cell lines was assessed by flow cytometry with human CD13-specific MAb SJ1D1. The THP-1 cell line, which is known to express CD13 on its plasma membrane, was used as a positive control. It was indeed positive for expression of CD13, with an approximately 2-log-unit signal displacement with MAb SJ1D1, indicating that our test was functional (Fig. 1). Expression of CD13 was also detected on L-132 cells, our reference lung cell line used for propagation of HCV-229E. The neuronal cell lines H4 and SK-N-SH were also positive for CD13 expression. For astrocytic cell lines, CD13 was found on the cell surface of GL-15 cells and at low levels on U-87 MG and U-373 MG cells. A low level of expression of CD13 was also found on the oligodendrocytic cell line MO3.13. However, we could not detect CD13 on the immortalized fetal microglial cell line CHME-5, as indicated by the absence of signal displacement with the anti-CD13 MAb SJ1D1. Less than 10% of the total cell population of ethidium bromide-labeled cells were found to be dead and, consequently, were excluded from the analysis (data not shown).

**Role of CD13 in HCV-229E binding to human neural cell lines.** Inhibition of attachment of $^{35}$S-labeled HCV-229E on human neural cell lines with the use of a human CD13-specific MAb was tested to determine the direct implication of CD13 in binding of HCV-229E to human neural cell lines. When monolayers of L-132 cells were exposed to increasing amounts of $^{35}$S-labeled HCV-229E, a monophasic, saturable virus binding curve was observed and the optimal amount of semipurified $^{35}$S-labeled HCV-229E to be used in the inhibition of attachment assay was found to be 100,000 dpm (Fig. 2). At this concentration, the CD13-expressing cells showed an increase of approximately threefold in binding of radiolabeled virus compared with the attachment seen when 30,000 dpm of radiolabeled virus was used.

The anti-CD13 MAb WM15 was also tested for its ability to interact with HCV-229E, which would have interfered with our attachment inhibition assay. No signal was observed when the anti-CD13 MAb WM15 was incubated onto HCV-229E antigen, unlike the positive-control anti-HCV-229E MAb 5-11H.6 (data not shown).

We were then able to test whether the use of the CD13-specific MAb WM15 could inhibit attachment of $^{35}$S-labeled HCV-229E onto human neural cell lines of various phenotypes. As shown in Fig. 3, the use of WM15 substantially inhibited the attachment of $^{35}$S-labeled HCV-229E onto cell lines of neuronal (H4 and SK-N-SH), astrocytic (GL-15, U-87 MG, and U-373 MG), and oligodendrocytic (MO3.13) phenotypes ($P < 0.05$; one-way ANOVA followed by a Tukey test). Similar results were observed with the use of excess unlabeled HCV-229E as a positive control. No significant inhibition of attachment was observed when control antibodies were used. Only the microglial cell line CHME-5 showed no inhibition with the use of MAb WM15. In this particular case, the observed attachment of $^{35}$S-labeled HCV-229E was equal to background noise when only $^{35}$S-labeled HCV-229E was added to a well containing only culture medium and no cells (data not shown).

The levels of attachment of $^{35}$S-labeled HCV-229E between the various cell lines were found to be variable. As shown in Fig. 4, we observed a correlation between the levels of attachment of $^{35}$S-labeled HCV-229E (Fig. 3) and the level of CD13 expression determined semiquantitatively by flow cytometry (Fig. 1).

**Role of CD13 in HCV-229E infection of human neural cell lines.** To further investigate the role of human CD13 in the susceptibility of human neural cell lines to HCV-229E infection, cell lines representative of the neuronal, astrocytic, and oligodendrocytic phenotypes were pretreated with the anti-CD13 MAb WM15 and were inoculated with HCV-229E. The presence of WM15 was able to drastically diminish the infection of L-132 cells, our reference cell line for growth of HCV-229E (Fig. 5A and B). Similar results were obtained for neuronal SK-N-SH cells (Fig. 5C and D), GL-15 astrocytes (Fig. 5E and F), and MO3.13 oligodendrocytes (Fig. 5G and H). The same neural cell lines pretreated with control antibodies showed approximately the same level of infection as the cells that had not been treated without antibodies and infected by HCV-229E (data not shown).

## DISCUSSION

HCV are recognized respiratory pathogens that can cause as much as 30% of common colds (33). However, some studies suggest their possible neurotropism. Human neural cell lines and primary cultures of human brain cells are susceptible to HCV-229E infection (1, 7, 44). Yeager et al. (51) have shown that hAPN (CD13) acts as a cellular receptor for infection of human lung fibroblasts. However, CD13 has been found on synaptic membranes, but not on glial cells of the human brain (27). Since virus tropism results in part from the nature, the number, and the distribution of cellular receptors, we asked whether CD13, which is present at the surfaces of many cells (2, 27, 35, 37), was indeed present at the surfaces of human neural cell lines and, consequently, whether strain 229E of HCV uses this protein for attachment and infection.

By flow cytometry, we were able to show expression of CD13 on cell lines of neuronal, astrocytic, and oligodendrocytic phenotypes but not of the microglial phenotype. Compared to the neuronal cell lines, the GL-15 astrocytic cell line showed an intermediate level of expression of CD13, which was nonetheless higher than that observed with the other two astrocytic cell

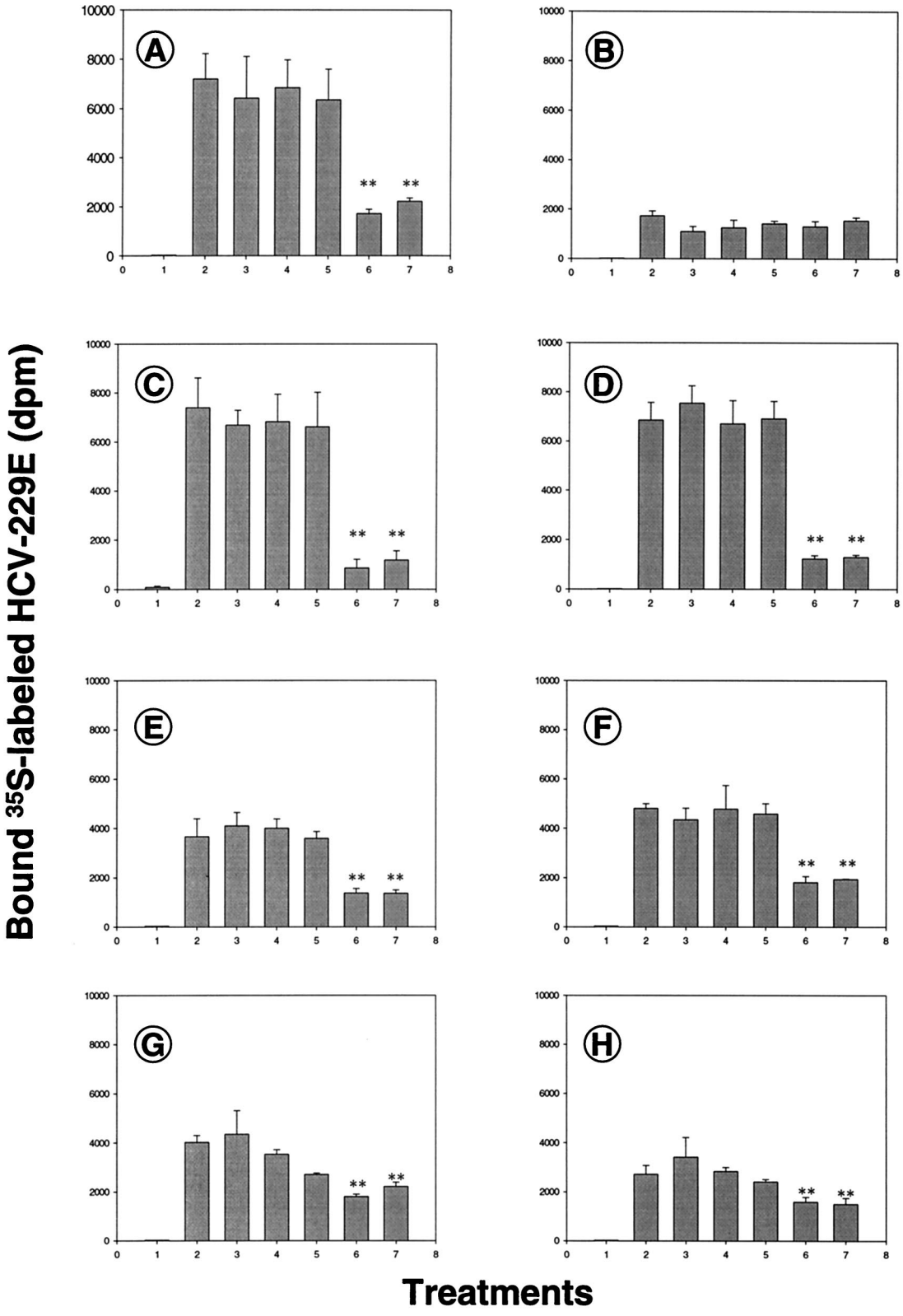



FIG. 3. Detection of binding of $^{35}$S-labeled HCV-229E on cultures of human neural cell lines pretreated with an anti-CD13 MAb or with various controls, all in triplicate. (A) L-132 human lung fibroblast cell line; (B) CHME-5 microglial cell line; (C) H4 neuronal cell line; (D) SK-N-SH neuronal cell line; (E) MO3.13 oligodendrocytic cell line; (F) GL-15 astrocytic cell line; (G) U-373 MG astrocytic cell line; (H) U-87 MG astrocytic cell line. Treatment 1, culture medium; treatment 2, $10^5$ dpm of HCV-229E; treatment 3, $10^5$ dpm of HCV-229E plus HLA-specific antiserum (15 μg); treatment 4, $10^5$ dpm of HCV-229E plus anti-TuMV MAb 6D (15 μg); treatment 5, $10^5$ dpm of HCV-229E plus anti-HCV-229E MAb 5-11H.6 (15 μg); treatment 6, $10^5$ dpm of HCV-229E plus anti-CD13 MAb WM15 (15 μg); treatment 7, $10^5$ dpm of HCV-229E plus unlabeled HCV-229E. These profiles are representative of two separate experiments. **, statistically significant difference according to Tukey's multiple comparison test, following an ANOVA test ($P < 0.05$).

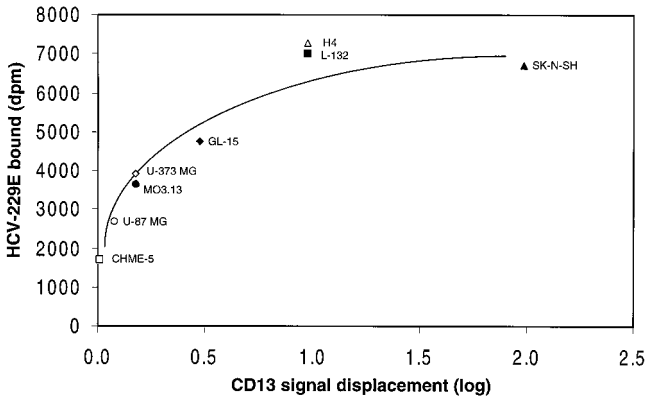



FIG. 4. Correlation between expression levels of CD13 on human neural cell lines and attachment of $^{35}$S-labeled HCV-229E. ■, L-132 human lung fibroblastic cell line; △, H4 neuronal cell line; ▲, SK-N-SH neuronal cell line; ◆, GL-15 astrocytic cell line; ○, U-87 MG astrocytic cell line; ◇, U-373 MG astrocytic cell line; ●, MO3.13 oligodendrocytic cell line; □, CHME-5 microglial cell line.

lines, U-87 MG and U-373 MG. This might explain our previous observation that the latter two astrocytic cell lines require a higher MOI for infection compared to the GL-15 cell line. Pig astrocytes were previously shown to express APN (4), but our results give the first report of APN expression on human astrocytes and oligodendrocytes. The absence of CD13 on the microglial cell line CHME-5 is surprising, because this protein is known to be present on macrophages (27). This result might be a consequence of the transformation of this cell line following its transfection with the simian virus 40 large T antigen (20). Nonetheless, this absence is consistent with the implication of CD13 as a cellular receptor on human neural cell lines since the CHME-5 cell line is not susceptible to HCV-229E infection (data not shown). Unfortunately, no other human microglial cell lines are to our knowledge available at this time, and further studies of HCV-229E infection of microglial cells will require either CD13 transfection into CHME-5 or the production of other immortalized cell lines.

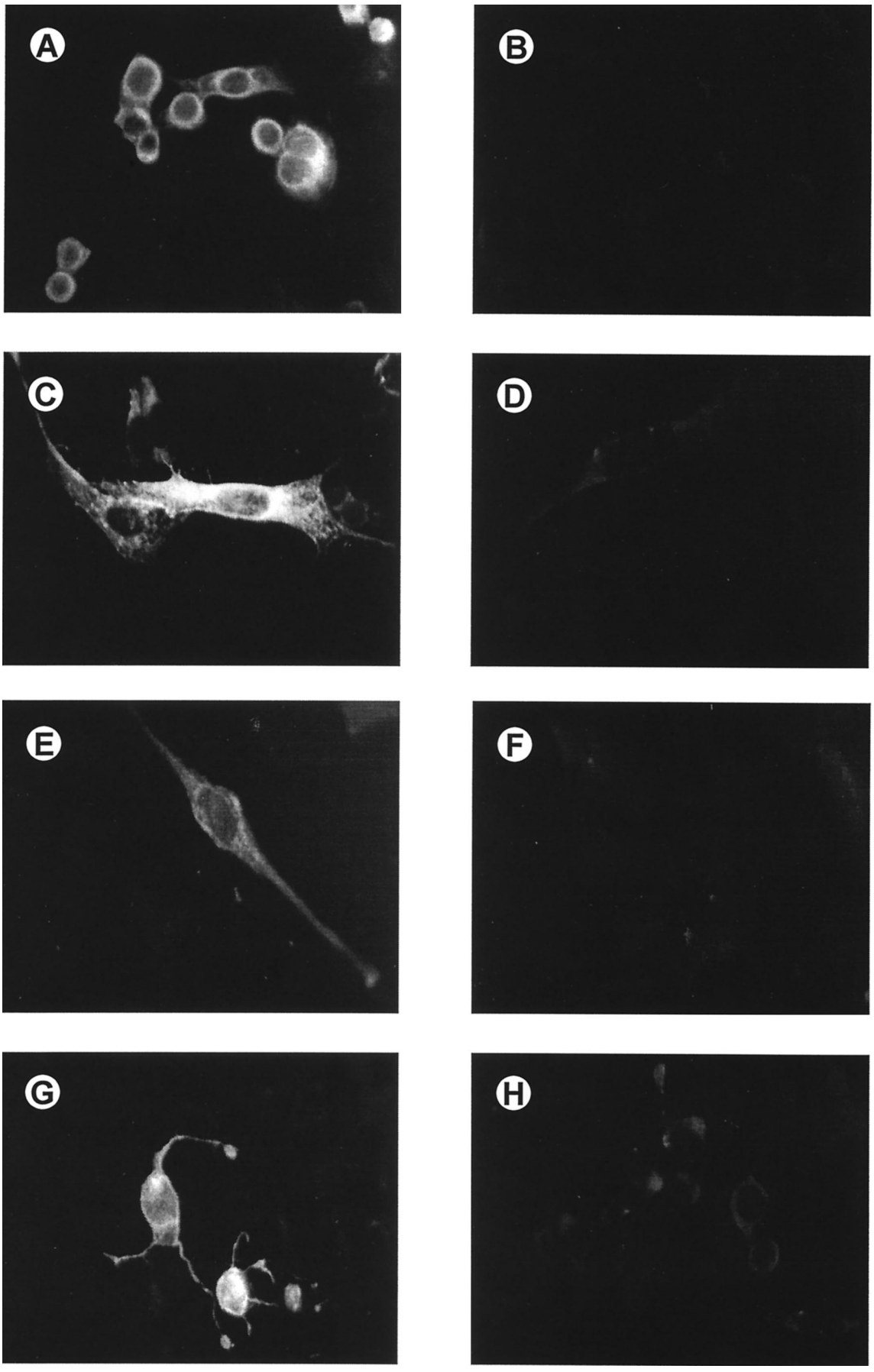
Having shown that CD13 is indeed expressed on human neural cells, the next step of this study was to verify whether this protein acts as a cellular receptor for HCV-229E infection of human neural cell lines. APN has been found to be a cellular receptor for HCV-229E infection of human lung fibroblast cell lines (51), but there had previously been no direct proof of the use of CD13 by HCV-229E for infection of human neural cells. Presumably, this virus could have used an alternative receptor, as has been shown for MHV (52). To evaluate the role of CD13 in infection of human neural cell lines by HCV-229E, we used a CD13-specific MAb to inhibit attachment of HCV-229E onto human neural cell lines and to inhibit infection of these cell lines by HCV-229E. The anti-CD13 MAb WM15 was able to substantially diminish attachment of HCV-229E to all human neural cell lines tested, except for the CHME-5 microglial cell line. The fact that WM15 did not recognize HCV-229E indicated that the inhibition seen was not a consequence of the association of this antibody with the virus that would block its attachment to the cells. Also, the inhibition did not appear to be caused by nonspecific attachment of antibodies to HCV-229E, since nonneutralizing anti-S antibodies against HCV-229E failed to inhibit HCV-229E attachment. Therefore, it seems that the presence of CD13 at the cell surface is necessary for the specific binding of HCV-229E to human neural cell lines of neuronal, astrocytic, and oligodendrocytic phenotypes (and presumably microglial cells also). These experiments also showed that virus binding was variable, depending on the cell lines used (H4 > SK-N-SH > MO3.13 > GL-15 > U-373 MG > U-87 MG). The correlation that was observed between the levels of expression of CD13 and HCV-229E binding to human neural cell lines seems to indicate that a higher-level expression of CD13 is accompanied by the binding of more viruses to these cells. This could explain our observation that a higher MOI is required for infection of some neural cell lines. However, an overexpression of CD13 does not necessarily translate into a more-efficient infection; overexpression of porcine APN at the cell surface results in a lower level of TGEV replication (17), a reduction that could be caused by the binding of porcine APN to the viral S glycoprotein, which apparently hindered virus maturation.

We also evaluated the role of CD13 in the initiation of a productive HCV-229E infection of human neural cell lines. Neuronal, astrocytic, and oligodendrocytic cells pretreated with the anti-CD13 MAb WM15 showed a marked decrease in the extent of HCV-229E infection. Like the inhibition of attachment experiment, control antibodies did not decrease the level of infection compared to cells untreated with MAb.

Overall, these inhibition of attachment and infection experiments suggest that, like human lung fibroblastic cell lines, CD13 acts as the cellular receptor for HCV-229E infection of human neural cell lines. It remains to be determined how and if HCV-229E could gain access to the brain to infect human neural cells. One possibility might be via the pericytes at the blood-brain barrier. The presence of CD13 on these cells (25) might provide an entry pathway for HCV-229E to the CNS. Indeed, primary cultures of human endothelial cells are susceptible to HCV-229E infection (12). This infection might be the initial step for the propagation of HCV-229E to the CNS via infection of astrocytes, which are cells that are susceptible to HCV-229E infection (7, 44).

Other coronaviruses, such as TGEV, FeCV, and FIPV, and human cytomegalovirus use APN for infection (15, 41, 46). The high levels of expression of this protein on the surfaces of epithelial cells of the respiratory and digestive tract could presumably confer on this protein a receptor function for several viruses. Therefore, it is possible that other viruses use APN as a cellular receptor, but that does not necessarily mean that the same region of APN might be recognized by different viruses. The anti-CD13 MAb WM15 used in this study was previously shown to block the enzymatic activity of CD13 (51), and we now show that it prevents HCV-229E infection of human neural cell lines. Also, molecules chelating the zinc ions required for CD13 enzymatic activity were shown to inhibit HCV-229E infection, but other molecules, such as bestanin and actinonin, which bind to the active site of CD13, were incapable of pre-

FIG. 5. Detection of viral antigens by indirect immunofluorescence in cultures of human neural cells pretreated with an anti-CD13 MAb and inoculated with HCV-229E. (A and B) L-132 human lung fibroblastic cell line; (C and D) SK-N-SH neuronal cell line; (E and F) GL-15 astrocytic cell line; (G and H) MO3.13 oligodendrocytic cell line. Cells were pretreated with 15 μg of an anti-CD13 MAb (B, D, F, and H) or with 15 μg of an isotypic control antibody (A, C, E, and G) and were inoculated with HCV-229E at an MOI of 0.1. After 22 h, the cells were fixed in cold acetone and examined by indirect immunofluorescence. The primary antibody used to detect viral antigens was a guinea pig antiserum against HCV-229E. This was followed by the addition of a fluorescein-conjugated goat affinity-purified anti-guinea pig secondary antibody Magnification, ×100. The results represent several fields, and observations at lower magnification yielded the following percentages of infected cells and qualitative levels of fluorescence (scale, 0 to 3+): 30% and 3+ (A and C), 10% and 1.5+ (E), 10 to 15% and 3+ (G), <5% and 1+ (B), <5% and 0.5+ (D and F), and negative (H).


A
B
C
D
E
F
G
H

venting infection by this virus, unlike human cytomegalovirus (41, 47, 51). Therefore, it seems that HCV-229E recognizes an epitope near the catalytic site of CD13. A recent report showed that a small region within the amino-terminal part of the feline APN is essential for HCV-229E infection (24).

Expression of CD13 on neurons, astrocytes, and oligodendrocytes would explain their susceptibility to HCV-229E infection. In addition, CD13 is a protein expressed on the surfaces of many cell types. This suggests that these cell types might be susceptible to HCV-229E infection. However, only human lung fibroblasts and CNS cells have so far been shown to be susceptible to HCV-229E infection (7, 44, 51). A complex relationship could exist between viral tropism and expression of CD13. For example, the poliovirus receptor is present on many cell types, even those not susceptible to poliovirus infection, suggesting that the presence of a coreceptor may be necessary for infection of permissive cells (40). Indeed, recent studies suggest that other postattachment factors might be necessary for poliovirus replication since CD44, a potential cellular receptor for this virus, is not required for poliovirus infection (8). The presence of a coreceptor for HCV-229E has not been described and may not be required, since we showed an apparently monophasic attachment of radiolabeled HCV-229E onto L-132 cells. However, such a putative coreceptor might be necessary only for virus penetration into the cell, something that cannot be detected by the above experiment. Similarly, even though transfection of CD13 into murine cells was sufficient to render these cells susceptible to HCV-229E infection (51), the possibility that these cells already expressed the putative coreceptor at their cell surface cannot be excluded. Nevertheless, our results do indicate that CD13 is essential for infection of the neural cell lines tested.

The results of our study are consistent with the possibility that CD13 plays an important role in the replication cycle of HCV-229E in the CNS, and its expression and use are consistent with the suspected neurotropism of this virus. Obviously, a better understanding of the role of this molecule will surely help the development of treatments to prevent human infections. This study relied on continuous human neural cell lines that may lose or gain functions during immortalization, although previous studies have shown a nice correlation between in vitro and in vivo coronavirus replication data (for example, see reference 34). Nevertheless, it will be interesting to verify the expression of CD13 on primary cultures of human neural cells and on human brain sections. The present study provides a strong rationale for such experiments aimed at the characterization of the neurotropism of human coronaviruses and their possible involvement in neurological diseases.

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