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Infection of Primary Cultures of Human Neural Cells by Human Coronaviruses 229E and OC43

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We evaluated the ability of human coronaviruses to infect primary cultures of human neural cells. Double immunofluorescence with antibodies to virus and cell markers showed infection of fetal astrocytes and of adult microglia and astrocytes by strain OC43. RNA amplification revealed infection of fetal astrocytes, adult microglia, and a mixed culture of adult oligodendrocytes and astrocytes by strain 229E. Infectious virus was released only from fetal astrocytes, with higher titers for OC43. Human coronaviruses have the capacity to infect some cells of the central nervous system, although infection of adult cells appears abortive.

Human coronaviruses (HCVs) cause up to one-third of common colds (14) and have been occasionally associated with multiple sclerosis (MS), an inflammatory demyelinating disease of the central nervous system (CNS), together with several other viruses (21). For example, coronaviruses SD and SK were isolated from brain samples of MS patients (1). Even though these isolates are likely murine contaminants (25), in vivo experiments in nonhuman primates have shown that the SD isolate can, after intracerebral inoculation, cause a subacute panencephalitis and that demyelination can be observed in brain sections from autopsy samples (13). Moreover, a neurotropic murine coronavirus was shown to enter the CNS of primates after peripheral inoculation (3). Other studies have reported the detection of HCV RNA in the CNS of MS patients (12, 20) and of coronavirus-myelin cross-reactive T cells in MS patients but not control subjects (24). Serological data showed a significant difference between MS patients and control subjects in titers of anticoronavirus antibodies in the cerebrospinal fluid (18). Although the neurotropism of coronaviruses in humans has not yet been proven (23), various continuous human neural cell lines have been shown to be susceptible to HCV infection (4, 22), and a preliminary study with HCV OC43 in mixed primary neural cultures suggested that human fetal astrocytes were possibly susceptible to infection (17).

In the present study, we used primary cultures of human brain tissue enriched for various neural cell types, an experimental system that was the closest possible to the in vivo situation, to determine the susceptibility of human neural cells to HCV infection. We report for the first time that some human neural cells are indeed infectable by both known strains of HCVs.

Preparation and viral infection of primary cultures. Dissociated human cultures of different neural cell types were obtained by previously described protocols for adult (27) or fetal (28) brains. Cell types and their purity were as follows: adult microglia, over 90% enriched; adult oligodendrocytes, between 80 and 95% enriched; adult astrocytes, low purity, 70% enriched at best; fetal astrocytes, over 95% purity; and fetal neurons, over 90% enriched. Typical morphologies of the various cell types are shown in Fig. 1. Both HCV strains, 229E and OC43, were initially obtained from the American Type Culture Collection (Rockville, Md.) and grown as described previously (7, 10). Cells were inoculated in either 60- by 15-mm or 100- by 15-mm petri dishes or in 16-well Lab-Tek dishes. Petri dishes were used when cells were seeded on microdiscs. Viral infections were performed for 2 to 7 days in a humidified chamber at 37°C and 5% (vol/vol) CO_2, using a multiplicity of infection (MOI) of 4 in petri dishes or 8 in Lab-Tek dishes.

Detection of HCV OC43 antigens by immunofluorescence. Preliminary experiments were performed in which only viral antigens were detected by single indirect immunofluorescence. This was followed by double indirect immunofluorescence with antibodies to viral antigens and cell-specific markers. To detect HCV 229E antigens, we used either a mouse immunoglobulin G1 (IgG1) monoclonal antibody (MAb) (5-11H.6) directed against the viral surface protein (1/10 dilution; 0.04 mg/ml) or a rabbit polyclonal antiviral antiserum (1/100 dilution). To detect HCV OC43 antigens, we used either a mouse IgG1 MAb (4-E11.3) directed against the nucleocapsid protein of the serologically related hemagglutinating encephalomyelitis virus of pigs (a gift from Serge Dea, Institut Armand-Frappier) (1/100 dilution; 0.014 mg/ml) or polyclonal antiviral antiserum produced in the guinea pig (1/10,000 dilution) or rabbit (1/100 dilution). A rabbit anti-cow glial fibrillary acidic protein (GFAP) antibody (DAKO, Carpinteria, Calif.; 1/100 dilution) identified astrocytes. To detect oligodendrocytes, we used a 1/100 dilution of either a hybridoma supernatant of an IgG3 mouse MAb against galactocerebroside (H8H9 or O1) or a rabbit polyclonal antibody against cyclic nucleotide phosphodiesterase, graciously provided by Peter Braun, McGill University, Montreal, Quebec, Canada. To identify microglia, we used 1/100 dilutions of either a mouse IgG1 MAb against CD68, designated KP1 (DAKO), a mouse IgG2b MAb specific for the alpha subunit of CD11c (anti-Leu-M5; Becton Dickinson, Mountain View, Calif.), or a mouse IgG1 MAb against lymphocyte function antigen that recognized CD11c (Boehringer Mannheim, Laval, Quebec, Canada). A rabbit anti-bovine neuron specific enolase antibody (DAKO; 1/100 dilution) identified human fetal neurons, which were also labeled with a mixture of MAbs to neurofilament proteins of 150 and 200 kDa.

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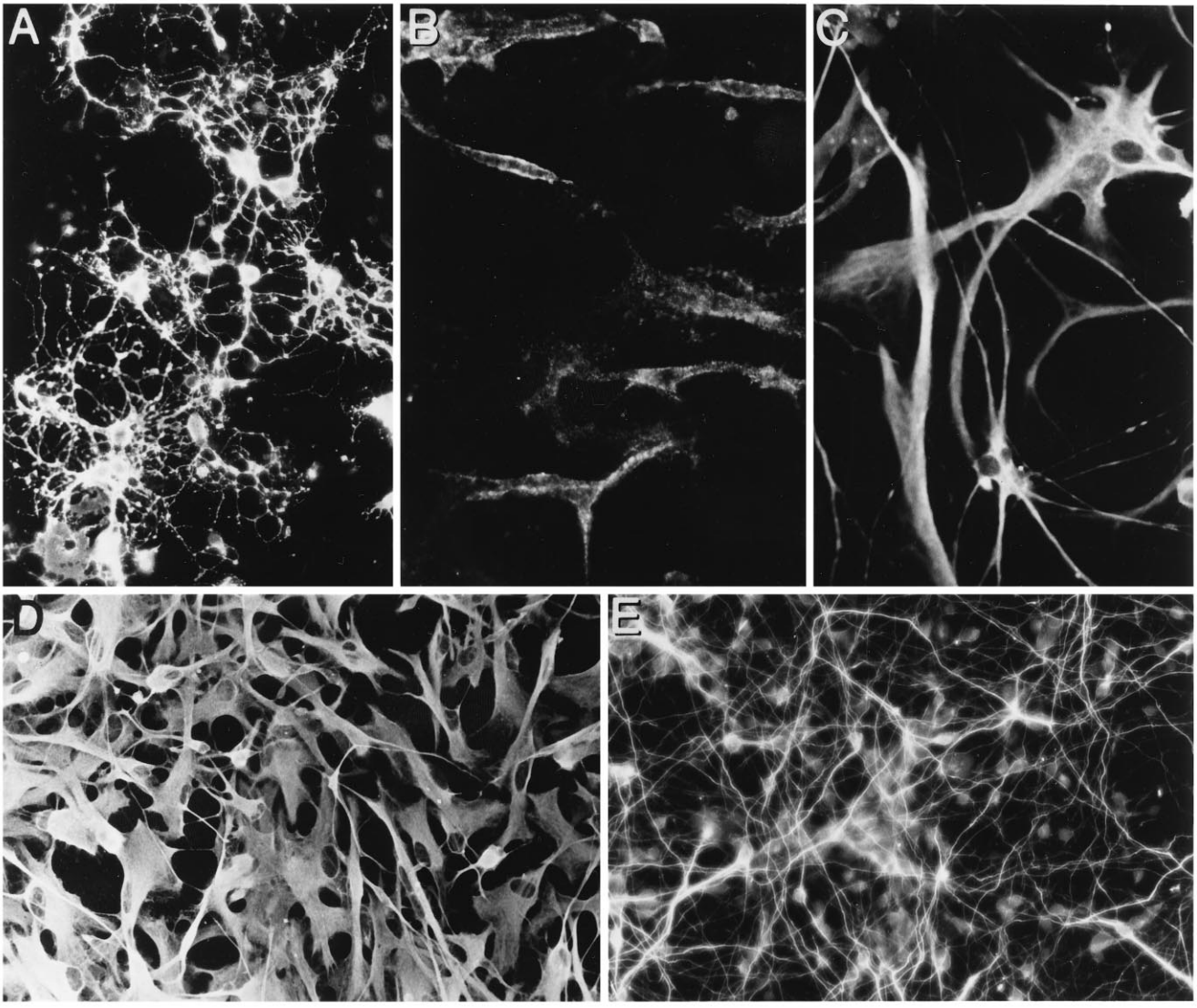

FIG. 1. Immunofluorescence labeling of cultures to identify various cell types. Cells in panels A to C are from adult human brain biopsy specimens, and cells in panels D and E are from human fetal brain tissues. (A) Oligodendrocytes (galactocerebroside staining with the O1 MAb); (B) microglial cells (CD11c staining with anti-Leu-M5 MAb); (C) adult astrocytes (GFAP staining); (D) fetal astrocytes (GFAP staining); (E) neurons (staining with a mixture of MAbs to neurofilament proteins of 150 and 200 kDa). Magnifications, ×450 (panel A), ×600 (panels B and C), and ×300 (panels D and E).

Fluorochrome-conjugated species-specific goat antibodies (Organon Teknika Corporation, Durham, N.C.; 1/50 to 1/200 dilutions) were employed as secondary reagents, with fluorescein being used for viral antigens and rhodamine being used for cell markers. Primary antibodies used in these assays originated from different animal species in order to avoid cross-recognition of both primary antibodies by the fluorochrome-conjugated secondary antibody. We also tested all the fluorochrome-conjugated antibodies for absence of cross-species reactivities. Since nonspecific fluorescence due to the attachment of the Fc portions of MAbs to proteins present at the cell surface was a potential problem (16), we eliminated the Fc portion by producing $F(ab')_2$ fragments (8). Also, to ensure that the immunofluorescence signals were specific to viral antigens, we used a rigorous set of controls for each experiment. The first was a negative control in which a double indirect immunofluorescence assay was performed on noninfected cells to verify any possible non-virus-specific fluorescence. The second control involved not including the antiviral antibody so as to detect a possible overlapping of the wavelengths of the two fluorochromes. A third control consisted of verifying the lack of nonspecific binding of antibodies to cells by replacing the antiviral antibody with a monoclonal isotypic control MAb (Myc 1-9E10.2, directed against human c-*myc*; American Type Culture Collection [5]) or with a preimmune serum. Finally, since the antiviral MAb would recognize only one epitope of a single viral protein and the synthesis of that protein may be modulated, we included in all tests an antivirus polyclonal antibody to broaden the spectrum of viral detection.

Cells were fixed in 70% (vol/vol) ethanol for 15 min (all incubations were at room temperature). Primary antibody was added, and the cells were further incubated for 45 min. This was followed by three washes in phosphate-buffered saline (PBS), pH 7.4, and by the addition of the fluorescein-conjugated secondary antibody, with which the cells were incubated for 45 min. After three washes in PBS, antibody to the appro-

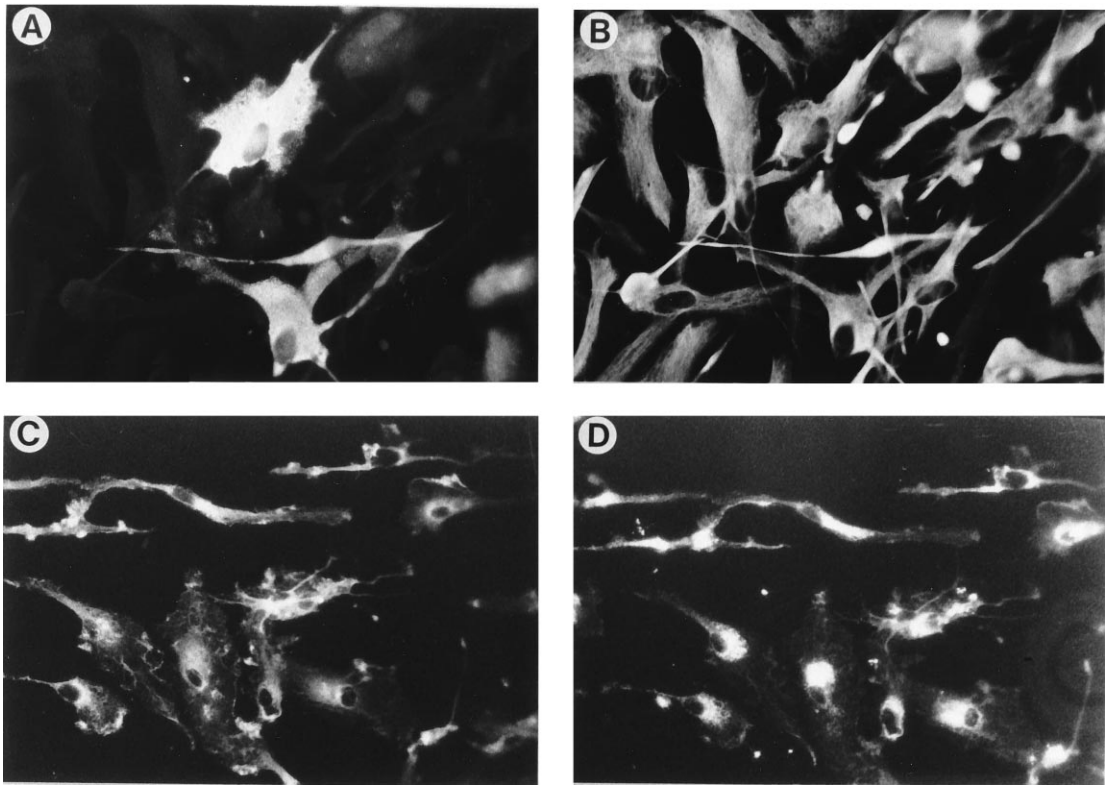

FIG. 2. Detection of viral antigens by indirect immunofluorescence in primary cultures of human neural cells infected with HCV OC43. The following primary cultures were used: fetal astrocytes (shown at 2 days postinfection) (A and B) and adult microglia (shown at 4 days postinfection) (C and D). Cells were inoculated with virus at an MOI of 8 (A and B) or 4 (C and D). After the indicated times, the cells were examined by two-color double indirect immunofluorescence. Primary antibodies to viral antigens and to cell markers were as follows: a mouse monoclonal antiviral antibody and a rabbit anti-GFAP (astrocyte marker) were used for panels A and B, respectively, and a guinea pig polyclonal antiviral serum and a mouse KP1 MAb (antimicroglial marker) were used for panels C and D, respectively. This was followed by fluorescein-conjugated goat anti-mouse or anti-guinea pig antibody and a rhodamine-conjugated goat anti-rabbit or anti-mouse antibody as secondary antibodies. (A and C) Detection of viral antigens; (B) detection of the GFAP astrocyte marker; (D) detection of the KP1 microglial marker. Magnifications, ×540 (panels A and B) and ×720 (panels C and D).

priate cell marker was added, and the cells were incubated for 45 min. After another round of washes in PBS, the species-specific rhodamine-conjugated antibody was added, and the cells were incubated for 45 min. After three more washes in PBS, nuclear yellow (Hoescht dye; DAKO) was added and the cells were incubated for 20 min. After three more washes in PBS and one in double-distilled and deionized water, the slides (or discs) were mounted with Gelvatol and stored at −20°C until they could be observed with a Reichert fluorescence microscope. Gelvatol was prepared by mixing 4 g of glycerol and 2 g of polyvinyl alcohol (Sigma, St. Louis, Mo.), leaving the solution at room temperature for 15 to 20 min, adding 14 ml of 0.1 M Tris-HCl (pH 8.0 to 8.3), stirring for 10 min, and incubating at 37°C for 20 to 24 h before clarification by low-speed centrifugation. When the cell marker antibody required live cells for staining (H8H9 or O1 antibodies to oligodendrocytes), the fixation step was omitted and the antimarker antibody incubation was performed first. After incubation with the appropriate fluorochrome-conjugated antibody, the cells were fixed and a single immunofluorescence detection of viral antigens was performed. In all cases, the percentage of infected cells was obtained by comparing the number of fluorescent cells and the total number of cells present in several fields.

Using double indirect immunofluorescence, viral proteins were reproducibly shown to be expressed in fetal astrocytes (Fig. 2A and B), adult microglia (Fig. 2C and D), and adult astrocytes (data not shown). Also, we observed an increase in the level of infection with time. At 2 days postinfection, about 10% of the fetal astrocytes were infected. This proportion increased to approximately 50% at 4 days and to between 70 and 100% at 7 days postinfection. For adult microglia, about 10% of the culture was infected at 2 days postinfection; at least 40% of the cells had viral antigens at 4 days, and 80 to 100% were positive at 7 days. Fetal neurons were negative for immunofluorescence (data not shown), as were adult oligodendrocytes; the only infected cells in the mixed cultures of adult astrocytes and oligodendrocytes were identified as astrocytes with the GFAP marker (data not shown). Cytopathic effects were not detected after viral infection of any of the primary cultures, and the virus-specific fluorescence observed was strictly cytoplasmic, which is typical of a coronavirus infection.

Visualization of progeny HCV OC43 virions by electron microscopy. Infected fetal astrocytes were fixed with 2.5% (vol/vol) glutaraldehyde (Mecalab, Montreal, Quebec, Canada) in 0.1 M sodium cacodylate buffer (pH 7.2; Mecalab) for 15 min. The solution was centrifuged at 12,000 × *g* for 3 min, and the pellet was washed three times with 3% (wt/vol) sucrose in sodium cacodylate buffer. After a 24-h incubation, the sample was fixed with 1.33% (wt/vol) osmium tetraoxide (SPI-Chem, West Chester, Pa.) in collidine buffer (Mecalab) for 2 h at 21°C. The sample was then dehydrated by 30-min incubations with 25, 50, 75, and 95% (vol/vol) acetone, followed by two incubations with 100% (vol/vol) acetone. The dehydrated sample was mixed 1:1 with Spurr resin (Mecalab) and incubated

for 24 h. The resin block was cut into 90- to 100-nm-thick sections with a microtome, and the samples were stained with 5% (wt/vol) uranyl acetate in 50% (vol/vol) alcohol and lead citrate (pH 12) (Mecalab) and observed with a Hitachi H-7100 electron microscope. Thin sections of infected fetal astrocytes showed progeny virions within intracellular vacuoles (Fig. 3), which confirmed viral replication in these cells.

Detection of HCV 229E RNA by RT-PCR and Southern blotting. Despite several attempts, proteins of HCV 229E could not be detected by immunofluorescence. Furthermore, electron microscopic examination of thin sections of fetal astrocytes did not reveal the presence of intracellular viral particles (data not shown), and cytopathic effects were not observed. These negative results were surprising since we had previously demonstrated infection of continuous neural cell lines by this virus (22). However, since we had previously reported the presence of HCV 229E RNA in brain samples from MS patients (20), we hypothesized that primary human neural cells may be infected at a low level not detectable by immunofluorescence. Therefore, we used a combination of reverse transcription-PCR (RT-PCR) amplification of bases 982 to 1265 of the nucleocapsid gene of HCV 229E and Southern blotting with an internal oligonucleotide probe to detect viral RNA within the cells, using a previously published protocol (20) in which the RNA extraction procedure was simplified as follows. Cells were gently scraped off with the aid of a pipette after three washes in PBS and treatment with 0.25% (wt/vol) trypsin (Gibco) at 37°C for 5 min, spun down at 5,900 × *g* for 10 min, and resuspended in 200 μl of 0.1% (vol/vol) diethyl pyrocarbonate (Sigma). The solution was boiled for 5 min, the cellular debris was eliminated by centrifugation for 2 min at 12,000 × *g*, and the supernatant was stored at −90°C.

We were able to detect HCV 229E RNAs in fetal astrocytes, in adult microglia, and in the mixed culture of adult oligodendrocytes and astrocytes (Fig. 4). Because the latter sample was composed of mixed cells, we could not determine if both cell types or only one was infectible, although it is likely that adult astrocytes were infected, given our observation of infection of fetal and adult astrocytes by HCV OC43. By using a short adsorption period on human fetal astrocytes (Fig. 4, lane 11) or on susceptible HeLa cells (data not shown), we confirmed that the positive RT-PCR signals were not from input virions and that viral replication thus had to have taken place before a signal was observed (adult microglia: Fig. 4, lane 3; adult astrocytes and oligodendrocytes: Fig. 4, lanes 5 and 7; fetal astrocytes: Fig. 4, lanes 9 and 12). Neurons were not assessed by this technique because of a shortage of available samples. Combined with the negative immunofluorescence results, we conclude that HCV 229E causes minimal infection of human microglia and astrocytes, at least when compared to HCV OC43.

Quantitation of release of infectious progeny virions by indirect immunoperoxidase assay. To verify the productivity of HCV infection of primary human neural cells, an immunoperoxidase assay was performed on coronavirus-susceptible cells. Susceptible cells (L132 or HRT-18) at 80% confluence were inoculated with logarithmic dilutions of infected cell culture supernatants in a 96-well Linbro plate (Flow, McLean, Va.). After 4 to 5 days of incubation in a humidified chamber at 33°C and 5% (vol/vol) CO_2, the cells were washed with PBS and fixed with 0.3% (vol/vol) hydrogen peroxide (Sigma) in methanol for 30 min. After being washed with PBS, they were incubated for 2 h at 33°C and 5% (vol/vol) CO_2 with an appropriate antiviral antibody (MAb 5-11H.6 or 4E11.3). The cells were then washed five times with PBS, and horseradish peroxidase-conjugated anti-mouse immunoglobulins (Cappel, Durham, N.C.) were added; this was followed by incubation for 2 h at 37°C without CO_2. The bound antibodies were detected by incubation in PBS supplemented with 0.025% (wt/vol) 3,3′-diaminobenzidine tetrahydrochloride (Bio-Rad, Richmond, Calif.) and 0.01% (vol/vol) hydrogen peroxide (Sigma). The color reaction was stopped with deionized water. Infectious-virus titers were calculated by the Karber method.

The results are shown in Table 1. After infection by HCV OC43, adult microglia did not produce significant amounts of infectious progeny viruses. The very low viral titer observed at 2 days postinoculation most likely corresponds to residual input virus, since infectious virus from these cells was not observed at 4 or 7 days post-viral inoculation. On the other hand, fetal astrocytes produced significant viral titers, and there was a clear increase in virus production over time, from a 50% tissue culture infectious dose ($TCID_{50}$) of 31,630/ml to a $TCID_{50}$ of 316,230/ml between 2 and 7 days postinfection. This correlated with immunofluorescence results. Our observations are in contrast with a preliminary study using human fetal tissue, in which HCV OC43 proteins, but not infectious virus, were detected in infected primary cultures, presumably astrocytes (17). However, specific GFAP labeling was not performed in that study, and the MOI was not indicated. Low titers of infectious virus were observed in the mixed culture of adult oligodendrocytes and astrocytes, but only at 2 days post-viral inoculation. As with adult microglia, this likely represents residual input virus. Given (i) the fact that these mixed cell cultures consisted of 80 to 90% oligodendrocytes and (ii) the immunofluorescence results showing that the only cells exhibiting fluorescence due to viral proteins were all positive for GFAP staining (the astrocyte marker), our results are consistent with an abortive infection of adult astrocytes, which is in contrast to a productive infection of fetal astrocytes. This is consistent with an age-related modulation of susceptibility to coronavirus infection. The much lower titers of infectious virus detected in the neuronal cultures at 2, 4, and 7 days postinoculation were most likely produced by infected astrocytes, which were shown by immunofluorescence to represent about 10% of the cells in the neuronal cultures (data not shown). Taken together with the negative immunofluorescence results, it appears that neurons are not infected by this virus. After infection by HCV 229E, only human fetal astrocytes were shown to produce some infectious progeny viruses. The titers obtained were very low but nevertheless increased with time (Table 1), which suggests a low-level productive infection; this could explain the negative results obtained in immunofluorescence and, at the same time, the positive results in RT-PCR–Southern blotting.

The difference in the degree of infectibility of the two HCV strains is not so surprising when one considers that a similar phenomenon has been reported in the mouse model with murine coronaviruses (6). It is unclear why HCV 229E infects neural cells poorly when compared to HCV OC43. A possible explanation would be a difference in receptors expressed in the neural cells. At this time, only the cellular receptor for HCV 229E has been identified (26), and its distribution in the CNS is not known, with the exception of a previously reported presence at nerve synapses. On the other hand, the cellular receptor used by HCV OC43 for infection of susceptible cells has not yet been identified. Another explanation could be the presence of penetration-associated or intracellular factors in one of the coronavirus strains that may facilitate replication.

The use of enriched primary human neural cell cultures enabled us to determine which cell type was susceptible to infection, at least for HCV OC43, and also to determine which

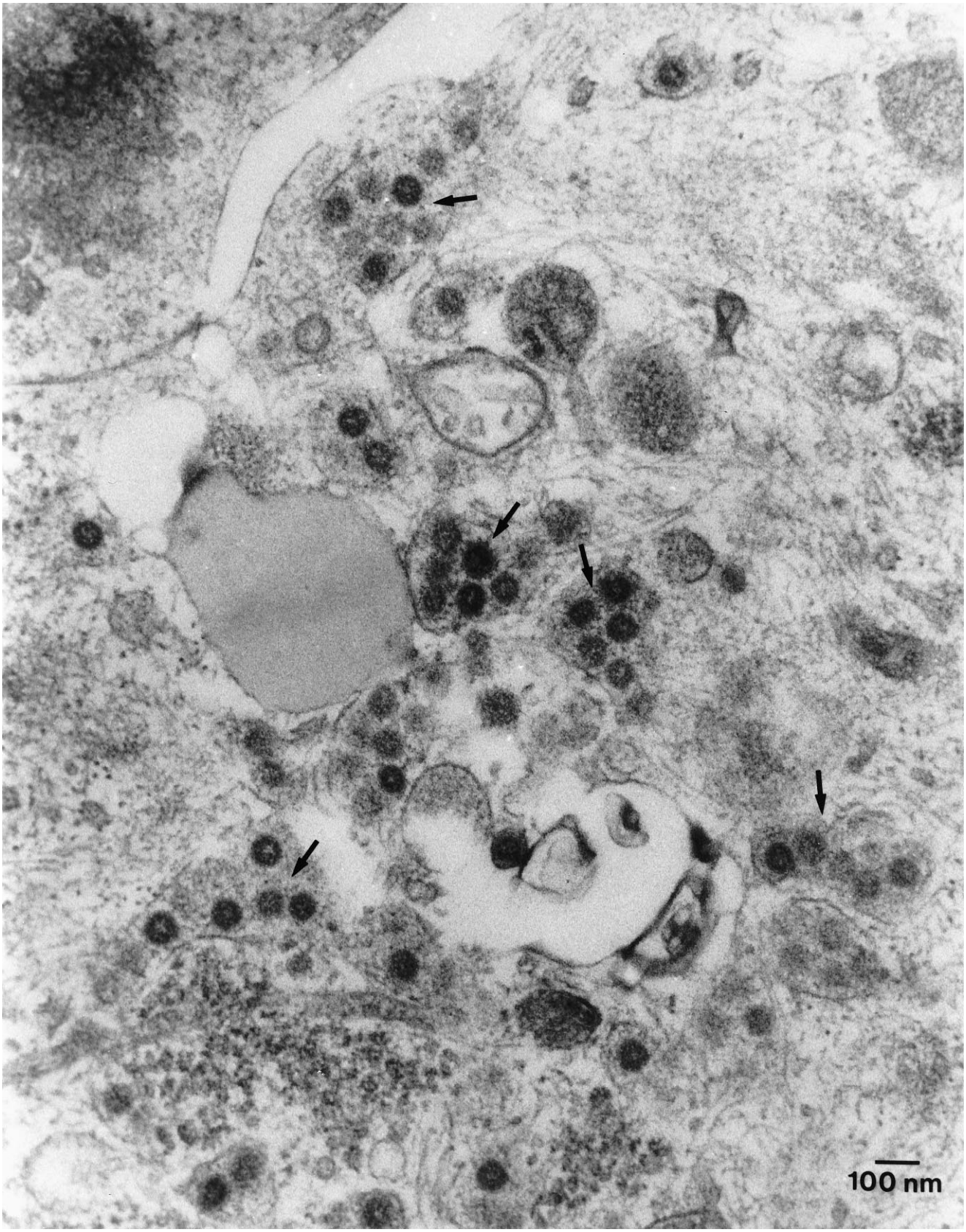

FIG. 3. Electron micrograph of an ultrathin section of a primary culture of human fetal astrocytes infected with HCV OC43 for 7 days. The arrows indicate coronavirions.

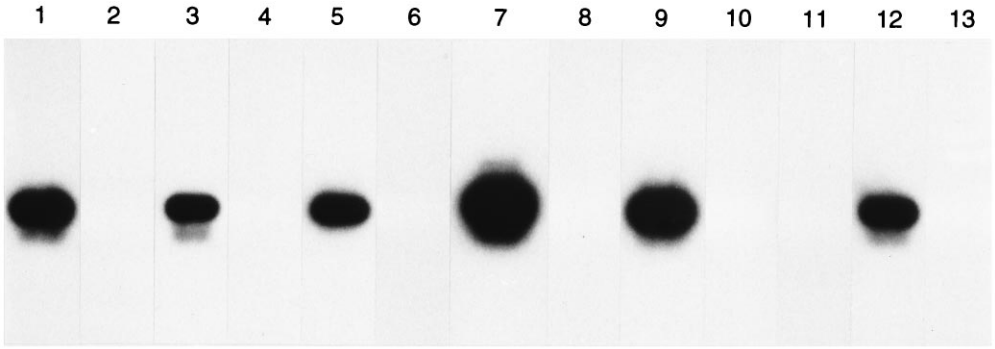

FIG. 4. Detection of HCV 229E RNAs in primary cultures of human neural cells inoculated at an MOI of 4. Lanes: 1, positive control of virus-infected L132 cells at 2 days postinfection; 2, negative control without RNA; 3, adult microglia infected for 4 days; 4, noninfected adult microglia; 5 to 8, mixture of adult oligodendrocytes and astrocytes (either noninfected [lanes 6 and 8] or infected for 4 days [lane 5] or 7 days [lane 7]); 9 to 13, fetal astrocytes (either noninfected [lanes 10 and 13], infected for 4 days [lane 9] or 7 days [lane 12], or inoculated with virus at 4°C for 2 h [lane 11]).

cell type produced infectious virus. To our knowledge, all previous studies on infection of primary human neural cultures, except for one study with human immunodeficiency virus (19), used mixed cell cultures. Our results are also important in view of the fact that the only available data on a possible infection of the human CNS by HCVs in vivo were from a study involving the detection of viral RNA in brain tissue of MS patients by RT-PCR (20). However, these results only indicated the presence of virus within the CNS and did not define the cell type in which the virus was replicating or if indeed it had been replicating within the CNS or had been carried there by viremia or within infected infiltrating immune cells.

The observation that microglial cells and astrocytes appear to be an important target for the replication of HCVs, at least from the in vitro evidence described here, can be correlated with data provided on two important viruses that are neurotropic in humans. Human T-cell lymphotropic virus was recently shown to replicate in cells that are probably astrocytes (9), and microglial cells were reported to be the main target for the replication of human immunodeficiency virus in the CNS (19), with astrocytes also implicated in another study (15). Importantly, infection of such glial cells has the potential to contribute to neuropathogenesis, including demyelination, which could, for example, be induced by secreted mediators, such as inflammatory cytokines (11), even in the absence of cytopathic effects on the infected cells or release of infectious progeny virions, as we have observed in the present study. Interestingly, primary cultures of human brain endothelial cells were recently shown to be susceptible to infection by HCV 229E (2). This could presumably provide a hematogenous route of entry into the CNS. Combined with the present study and previous reports on the detection of coronavirus RNA and proteins in human brain tissue (12, 20), it is tempting to speculate that coronaviruses may have neurotropic potential in humans. If confirmed by in situ detection in human brain sections, the neurotropism of HCVs would become an added incentive to pursue studies of their possible involvement in neurologic diseases such as MS.

TABLE 1. Infectious-virus titers recovered from the extracellular medium covering primary human neural cell cultures infected with HCV OC43 or 229E

Cell type	Time postinfection (d)	Infectious titer ($TCID_{50}$/ml) of:	
		HCV OC43	HCV 229E
Fetal astrocytes	2	31,630	<100
	4	31,630	320
	7	316,230	560
Adult microglia	2	1,000	ND[a]
	4	<30	<30
	7	<30	<30
Mixed culture of adult astrocytes and oligodendrocytes	2	560	ND
	4	<60	<30
	7	<100	<30
Fetal neurons	2	1,200	190
	4	2,500	<20
	7	800	<20

[a] ND, not done.

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