JOURNAL OF VIROLOGY, Sept. 1996, p. 6395–6401 Vol. 70, No. 9
0022-538X/96/$04.00+0

Persistent Poliovirus Infection of Human Fetal Brain Cells

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Received 5 February 1996/Accepted 17 May 1996

It has been suggested that poliovirus (PV), the causative agent of poliomyelitis, could persist in surviving patients. We have previously shown that PV can persistently infect some human cell lines in vitro, particularly neuroblastoma cell lines. We report here an ex vivo model in which PV can persistently infect primary cultures of human fetal brain cells. Two mutations involving capsid residues 142 of VP2 and 95 of VP1 were repeatedly selected during the persistent infections. These residues are located in capsid regions known to be involved in interactions between PV and its receptor. During the first week after infection, viral antigens were found in cells of both the neuronal and glial lineages. In contrast, 2 weeks after infection, viral antigens were detected almost exclusively in cells of the neuronal lineage. They were detected predominantly in cells expressing a marker of early commitment to the neuronal lineage, MAP-5, particularly in neuroblasts. Viral antigens were also found in immature progenitors expressing a neuroepithelium marker, nestin, and in cells expressing a marker of postmitotic neurons, MAP-2. The presence of viral antigens in postmitotic neurons suggests that PV can persist in neurons of patients who have survived poliomyelitis.

Poliovirus (PV), the etiological agent of paralytic poliomyelitis, belongs to the *Enterovirus* genus of the *Picornaviridae* family. It has a single-stranded RNA genome of positive polarity, about 7.5 kb long, enclosed in an icosahedral capsid composed of 60 copies of each of the four proteins VP1 to VP4. The genomes of wild-type and attenuated strains of the three serotypes have been sequenced, and the three-dimensional structure of the capsid has been determined to a resolution of 0.29 nm (20–22).

Post-poliomyelitis syndrome (PPS) is frequent, at least in North America, among survivors of acute paralytic poliomyelitis, after decades of clinical stability (16). There have been several nonexclusive explanations for PPS (15), including the persistence of PV. Several groups looked for signs of PV persistence in patients suffering from PPS, with positive results (28, 29, 34, 40).

At the present time, no convenient animal model is available for studying PPS. We and others have developed in vitro models in which PV persistently infects human cell lines (2, 11), particularly neuroblastoma cell lines (12). Several weeks to a few months after infection, the genome of viruses isolated from persistently infected neuroblastoma cells is highly mutated and the phenotype of these PV mutants, called PVpi, is very different from that of the parental strains (36). One interesting property is that the PVpi can persistently infect cells of nonneural origin, such as HEp-2c cells (36). The molecular determinants of this phenotype were mapped to the region encoding the capsid proteins (8). Moreover, some of the PVpi mutations conferred the capacity to infect the central nervous system (CNS) of mice to a wild-type PV strain (13, 14).

However, observations of infected cell lines, particularly cell lines derived from tumors, do not always reflect the pathways of in vivo infections. We therefore investigated the possibility of establishing persistent PV infections in human brain cells. A relevant model for this kind of study has been recently developed by using primary cultures of proliferative neural progenitors from human fetuses (7). The cells coexpress the characteristic markers nestin and vimentin and have the potential expected of neuroepithelial cells: they differentiate exclusively into derivatives of the neuronal and glial lineages, including neurons, astrocytes, and oligodendrocytes (7, 39).

Persistent PV infection in primary cultures of human fetal brain cells. To study the capacity of PV to persistently infect human CNS cells, we prepared cultures of human neural progenitors from germinative zones of the brains of 6- to 8-week-old fetuses obtained by legal abortions (P. Blot and J. F. Oury, Hôpital R. Debré, Paris, France). The brains were separated into an anterior fraction containing the telencephalic vesicles and diencephalon and a posterior fraction containing the mesencephalon, pons, and cerebellar enlage. Single cells were obtained by trypsinization as described previously (7). The phenotype of more than 90% of the cell population was initially that of neuroepithelial and/or neuroblastic cells (7). The human CNS progenitors could be propagated in vitro for one to three passages as a quasihomogeneous population of neuroblasts (7, 39), in defined serum-free N2 medium (modified from the method of Bottenstein and Sato [6]) containing basic fibroblast growth factor (bFGF; 10 ng/ml) (Boehringer). Brain cells were infected (1×10^5 to 5×10^5 cells per 1.8 cm^2 well) with wild-type Mahoney or attenuated Sabin 1 strains of PV type 1 (PV1) at four multiplicities of infection (MOI): 0.01, 1, 10, and 100 50% infectious doses (ID_{50}) per cell. To monitor the kinetics of virus synthesis, the inoculum was replaced by N2 medium containing 0.3% heat-inactivated fetal bovine serum, 30 min after infection, and then cells were incubated at 37°C. Cells were refed by replacing 60% of the medium twice a week. The omission of bFGF and the addition of a low concentration of fetal bovine serum favored progressive differentiation of the nestin-positive progenitors into neuronal and glial derivatives (7, 39).

Extracellular virus infectivity titers were determined on HEp-2c cells at the times indicated up to 3 weeks after infection for the MOI of 10 ID_{50} (Fig. 1). During the first 2 days postinfection, there was massive production of virus, almost 10-fold greater for the wild-type Mahoney strain than for the

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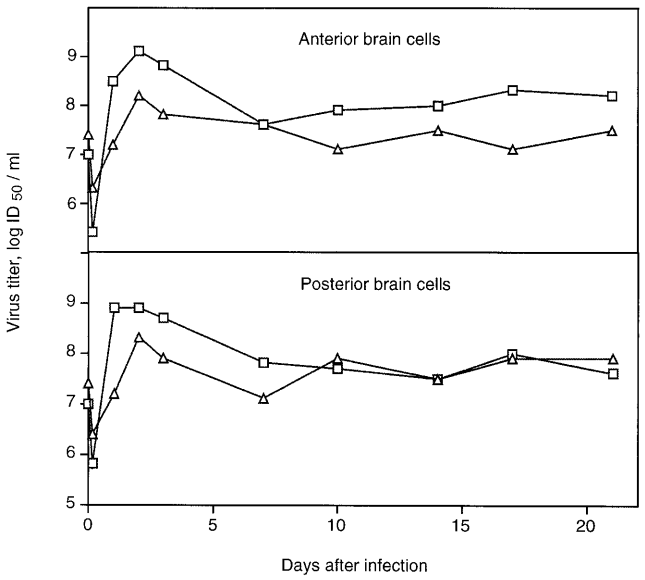

FIG. 1. PV titers in supernatants of human fetal brain cells infected at an MOI of 10 ID_{50} per cell. Cultures of human neural progenitors were derived from the anterior or posterior part of the brain of a 6.5-week-old fetus. Cells were infected at 37°C with wild-type Mahoney (squares) or attenuated Sabin 1 (triangles) PV1 strains. Sixty percent of the medium was replaced twice a week. The titers of extracellular virus were determined on HEp-2c cells.

attenuated Sabin 1 strain. In agreement with this, cytopathic effects appeared a few hours earlier and were more substantial in cells infected with the wild-type Mahoney strain than in cells infected with the thermosensitive, attenuated Sabin 1 strain (data not shown). This indicates that early after infection, the Sabin 1 strain did not grow as well as the Mahoney strain in brain cultures. This could have been due to a low level of replication of viruses carrying attenuating mutations in the 5′-noncoding region of their genome, as previously shown in some neuroblastoma cell lines (1, 26). After the initial massive production of PV, virus synthesis and liberation into the cell culture medium remained high and stable for cells in both anterior and posterior brain fractions, at about 10^8 ID_{50}/ml, despite the renewal of the culture medium. The level of virus production 2 to 3 weeks after infection was about threefold higher for the Mahoney strain than for the Sabin 1 strain in anterior brain cells. This difference was observed in three of four experiments, which is in agreement with the results obtained in neuroblastoma cells (1, 26), but it was not observed in posterior brain cells (Fig. 1). A stable production of virus was obtained in three independent experiments for the MOI of 10 ID_{50}, indicating that the viruses present in cell supernatants 3 weeks after infection were the result of continuous synthesis of virus. Similar results were also obtained for the MOI of 0.01 ID_{50} (data not shown). Cells survived infection even when MOIs as high as 100 ID_{50} per cell were used, and viruses were recovered for characterization up to 3 weeks after infection (see below). One experiment was continued even longer, and a persistent PV infection, established in anterior brain cells infected at an MOI of 10 ID_{50} per cell with the Mahoney strain, was maintained up to 7.5 weeks after infection, with virus production (data not shown). Thus, we demonstrated that attenuated and wild-type strains of PV could establish persistent infections in anterior and posterior fetal brain cells.

Selection of PV mutants during persistent infection of fetal brain cells. To determine whether mutations were selected in human fetal brain cells persistently infected with PV, we sequenced genomes of Mahoney-derived viruses in the supernatant of infected brain cells. The genomic P1 region encoding the capsid proteins was first chosen for sequencing because mutations previously shown to be involved in persistent infections were found in this genomic region for several picornaviruses, including PV, foot-and-mouth disease virus, and Theiler's virus (8, 17, 31). Viruses were amplified once on HEp-2c cells. Total cytoplasmic RNA from PV-infected HEp-2c cells (10 μg) was used for sequencing with avian myeloblastosis virus reverse transcriptase and primers specific for the Mahoney strain, as previously described (19).

Virus was isolated 3 weeks after infection from anterior brain cells infected at an MOI of 10 ID_{50} per cell with the Mahoney strain. The entire genomic P1 region of this virus was sequenced, and only two point mutations were found. The mutations were at positions 1373 and 2762, corresponding to residue 142 of VP2 (His→Tyr) and residue 95 of VP1 (Pro→Ser), respectively. These two substitutions have been observed in the Mahoney-derived PVpi isolated from neuroblastoma cells (10, 14), but they have never appeared in viruses growing on HEp-2c cells. The first substitution, at amino acid 142 of VP2, was also selected in the Sabin 1-derived PVpi S11 isolated from neuroblastoma cells (5). The second substitution, at position 95 of VP1, corresponds to a Sabin 1 residue which has been conserved in the PVpi S11 (5). In order to determine if other mutations selected in PVpi isolated from neuroblastoma cells could also be selected in human fetal brain cells, we looked for the presence of mutations at nucleotide positions frequently mutated in Mahoney-derived PVpi, i.e., nucleotide positions 1373, 1944, 2607, and 2762, in viruses recovered from four independent experiments. Each of these independent experiments included four MOIs (0.01, 1, 10, and 100 ID_{50}), and in total, 19 viral isolates were analyzed after infection of anterior or posterior brain cells at various times postinfection. In the viral genomes, only the two missense mutations at nucleotide positions 1373 and 2762 were found repeatedly and no additional mutations were detected (Fig. 2). We looked for the time of first detection of the two mutations. The timing of the appearance of the mutations at positions 1373 and 2762 indicated that they were selected 2 weeks after infection in most viral isolates (10 of 13 and 8 of 11, respectively), independently of the origin of the cells (anterior or posterior part of the brain) and of the MOI. Either of the two mutations could be selected independently, or they could be selected simultaneously (Fig. 2). At the time of initial detection of the mutations, the percentage of the viral population bearing the mutant sequence varied between 20 and 100%. These results were confirmed by sequencing directly reverse transcription-PCR products obtained with the supernatant of persistently infected brain cells. In all cases, this percentage reached 100% (the wild-type sequence was no longer detected) a few days after the initial detection of the mutated nucleotide.

The two missense mutations repeatedly selected during the persistent infection of fetal brain cells caused substitutions of amino acid residues located at the surface of the capsid. Residue 142 of VP2 is in the E-F loop, in the canyon, which is thought to be the site of attachment of the virus to its receptor (21, 22). Residue 95 of capsid protein VP1 is in the B-C loop at the fivefold axis of symmetry. Several arguments suggest that these residues are involved in the early steps of the virus cycle: (i) they fall within the footprint of the receptor on the virus and at the periphery of the footprint, respectively (14); (ii) they enable PV to utilize defective receptors (13); and (iii) both residues are located in regions involved in PV adaptation to

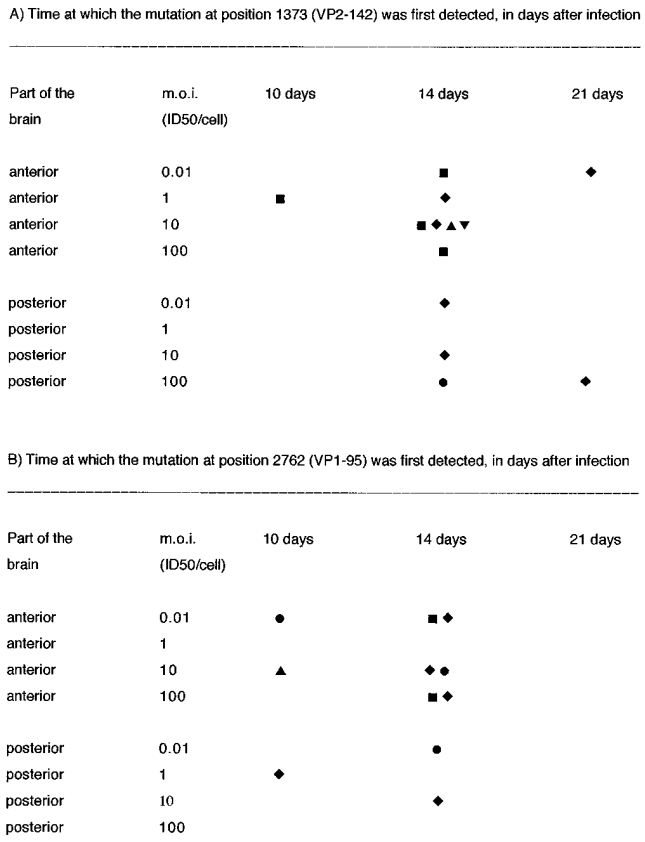

FIG. 2. Selection of viral mutants in fetal brain cells persistently infected with the Mahoney strain of PV1. The times at which mutations at nucleotide positions 1373 and 2762 were first detected are indicated in panels A and B, respectively. Different symbols represent the detection of mutations in independent wells, in four independent experiments. Similar symbols in panels A and B correspond to mutations identified in the same well. In three of four experiments (squares, triangles, and circles), viruses were tested for mutations up to 14 days after infection. The fourth (diamonds) was monitored up to 21 days after infection. Mutations at nucleotide positions 1373 and 2762 were generally first detected 2 weeks after infection, indicating that PV multiplication continued more than 10 days after infection.

the murine CNS (14, 30, 35). PV restriction in the murine host is believed to involve interactions between PV and its receptor.

Our results suggest that mutant viruses able to progress efficiently through the early steps of the virus cycle were rapidly selected in brain cells. The PV receptor, or a molecule interacting with it, may be slightly different in brain cells from that found in HeLa or HEp-2c cell lines. If, as previously proposed (33), interactions between PV and the PV receptor control cell killing through cell signaling pathways, a structural difference in the PV receptor at the surface of human brain cells (or in a protein interacting with it) could be responsible for modified PV-PV receptor interactions. This would prevent cell killing and therefore allow persistent infection to occur.

Since mutations in the 5′-noncoding region are associated with attenuation in neuroblastoma cells (1, 26), the presence of mutations at nucleotide position 480 was checked for in five isolates of anterior or posterior fetal brain cells 2 to 3 weeks after infection with the Mahoney strain (MOI, 10^{-2}, 1, and 10). No mutations were found at this position. Nucleotide positions 525 and 606, which were mutated in some PVpi (5), were not mutated in the genomes of viruses isolated from fetal brain cells. For one isolate, however, one transition mutation was found at position 667, creating an 8-nucleotide duplication (positions 662 to 669 and 689 to 696). The significance of this mutation is unknown, because it belongs to a region preceding the start codon, which can be deleted without altering the viability of the virus (24, 25, 37). The selection of a mutation at position 667 may indicate that tissue-specific factors interact with this region of viral RNA in brain cells.

TABLE 1. Detection by cytofluorometry of the PV receptor at the surfaces of cells

Cells	MAb 280[a]	% of fluorescent cells	Mean fluorescence/positive cell[b]
HEp-2c	+	86.2	311
	−	4.6	
LM[c]	+	7	
Brain cells[d]	+	38.8	192

[a] Monoclonal antibody against the PV receptor (32). The monoclonal antibody was added (+) or omitted (−) in the reaction.
[b] In arbitrary units.
[c] LM cells are PV receptor-negative mouse cells, used as a negative control.
[d] Fetal anterior brain cells.

Identification of persistently infected cells. Mock-infected cultures were not homogeneous: star-shaped, flat cells were observed among a majority of cells with a network of neurites. In contrast, most surviving cells in infected cultures were neurite-bearing cells, with a neuroblast-like morphology. To identify the fetal brain cells persistently infected by PV, we characterized anterior brain cells during the first days and 2 weeks after infection by the PV1 Mahoney strain, when persistent infections were maintained with stable virus production.

We used immunofluorescence labeling to identify infected cells in the cell cultures. Cells were first fixed with paraformaldehyde and permeabilized with Triton as described previously (7). Viral antigens were detected by using the neutralizing monoclonal antibody C3 (3) (kindly provided by B. Blondel, Institut Pasteur, Paris). The C3 antibody specifically binds to neutralization antigenic site 1 of the Mahoney strain of PV1, corresponding to amino acids 89 to 105 of the capsid protein VP1 (42). The binding also occurs with the Sabin 1 strain (4) and with the point mutant Mah-KKVP1S95 (14), which both have a Ser at position 95 of VP1. A mouse monoclonal antibody, IgG1 K (Sigma), was used as a negative control for the C3 antibody. The secondary antibody was a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG) (TEBU). Cell fluorescence was either examined directly by fluorescence microscopy, using a BX60 microscope (Olympus), or analyzed with a confocal laser-scanning microscope (LEICA; Laser Teknik, Heidelberg).

The percentage of cells harboring viral antigens was calculated at various times after infection (from photographs, with 600 to 1,000 cells for each time). The percentage of positive cells decreased with time; at 1, 3, 7, 10, and 14 days after infection, it was 39, 32, 14, 10, and 9%, respectively.

It has been shown that the susceptibility of primate cells grown ex vivo to PV depends on virus receptor expression (23). We investigated the presence of the PV receptor at the surfaces of fetal brain cells by indirect immunofluorescence as described previously (9), using the monoclonal antibody 280 (32) specific for this protein (a kind gift from P. Minor, National Institute for Biological Standards and Control, Potters Bar, United Kingdom). Cell fluorescence was quantified with a fluorescence-activated cell sorter (FACS) (Coulter Elite E. S. P.). At the time of infection, fetal brain cells expressed lower levels of PV receptor than the fully permissive HEp-2c cell line

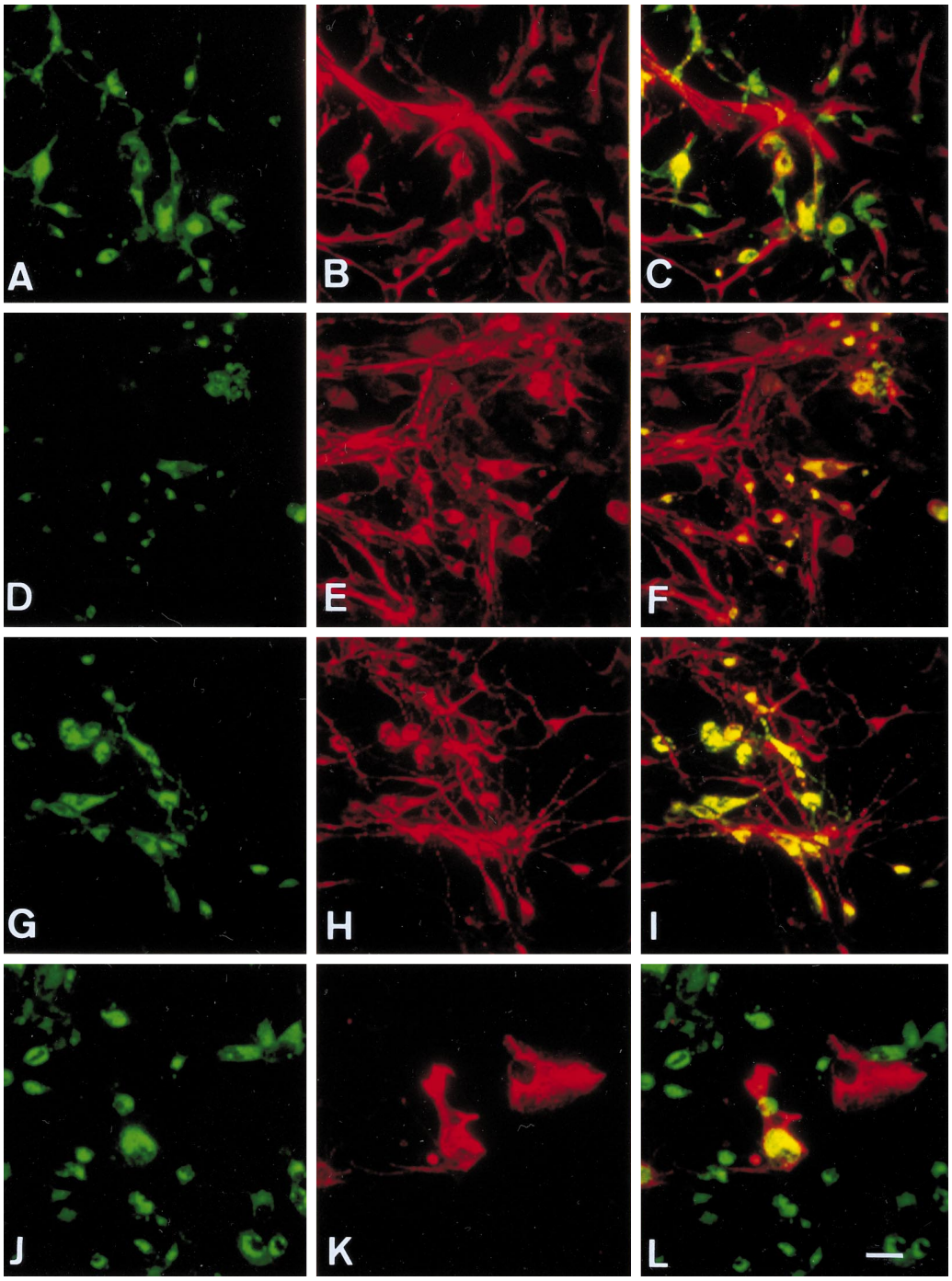

FIG. 3. Detection of viral and cellular antigens by confocal microscopy in fetal anterior brain cells 1 day after infection with the wild-type Mahoney PV1 strain. Viral antigens were detected by antibody C3 and stained in green with FITC-labeled secondary antibodies (A, C, D, F, G, I, J, and L). Cellular antigens were detected by antibodies against nestin (B and C), MAP-5 (E and F), MAP-2 (H and I), and GFAP (K and L) and stained in red with CY3-labeled secondary antibodies. The stainings of viral and cellular antigens were superimposed (C, F, I, and L). The superimposition of green and red fluorescences appears yellow. Some nestin-, MAP-5-, MAP-2-, and GFAP-positive cells were found to harbor viral antigens. Bar, 20 μm.

(Table 1). It is possible that a limiting amount of PV receptor could contribute to PV persistence. Several explanations can be proposed to understand the low percentage of infected cells in the cultures at 2 weeks after infection. The majority of surviving cells could have been resistant to PV infection because of a low level of expression or a modification of the virus receptor or because of particular intracellular factors. It is also possible that the amount of viral antigens was very small in the majority of cells and thus not detected.

To determine which cells were infected and to characterize cells surviving infection, double-labeling experiments were done by performing two successive single labelings with the C3

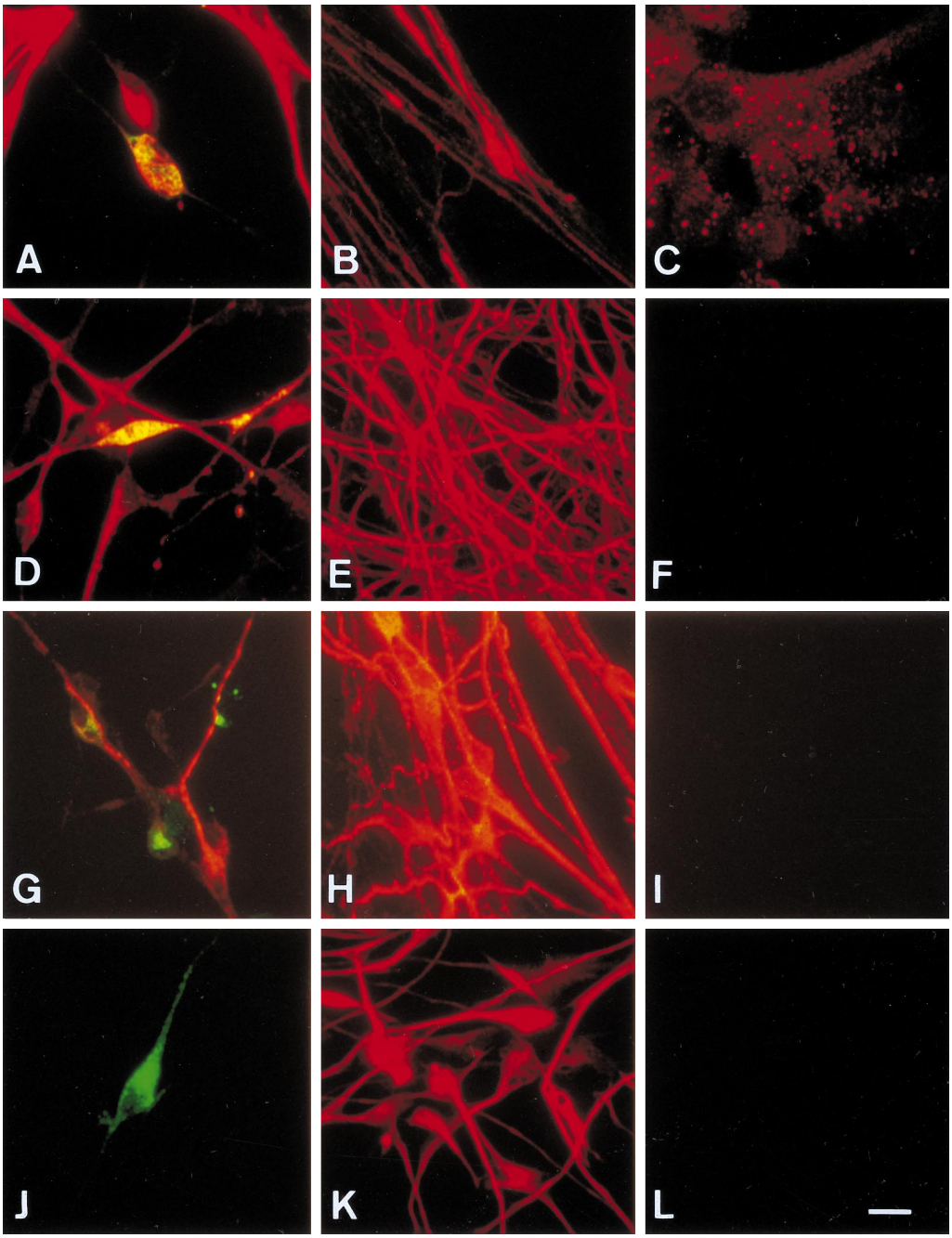

FIG. 4. Detection of viral and cellular antigens by confocal microscopy. (Left panels) Fetal anterior brain cells 2 weeks after infection with the wild-type Mahoney PV1 strain. (Center panels) Uninfected fetal anterior brain cells. (Right panels) Uninfected HEp-2c cells. (A, D, G, and J) Viral antigens detected by antibody C3 and stained in green with FITC-labeled secondary antibodies. (A, B, and C) Nestin, (D, E, and F) MAP-5, (G, H, and I) MAP-2, and (J, K, and L) GFAP markers stained red by immunofluorescence using CY3-labeled secondary antibodies. The superimposition of green and red fluorescences appears yellow. Nearly 100% of C3-positive infected brain cells were MAP-5 positive. A few C3 positive cells were either nestin or MAP-2 positive. No C3-positive cell was GFAP positive. Bar, 10 μm.

antibody and with antibodies against markers of neural cells, at 1, 3, 7, 10, and 14 days after infection (Fig. 3 and 4). Cells of the neuronal lineage were stained either with polyclonal rabbit anti-nestin 129 (a kind gift from R. McKay, National Institutes of Health, Bethesda, Md.) or with mouse monoclonal antibodies: anti-microtubule-associated protein 5 (MAP-5; Sigma), anti-β3-tubulin (Sigma), and MAP-2 (Sigma). The intermediate filament protein nestin is a classical neuroepithelium marker (27), MAP-5 and β3-tubulin are markers of early commitment to the neuronal lineage (18, 41), and MAP-2 is found in postmitotic neurons (41). To attempt to characterize the flat cells in the cultures, we used a polyclonal rabbit serum anti-glial fibrillary acidic protein (GFAP) (38) (Dako), a marker of astrocytes and preastrocytes. HEp-2c cells were used as negative controls in the characterization of neural cells. Species-specific secondary antibodies used were CY3-conjugated goat anti-

rabbit IgG (Jackson Immunoresearch Laboratories), FITC-conjugated goat anti-mouse IgG (TEBU), and CY3-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories). Negative controls were performed by omission of the primary antibody in the second labeling. Absence of cross-reactivity of the secondary antibodies and of significant spilling over was verified.

The results are shown for day 1 (Fig. 3) and day 14 (Fig. 4) after infection. During the first week after infection, cells positive for viral antigens were either of the neuronal or of the glial lineage (Fig. 3), indicating that cells of both lineages could be susceptible to PV early after infection. The presence of C3-positive cells that were either nestin-positive or MAP-2-positive (Fig. 3) indicated that both immature derivatives of the neuronal lineage and more mature young neurons could be infected. Among C3-positive cells which were also nestin, MAP-5, or MAP-2 positive, some cells were rounded (Fig. 3), suggesting that they would not have survived infection, while others were apparently healthy and could therefore have been persistently infected. The presence of C3-positive, GFAP-positive cells indicated that astrocytes or preastrocytes were infected at early times postinfection. Since these cells progressively disappeared in infected cultures, it seems probable that they were lytically infected during the first days.

Two weeks after infection, the majority of cells were MAP-5 and β3-tubulin positive (not shown). Among C3-positive cells, the majority of them were MAP-5 positive (Fig. 4). The presence of a few C3-positive cells that were either nestin or MAP-2 positive was detected (Fig. 4). At low magnification, several C3-positive, MAP-2-positive cells, which were apparently healthy, could be seen simultaneously (not shown). Therefore, persistently infected cells seemed to be neuroepithelial precursors, neuroblasts, and young neurons, although the majority of them were probably neuroblasts. Our results indicate that 2 weeks after infection, cells strongly positive for viral antigens were mostly, if not exclusively, cells of the neuronal lineage. More specifically, the presence of viral antigens in some of the MAP-2-positive cells suggests that PV can persist in postmitotic neurons.

In conclusion, this is the first report of a persistent PV infection in primary cultures of human cells explanted from the CNS. Two mutations modifying capsid residues, probably involved in virus-receptor interactions, were repeatedly selected for in independent infections. We have found that the cells permitting PV multiplication during the first days after infection belonged to both the neuronal and glial lineages, and our results suggest that the latter cells were lytically infected. At later times, 2 weeks after infection, infected cells expressed predominantly markers of the neuronal lineage. The presence of viral antigens in postmitotic neurons is in agreement with PV being able to persist in postmitotic motoneurons of patients who have survived poliomyelitis. The persistent PV infection of primary cultures explanted from the human CNS, which can be maintained for more than a month postinfection, may therefore be a useful ex vivo model for rare chronic PV infections and possibly for PPS.

Our particular thanks to R. McKay, P. Minor, and B. Blondel for their generous gifts of anti-human nestin 129 antibodies, anti-PV receptor monoclonal antibody 280, and anti-PV1 monoclonal antibody C3, respectively. We thank R. Hellio for expert advice in confocal microscopy and H. Kiefer and M.-C. Wagner for FACS analyses. We are grateful to T. Couderc, G. Duncan, I. Pelletier, B. Blondel, F. Delpeyroux, and A. Edelman for critical readings of the manuscript.

The Ministère de l'Enseignement Supérieur et de la Recherche is acknowledged for the fellowship awarded to N.P. This work was supported by the Institut Pasteur, the Centre National de la Recherche Scientifique, and the Association Française contre les Myopathies (contrat 3720).

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