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Persistent poliovirus infection of human neuroblastoma cells

(poliomyelitis/motor neuron disease/chronic infection)

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ABSTRACT **Two human neuroblastoma cell lines were persistently infected with poliovirus strains of all three serotypes. In persistently infected IMR-32 cells, which were studied in greatest detail, viral antigens were present in most cells, and over a 9-month period virions were found in the medium at high titers. Persistently infected cells were resistant to superinfection by Sabin 1, 2, and 3 poliovirus but sensitive to coxsackievirus B3. The viruses recovered from persistently infected cells were studied for conservation of epitopes, host cell specificity, and temperature resistance phenotype. The antigenic site 1 carried by the major capsid protein VP1 was modified on the persistent viruses of all three serotypes. This was confirmed for one virus by sequencing the corresponding genomic region in which two mutations were detected. The titers of persistent viruses were 1–3 $\log_{10}$ units higher on IMR-32 cells than on nonneuronal HEp-2 cells, while parental viruses had similar titers on both lines. When thermosensitive viruses were used to initiate the infection, the persistent viruses were found to be thermoresistant at 39°C. Together the results indicate that the persistent infection correlated with the selection of highly mutated viral strains. Poliovirus-infected neuroblastoma cell lines thus constitute an *in vitro* model of chronic viral infections, which are increasingly implicated in human neural diseases.**

Poliomyelitis paralyses are caused by poliovirus (PV)-induced necroses of motor neurons (1, 2). A late postpolio syndrome has been described: new focal motor neuron deterioration emerges after an average period of 30 years from initial infection (3, 4). One of the hypotheses proposed to account for this syndrome is a persistent viral infection (3, 4). Several members of the picornavirus family are effectively able to induce a persistent infection of cells *in vivo* and *in vitro* (5–11). It is assumed that the selection of viral mutants, viral interference, and interferons play a role in this persistence (12, 13). For PV, the viral cytolytic action was shown to be limited by the intercellular matrix (14). Cultures in which only a small fraction of cells were susceptible to PV have been described (15–17). In a more recent study, Kaplan *et al.* (18) isolated PV-infected HeLa cell lines blocked at different steps of the virus life cycle which underwent periodic crises with cytopathic effects (CPE).

In cell lines of neuronal origin, PV–cell interactions have been studied only in one-step growth experiments during the first 8 hr post infection (p.i.) (19). We report here the establishment of a persistent PV infection of human neuroblastoma cells. Most cells harbored viral antigens and cultures produced virions for months at high titers in the absence of detectable CPE. Persistently infected cell lines and the resulting PV mutants are described.

MATERIALS AND METHODS

Materials. The Leon 37, vFG68 (20), LSc2ab (S1), P712 Ch 2ab (S2), and P3/Leon 12alb (S3) Sabin strains (21) of PV were used. Another enterovirus, coxsackievirus B3, was used as control in some experiments. The human neuroblastoma cell lines IMR-32, described as a relatively poor host for virus replication (22), and SK-N-MC (23) were purchased from the American Type Culture Collection and cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Monoclonal antibody (mAb) 1o (24) and rabbit anti-type 3 PV serum (20) were used for immunofluorescence. Anti-human hepatitis A virus mAb 1B5, and anti-human cytomegalovirus rabbit serum were used as controls. mAb 2F/11, which stains the 70- and 200-kDa polypeptides of human neurofilament, was purchased from Sanbio (Uden, The Netherlands). mAbs Ib, 1c, 1o, Io, and C_3 (24, 25) were used for neutralization epitope mapping.

Immunofluorescence and Seroneutralization. Immunofluorescence staining was performed with all antibodies according to the instructions of Sanbio. Briefly, cells were fixed in 3% paraformaldehyde, treated with 0.5% Triton X-100, washed, incubated in 50 mM NH_4Cl, and washed again. Fixed cells were then successively incubated with the first antibodies, then with fluorescein-labeled anti-immunoglobulin serum, each for 60 min at 37°C. For neutralization, virus was titered in the presence of a constant concentration of antibodies.

Infectious Center Assay. Extracellular virus was eliminated by neutralization, extensive washing, or both. Ten to 50 infected cells were allowed to adsorb for 2.5 hr at 37°C onto a confluent monolayer of IMR-32 cells, which was then overlaid with a medium containing 1.6% carboxymethylcellulose (BDH).

RNA Sequencing. The RNA genome of vFG68, isolated from persistently infected cells, was sequenced in the region of the VP1 capsid protein (20), using the dideoxy chain-termination method (26).

RESULTS

Establishment of a Persistent Infection of Human Neuroblastoma Cells with PV. When IMR-32 human neuroblastoma cells were inoculated with PV at a multiplicity of infection (moi) higher than 100 ID_{50} per cell, all the cells were infected and died after virus multiplication, indicating that they carry a receptor for PV. At an moi of 1–10, 95% of the cells rounded up within 2 days, while the others started to grow. These results were independent of the virus strain and incubation temperature (37°C for wild-type PV, 34°C for the attenuated strains). For one month p.i., a large proportion of the cells died and there was concomitant virus production (Fig. 1). Forty days p.i., there remained only a few colonies per flask. From this time on, no more CPE were observed and cells reached confluency with persistent virus synthesis. Similar results were obtained with wild-type PV (not shown) and Sabin strains (Fig. 1). Between 2 and 9 months p.i., persis-

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Abbreviations: CPE, cytopathic effects; mAb, monoclonal antibody; moi, multiplicity of infection; p.i., post infection; PV, poliovirus.
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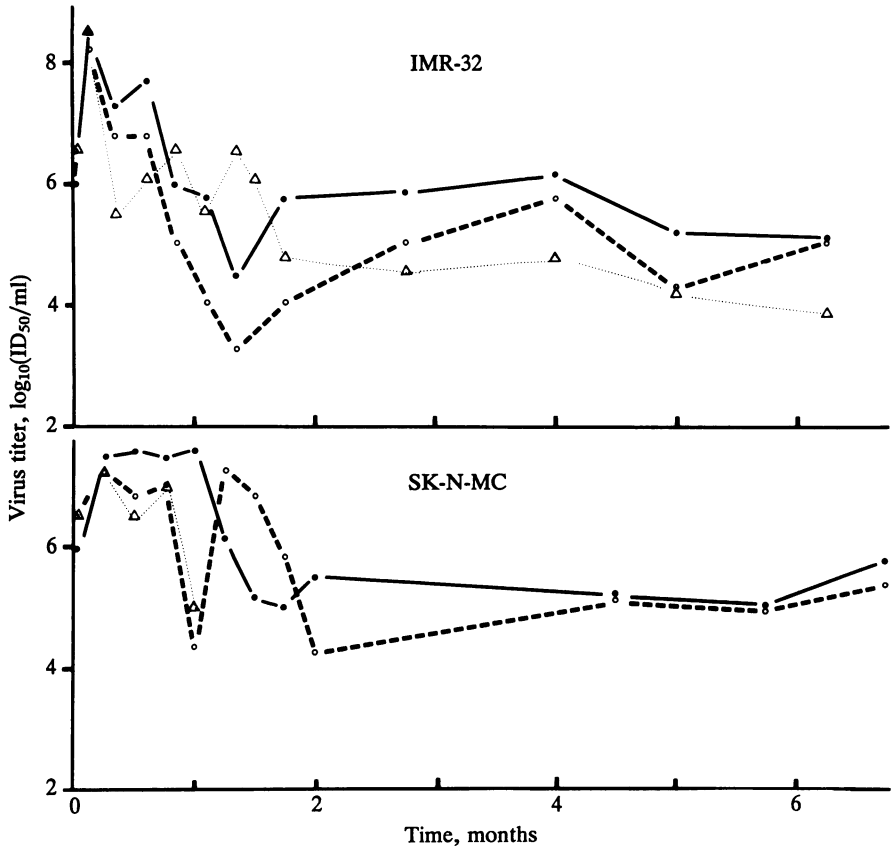

FIG. 1. PV titer in cultures of persistently infected human neuroblastoma cells. The IMR-32 and SK-N-MC cells were infected at 34°C with the Sabin 1 (● — ●), Sabin 2 (○--○), or Sabin 3 (△···△) PV strain at an moi of 5 ID_{50} per cell. Cultures were transferred to 37°C 1 week p.i. and extracellular virus was titered on HEp-2 cells at the indicated times p.i.

tently infected IMR-32 cells were trypsinized and the cultures were split 1 to 3 weekly.

To find out if a persistent infection could be established with another human neuroblastoma cell line, SK-N-MC cells were infected with each of the three attenuated Sabin strains at an moi of 5 ID_{50} per cell. During the first weeks p.i., high virus titers were found in the medium, while the majority of cells survived, especially with the Sabin 1 and 2 strains. However, 1 month p.i., cells started to die and in some experiments those infected with the Sabin 3 strain were lost (Fig. 1). Continuous virus production was regularly demonstrated in infected cultures, but, contrary to IMR-32 cells, persistently infected SK-N-MC cells never reached confluency. Colonies of cells could be maintained for more than 9 months by changing the medium weekly. Virus production was stable and titers were similar in the IMR-32 and SK-N-MC persistently infected cell lines (Fig. 1). Because they are easier to grow, we chose the IMR-32 cells to study the persistent infection further.

IMR-32 cells persistently infected with the Sabin strains of serotypes 1, 2, and 3 were designated IMR/S1, IMR/S2, and IMR/S3, respectively. Similarly, others were designated IMR/ followed by the name of the virus. Viruses recovered from IMR/S1, IMR/S2, and IMR/S3 cells were designated S1 (IMR), S2 (IMR), and S3 (IMR), respectively.

To demonstrate that the virus that was synthesized by persistently infected cells was indeed the virus that was used for inoculation 6 months earlier, the virus type was confirmed by neutralization with type-specific anti-PV sera. Since some cells were infected with vFG68 (20), a Sabin 3 PV mutated by a 9-nucleotide insertion in the region of the capsid polypeptide VP1, the genome of the virus from IMR/vFG68 cells was sequenced in the region of the insertion. The insertion was still present in the viral genome, although two additional point mutations were detected within the insertion. Arginine residues at positions 100 and (100+3) of VP1 (20) were replaced by a glutamine and a tryptophan residue, respectively (not shown).

Phenotypic Analysis of Persistent Viruses. The high mutation rate suggested by the results mentioned above was confirmed by further characterization of persistent viruses. In some experiments, the thermosensitive Sabin strains, which are normally grown at 34°C (21), were used to establish the infection at 34°C. Since the infected IMR-32 cells were transferred to 37°C 1 week later and maintained at this temperature thereafter, the thermosensitivity of the persistent viruses was studied. The difference in titer [$\log(ID_{50}/ml)$] in IMR-32 cells after 3 days of incubation at 34°C and 39°C was determined. While the control S3 strain had a negative reproductive capacity at supraoptimal temperature (rct) (27) (difference greater than 4), S1 (IMR), S2 (IMR), and S3 (IMR) viruses had a positive rct (difference lower than 2).

Other phenotypic changes were noted in the persistent viruses. The antigenic sites 1 and 2A, corresponding to the amino acids 93–100 and 221–223 of capsid protein VP1, respectively, were modified for S1 (IMR) (Table 1). Similar results were obtained with S2 (IMR) and S3 (IMR) (not shown). The host cell specificity of persistent viruses was also modified. Virus synthesized by IMR/S3 cells 3 months p.i. had a titer 3 logarithmic units higher on IMR-32 than on HEp-2 cells (Table 2). Similarly, S1 (IMR) and S2 (IMR) had a titer 1–2 logarithmic units higher on IMR-32 than on HEp-2 cells. This difference in titer could have resulted from a viral interference phenomenon on HEp-2 cells. To test this hypothesis, parental S3 virus was titered on HEp-2 cells in the presence or in the absence of a constant amount (10^3 ID_{50}) of

Table 1. Neutralization epitope map of the virus recovered from IMR/S1 cells

Type 1 PV strain	Presence of neutralization epitope				
	mAb C3 (Site 1, VP1)	mAb Ib (Site 2A, VP1)	mAb 1c (Site 2C, VP2)	mAb 1o (Site 3B, VP3)	mAb Io (Site 3B, VP3)
Mahoney	+	+	+	−	+
Sabin 1	+	+	+	+	−
S1 (IMR)	−	−	+	+	−

Column headings give the mAb and, in parentheses, the antigenic site and the PV capsid polypeptide in which the site occurs. The neutralization index (NI) was defined as the difference of the logarithm of the virus titer in the absence and presence of each mAb (24). NI ≥ 2 indicates the presence (+), while NI < 2 indicates the absence (−), of the neutralization epitope defined by the respective mAb.

S3 (IMR). The titer of parental virus could be determined by an early reading of plates because of the faster multiplication of this virus in HEp-2 cells. No viral interference was noticed under these conditions, since the titer of S3 was not changed significantly by the presence of an excess of S3 (IMR). The fact that the host cell specificity of persistent viruses was unchanged after virus cloning on IMR32 cells (not shown) confirmed the genetic character of their cell tropism.

Viral Antigens and Virion-Producing Cells in Persistently Infected IMR-32 Cultures. The synthesis of viral antigens in persistently infected IMR-32 cells was studied by indirect immunofluorescence with a Sabin 1-specific mAb, 1o (24). A granular cytoplasmic fluorescence was observed in almost all IMR/S1 cells treated with mAb 1o (Fig. 2 *a* and *b*) and was not observed in IMR/S3 cells with the same mAb or in IMR/S1 cells treated with an anti-human hepatitis A virus mAb (Fig. 2*d*). In IMR/S1 cells, the number of mAb 1o-fluorescent dots per cell varied from one to confluency (Fig. 2 *a* and *b*) and the percentage of brilliantly fluorescent cells was estimated to be 5–7% on pictures at low magnification. Cells in which brilliant perinuclear fluorescence was observed were sometimes rounded, as shown by using a polyclonal anti-PV type 3 serum on IMR/S3 cells (Fig. 2*c*). Because the establishment of persistent infection may represent strong cell selection, the neuronal origin of persistently infected IMR/S1 and IMR/S3 cells was ascertained with a mAb that stains the 70- and 200-kDa polypeptides of human neurofilaments. Almost 100% of the cells were found to have characteristic cytoplasmic fluorescence (Fig. 2*e*).

Table 2. Comparative titration on neuroblastoma and HEp-2 cells of the extracellular virus from IMR/S3 cells

Time p.i., weeks	Average virus titer, $\log_{10}(ID_{50}/ml)$	
	IMR-32 cells	HEp-2 cells
1	8.8	9.2
3	6.3	6.5
6	7.3	6.0
11	7.7	4.6
20	7.0	4.2

The proportion of cells able to liberate virions was estimated by infectious center assays, as described in *Materials and Methods*. Five percent of IMR/S1 cells and 12–25% of IMR/S3 cells were found to synthesize and liberate virions. In IMR/S3 cells, no DNA complementary to the PV genome was detected by Southern blotting (28) under conditions in which 0.5 viral DNA molecule per cell would have been easily detected (not shown).

The kinetics of virion synthesis and liberation was studied in IMR/S3 cells over a period of 7 days between two cell trypsinizations. As shown in Fig. 3, virus synthesis did not follow the exponential cell growth curve. It occurred mainly between 1 and 4 days after trypsinization (Fig. 3).

Resistance of Persistently Infected IMR-32 Cells to PV Superinfection. Subconfluent IMR/S3 cells were superinfected at an moi of 6 ID_{50} per cell for 30 min at 37°C with each of the three Sabin strains to study the susceptibility of persistently infected cells to PV superinfection. No CPE was observed during the following days. One week after superinfection, no increase in virus titer could be detected in superinfected cells as compared to mock-superinfected IMR/S3 control cells. Since the second virus might have multiplied at a low level, titers of persistent virus and of the superinfecting virus were determined after type-specific seroneutralization. In IMR/S3 cells superinfected with PV Sabin 1 or Sabin 2, no multiplication of the superinfecting virus could be demonstrated. Similarly, no multiplication of PV Sabin 2 could be demonstrated in IMR/S1 cells superinfected at an moi of 6 or 120 ID_{50} per cell. Another enterovirus, coxsack-

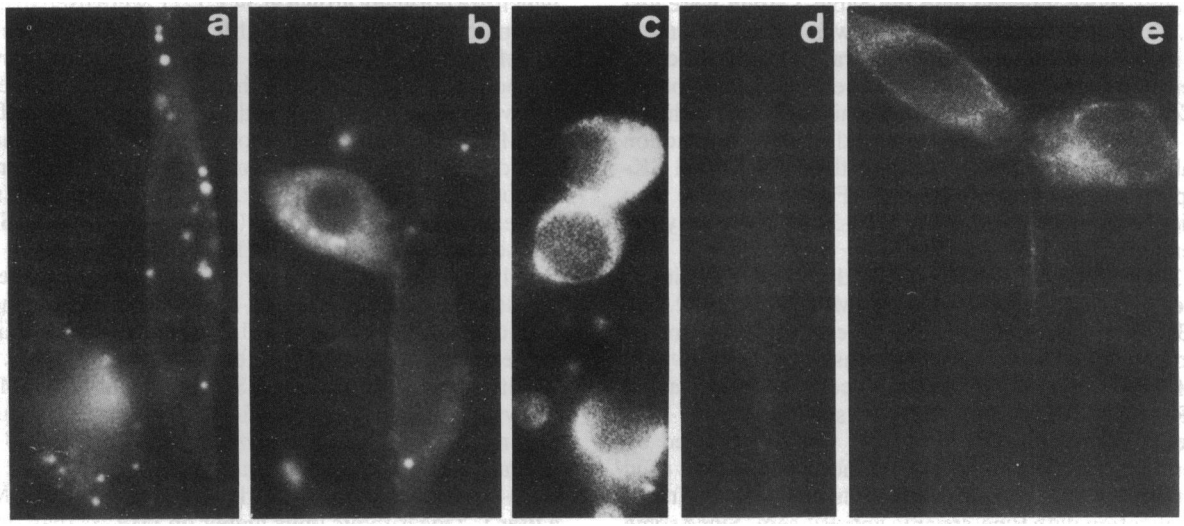

FIG. 2. Detection by indirect immunofluorescence of PV antigens and human neurofilaments in IMR-32 cells persistently infected with PV. (×450.) Viral antigens were detected with Sabin 1-specific mAb 1o in IMR/S1 cells (*a* and *b*) and with PV type 3 rabbit antiserum in IMR/S3 cells (*c*). No fluorescence was observed when mAb 1B5 (anti-human hepatitis A virus) was used as a control in IMR/S1 cells (*d*). Neurofilaments were stained with mAb 2F/11 in IMR/S1 cells (*e*).

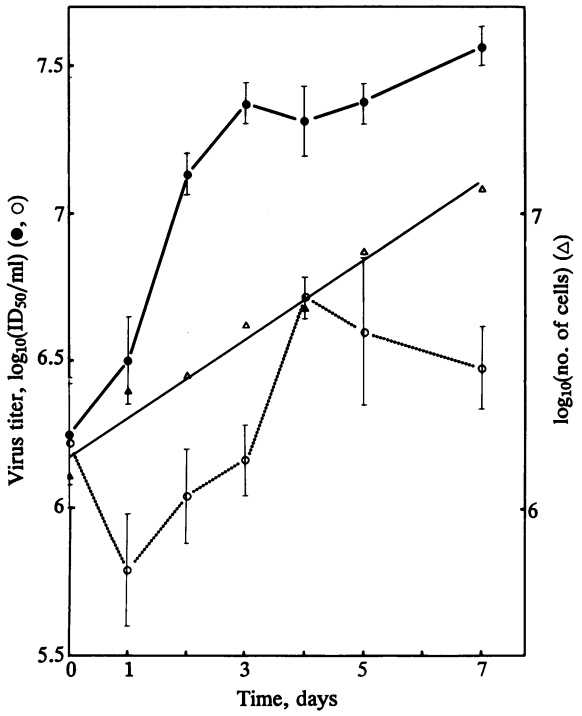

FIG. 3. Kinetics of virus synthesis in IMR/S3 cells between two subcultures. IMR/S3 cell cultures were split in a ratio of 1:3. At 3 hr (day 0) and the indicated days after trypsinization the culture medium was collected from two flasks, clarified, and frozen. Cells were counted (△ — △) and the intracellular virus was recovered by freezing and thawing. Extracellular (● — ●) and intracellular (○···○) virions were titered on IMR-32 cells. Results represent the mean of two independent experiments. The standard deviation is indicated by vertical bars.

ievirus B3, was found to multiply in PV-persistently infected IMR-32 cells (not shown).

DISCUSSION

PV, whether virulent or not, is usually cytolytic in primate tissue culture. At the end of the viral growth cycle, virions are liberated in a burst, when host cells lyse (29). We have shown here that PV can, however, induce a persistent infection of two human neuroblastoma cell lines. Persistently infected IMR-32 cells were obtained with the three serotypes of PV (Fig. 1) and with both virulent and attenuated strains. The neuronal origin of cells was ascertained by the detection of neurofilaments (Fig. 2). All IMR-32 cells have a receptor for PV, since at an moi higher than 100 ID_{50} per cell PV killed 100% of cells. This was confirmed by the observation that, in persistently infected cells obtained after infection at a lower moi, viral antigens were detected in all cells (Fig. 2). This type of infection thus differs from the carrier states previously reported (16, 17, 30). Virus titers in the medium of persistently infected cells were high ($10^{7.5}$ ID_{50}/ml when titered on IMR-32 cells), although an average of only one intracellular ID_{50} per cell was found 4 days after cell trypsinization (Fig. 3). All infected cell lines produced virus over a 9-month observation period. Since only 5–7% of IMR/S1 cells were brilliantly stained with anti-PV antibodies, the liberation of the majority of virions may occur in this percentage of cells at the end of a normal viral cycle, as previously shown to be the case for isolated monkey kidney cells infected with PV (29). The results of infectious center assays are in agreement with this hypothesis.

Persistently infected cells were resistant to superinfection by Sabin 1, 2, and 3 PV, but sensitive to another enterovirus, coxsackievirus B3. Because PV and coxsackieviruses have different receptors, our results suggest that one of the early steps of the PV growth cycle—i.e., adsorption, penetration, decapsidation—is probably involved in the resistance to PV superinfection. In contrast to results reported for persistent rabiesvirus infection (31), the synthesis of α or β interferon was not detected in persistently infected IMR-32 and SK-N-MC cells, since less than 3 units/ml was found in cell supernatants (J. L. Virelizier, personal communication).

The genome of the virus from persistently infected cells was highly mutated as shown by sequence analysis, epitope mapping, host cell specificity, and temperature resistance phenotype. Similar observations were reported with other viruses isolated from persistent infections *in vitro* and *in vivo* (5, 10, 32, 33). It has been proposed that progressive selection of viral variants contributes to the establishment of persistent infection (12, 18). The virus recovered from our persistently infected cells had a titer 1–3 logarithmic units higher on IMR-32 than on HEp-2 cells. This is reminiscent of an earlier observation: when a PV was serially passed in the monkey central nervous system, the recovered virus still induced poliomyelitis by intracerebral inoculation but was unable to grow in nonneural tissue culture (34). These results suggest that host cell-range mutants of PV can be selected in neural cells.

In conclusion, we have shown that PV, typically a cytolytic virus, can induce persistent infection of human cells of neuronal origin. This constitutes an *in vitro* model for investigating the relationships between neuronal cells and PV or other viruses, which are increasingly implicated in human neural diseases.

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