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Isolation and Characterization of HeLa Cell Lines Blocked at Different Steps in the Poliovirus Life Cycle

GERARDO KAPLAN, ALLISON LEVY, AND VINCENT R. RACANIELLO*

Department of Microbiology, Columbia University College of Physicians & Surgeons, New York, New York 10032

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Cotransfection of poliovirion RNA and R1, a poliovirus subgenomic RNA containing a deletion of nearly all of the capsid region, resulted in surviving cells, in contrast to the complete cell death observed after transfection with viral RNA. Cells that survived the cotransfection grew into colonies, produced infectious poliovirus, and underwent cycles of cell lysis (crisis periods) where less than 1% of the cells survived, followed by periods of growth. Poliovirus evolved during the persistent infection as judged by changes in plaque size. After passage for 6 months, a stable line called SOFIA emerged that no longer produced infectious virus and did not contain viral proteins or viral RNA. Cells frozen in liquid N_2 while still in crisis and recultured 4 months later (named SOFIA N2) were also stabilized. After infection with poliovirus, SOFIA N2 cells showed a delay in the development of cytopathic effect, viral production, and cellular death when compared with HeLa cells. In contrast, SOFIA cells did not develop cytopathic effect and produced 10,000 times less virus than SOFIA N2 or HeLa cells. Viral production was delayed in SOFIA and SOFIA N2 cells transfected with poliovirus RNA when compared with HeLa cells, suggesting the presence of an intracellular block to poliovirus replication. Analysis of the cellular receptor for poliovirus by virus binding, an enzyme-linked immunosorbent assay, and in situ rosette assays with an antireceptor monoclonal antibody showed that receptors were expressed in SOFIA N2 cells but not in SOFIA cells. Echovirus 6, an enterovirus which uses a different cellular receptor, formed small plaques on SOFIA cells. Vesicular stomatitis virus formed plaques of similar size on SOFIA and HeLa cells, suggesting that the intracellular block was specific for enteroviruses. Cotransfection of the subgenomic replicon R1 with poliovirion RNA therefore resulted in the selection of HeLa cell variants containing blocks to poliovirus replication at the level of receptor and within the cell.

Poliovirus infection of susceptible cells results in the rapid development of cytopathic effect (CPE), production of progeny virus, and cellular death. Although the viral requirements for replication have been studied for over 30 years and are beginning to be understood, far less is known about specific cellular requirements for infection. As obligate intracellular pathogens poliovirus and all viruses clearly require many host cell functions. It is known that poliovirus requires a cellular receptor to initiate infection, that at least two host proteins are involved in viral RNA synthesis in vitro, and that components of the translation machinery are employed to synthesize viral proteins (reviewed in reference 32). However the details of these requirements and whether other cell functions are needed for replication are not known.

One approach to studying host requirements for poliovirus infection is to isolate cell lines that are defective in the ability to support viral replication. Cell lines that are partially or totally resistant to picornavirus infection have been reported, although the mechanism of resistance is not known (1, 5, 10, 21, 22, 30, 31). In this study we selected HeLa cells resistant to poliovirus infection by cotransfecting an in vitro-synthesized subgenomic replicon (14) and poliovirion RNA. A cell line emerged after cotransfection which was unstable and produced virus during serial passage for 6 months. During this time the cells underwent alternating cycles of growth and death, stabilizing after 6 months coincident with the disappearance of the virus. Stabilization also resulted from freezing an early passage in liquid nitrogen and subsequent reculturing. Cell lines were isolated that supported poliovirus infection with different levels of efficiency. One line, SOFIA, was resistant to virus infection by virtue of a block to virus entry and an intracellular block to viral replication. Another line, SOFIA N2, contained only an intracellular block to viral replication and showed delayed CPE and low virus yields after infection. Study of such variants that differ in susceptibility to infection will permit elucidation of cellular functions needed for poliovirus replication.

MATERIALS AND METHODS

Cells and viruses. HeLa S3 cells were grown in spinner cultures and plated in 6-cm (3×10^6 cells per dish) or 15-cm (1×10^7 cells per dish) plastic cell culture dishes 24 h before use (16). L cells were grown in monolayers and maintained in Dulbecco modified Eagle medium (DMEM) containing 10% calf serum. Stocks of poliovirus P1/Mahoney, P2/Lansing, echovirus 6, and vesicular stomatitis virus (VSV) Indiana strain were prepared in 15-cm dishes containing confluent HeLa cell monolayers (16).

For infectious centers assay, monolayers were infected with poliovirus at a multiplicity of infection of 10. At 2 h postinfection, cell monolayers were dispersed by trypsin treatment, and 1 ml of 10-fold dilutions of cells in DMEM–0.9% Bacto-Agar (Difco Laboratories) was placed in another cell culture dish containing a subconfluent monolayer of HeLa cells. After hardening, 4 ml of DMEM–0.9% Bacto-Agar was added, and cells were incubated at 37°C for 48 h before staining with crystal violet (23).

Antisera. Anti-$3D^{pol}$ antiserum was obtained from rabbits immunized with a *trpE*-$3D^{pol}$ fusion protein produced in *Escherichia coli* (14). Rabbit anti-2C (3) and anti-$2A^{pro}$, gifts from D. Baltimore, were obtained from rabbits immunized with viral proteins purified from infected cells. Monoclonal

* Corresponding author.

antibody D171 directed against the HeLa cell poliovirus receptor (20) was purified by chromatography on protein A-Sepharose (Pharmacia Fine Chemicals) and diluted to a concentration of 1 mg/ml.

Transfection. Transfections and cotransfections of HeLa cells were performed as described previously (14). Briefly, 1/5 of a T7 transcription reaction (4 to 5 μg of RNA) was used to transfect each 6-cm cell culture dish by using DEAE-dextran as a facilitator. Cotransfections were performed with 1/5 of the T7 transcription reaction plus 500 ng of P1/Mahoney poliovirion RNA. Poliovirion RNA was prepared as described, by phenol–chloroform–1% sodium dodecyl sulfate extraction of purified poliovirus grown in HeLa cell spinner cultures (3, 23, 28).

ELISA. For the enzyme-linked immunosorbent assay (ELISA), confluent monolayers grown in 96-well plates were blocked with 3% bovine serum albumin–1% sodium azide in phosphate-buffered saline (PBS). Twofold dilutions of monoclonal antibody D171 in PBS were added to duplicate wells and incubated for 2 h at room temperature. Monolayers were washed with 1% bovine serum albumin in PBS, and 100 μl of a 1:350 dilution of anti-mouse polyvalent immunoglobulins-peroxidase conjugate (Sigma Chemical Co.) per well was added and incubated for 1 h at room temperature. *O*-Phenylenediamine dihydrochloride (Sigma) was used as a substrate and developed as recommended by the manufacturer.

In situ rosette assay. Poliovirus receptor-positive cells were visualized on monolayers by a modified in situ rosette assay (17). Subconfluent monolayers in 6-cm plates were treated with 3 ml of a 1:5,000 dilution of monoclonal anti-receptor antibody D171 in PBS. After 1 h of incubation at room temperature, monolayers were washed with PBS and treated with 50 μl of immunobeads coupled to rabbit anti-mouse immunoglobulin (Bio-Rad Laboratories) in 1 ml of PBS for 1 h at room temperature. Unbound beads were washed away with PBS, and receptor-positive cells were visualized with a phase-contrast microscope.

Cell protection assay. Confluent monolayers in 3-cm dishes were treated with 40 μg of monoclonal anti-receptor antibody D171 in 0.1 ml of PBS for 30 min at 37°C. Cells were infected with P1/Mahoney at a multiplicity of infection of 10 for 30 min at 37°C. Antibody and unadsorbed virus were washed away with PBS, medium was added, and monolayers were incubated at 37°C. Samples of the culture medium were taken at different times, and the virus titer was determined in HeLa cells.

Poliovirus binding assay. Confluent monolayers grown in 10-cm plates were infected with 10^4 PFU of P1/Mahoney in 0.3 ml of DMEM. Samples of the cell culture medium were taken at 0, 1, and 2 h postinfection for virus titration. The percentage of adsorbed virus was calculated as the mean of five independent experiments.

Analysis of [^{35}S]methionine-labeled polypeptides. Infected or persistently infected cell monolayers were washed with PBS and incubated for 45 min at 37°C in 2 ml of methionine-free DMEM, containing 50 μCi of [^{35}S]methionine (Amersham Corp.; 1,383 Ci/mmol). Cytoplasmic extracts were prepared and immunoprecipitated as described previously (14). Proteins were fractionated in 15% polyacrylamide–sodium dodecyl sulfate gels, stained with Coomassie blue, impregnated with Autofluor (National Diagnostics), dried, and exposed to Kodak X-Omat AR film with an intensifying screen for 3 to 10 days.

Analysis of RNA by blot hybridization. Cytoplasmic extracts prepared as described previously (24) were extracted with phenol–chloroform–1% sodium dodecyl sulfate and denatured with an equal volume of 22% formaldehyde–8× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C for 15 min. Samples were filtered onto nitrocellulose paper by using a slot blot apparatus (Schleicher & Schuell Co.). Filters were baked, prehybridized, and hybridized (18) to 5′-end-labeled synthetic oligonucleotides complementary to bases 711 to 728 of the P2/Lansing genome (5′-TAA CAA TGA GGT AAT TCC-3′) or complementary to bases 686 to 703 of the P1/Mahoney genome (5′-TAC TTA GAG TAA ACA CAC-3′).

For Northern (RNA) blot analysis, total cell RNA was prepared by the guanidine thiocyanate–CsCl technique (18), fractionated in 1% agarose–formaldehyde gels, transferred to nitrocellulose paper, and hybridized with a double-stranded cDNA labeled with ^{32}P by random priming as described (14). The cDNA fragment used as template extended from a unique *Bgl*II site (nucleotide 5,601) to the 3′ end of P1/Mahoney/41.

TABLE 1. Poliovirus titers at different passages

Passage	Viral titers (PFU/ml) during:	
	Normal growth	Crisis[a]
0	1.5×10^6	ND[b]
1	7.0×10^7	ND
2	4.0×10^6	ND
3	4.0×10^6	3.0×10^7
6	2.0×10^6	1.1×10^7
7	1.5×10^5	2.8×10^6
8	1.6×10^3	1.7×10^3[c]
9	4.0×10^2	—[d]
10	$<2.0 \times 10^2$	—[d]
12	$<2.0 \times 10^2$	—[d]
15	$<2.0 \times 10^2$	—[d]

[a] Except where indicated, 99% of cells died.
[b] ND, Not determined.
[c] In passage 8, 20% of the cells died.
[d] —, No crisis.

RESULTS

Establishment of a persistent poliovirus infection in HeLa cells. HeLa cells were cotransfected with poliovirion type 2 RNA and R1, an in vitro-synthesized poliovirus subgenomic replicon which contains a deletion of nearly all the capsid region (14). At 48 h posttransfection several surviving cells remained, whereas no cells were observed in control plates transfected only with poliovirion RNA. The culture medium was changed, and surviving cells were incubated at 37°C. The cells grew to colonies 1 to 5 mm in diameter and underwent a crisis period during which 99% of the cells died. The surviving cells recovered and grew again until the next crisis period; this pattern continued for 6 months, during which time cells were subcultured 1:2 every 20 days. Cells from passage 3 were frozen in the presence of 10% dimethyl sulfoxide and stored in liquid nitrogen.

No cell death was observed after passage 8, even though small amounts of virus were present in passage 9 (Table 1). An infectious center assay revealed that only 1 out of 10^5 cells was infected at this passage, characteristic of a carrier state type of persistent infection (13). After 6 months (passage 9) the cells grew without periods of crisis, and starting with passage 10 fewer than 2×10^2 PFU could be detected in

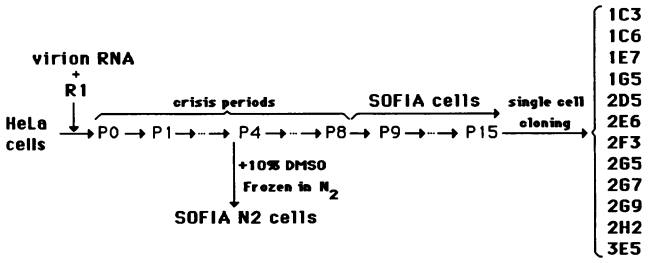

FIG. 1. Origin of SOFIA and SOFIA N2 cell lines. HeLa cells were cotransfected with virion RNA and the in vitro-synthesized subgenomic replicon R1, and the surviving cells were propagated. Crisis periods occurred in the first eight passages (P0 to P8); SOFIA N2 cells were isolated from cells that had been frozen at passage 3. Cells stabilized in passage 9 (SOFIA cells), from which 13 single cell clones were derived at passage 15. DMSO, Dimethyl sulfoxide.

cell supernatants. This cell line was named SOFIA (Fig. 1). Curiously, when frozen cells from passage 3 were thawed, they also grew without crisis periods and without producing virus, an unexpected finding since at this passage virus was present before freezing (Table 1). This cell line was named SOFIA N2 (Fig. 1).

Viral evolution during the persistent infection. Titers of cell supernatants from different passages were determined by plaque assay in HeLa cells. During the first six cell passages viral production remained constant (approximately 10^6 to 10^7 PFU/ml), and decreased by 4 $\log_{10}$ PFU in the next three passages before falling to undetectable levels in passage 10 (Table 1). Viral titers rose by approximately 1 $\log_{10}$ unit during crisis periods, in which 99% of the monolayer was destroyed, except during the crisis in passage 8, where only 20% of the cells were destroyed. The observation that stabilization coincided with loss of virus suggested that cell destruction during the previous crisis periods was due to viral replication.

Rapid evolution of viral populations during persistent infections has been reported previously (33). In the system described here similar variation was observed, as measured by changes in plaque morphology. Virus produced during the first passages of the persistent infection contained a higher proportion of small plaque variants compared with the parental P2/Lansing (Fig. 2; passage 2). Supernatants from passage 8 formed plaques of heterogeneous size and caused diffuse destruction of the monolayer, presumably due to the presence of minute plaque variants (Fig. 2). The appearance of such minute plaque variants preceded the stabilization of the cells and elimination of virus. These variants are clearly defective in some step of viral replication, and their presence may be related to the stabilization of the cells (33).

Analysis of viral proteins and RNA in persistently infected cells. Cells from passages 5, 7, and 8 were labeled with [^{35}S]methionine for 45 min, and cytoplasmic extracts were prepared and immunoprecipitated with anti-3D^{pol} and anti-2C antisera (Fig. 3). Viral polypeptides from the P2 and P3 regions of the viral genome, as well as from the P1 region. (data not shown), were identified by comparison with proteins in HeLa cells infected with wild-type virus. For example, 3CD, 3D^{pol}, and 2C were all readily detected in cells of passages 5, 7, and 8 (Fig. 3A, lanes 1 and 4; Fig. 3B, lanes 1, 2, 5, and 6). In addition immunoprecipitation with anti-2A^{pro} antisera showed that 2A^{pro} from the persistently infected cells comigrated with that produced in infected HeLa cells (data not shown). It appears that in passage 5 the precursor

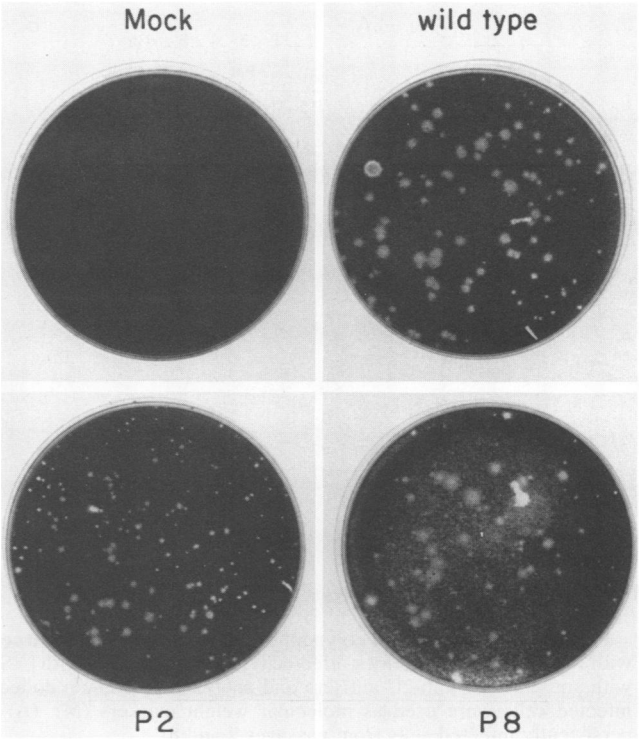

FIG. 2. Viral evolution during persistent infection. Plaque morphology of P2/Lansing and virus present in persistently infected cells at passages 2 (P2) and 8 (P8) on HeLa cell monolayers. Note the diffuse cell destruction caused by the passage 8 supernatant, presumably caused by a minute plaque variant.

3CD accumulates with respect to 3D^{pol}, 3C′, and 3D′, when the persistently infected cells are compared with infected HeLa cells (Fig. 3A, compare lanes 1 and 2). Perhaps the limited proteolysis of such a precursor plays a role in the establishment and maintenance of the persistent infection. No viral proteins were detected in passage 10 (data not shown), which correlates with the absence of virus in the cells at this passage (Table 1).

To determine whether replicon R1 sequences were present in persistently infected cells, total RNA from passages 7 and 8 and RNA from infected HeLa cells were subjected to Northern blot analysis. A similar size distribution of poliovirus-specific sequences was observed, beginning with material migrating at the position expected for full-length genomes and smearing down to molecules less than 2 kilobases in length (Fig. 4, compare lanes 1 and 2 with lanes 3 and 5). No specific band corresponding to R1 (5.1 kilobases) or smaller was detected in passages 7 and 8. Note that nonspecific trapping by 28S and 18S rRNAs is responsible for the appearance of RNAs at these positions in passages 7 and 8 as well as in infected HeLa cells. Slot blot analysis of total RNA obtained from cytoplasmic extracts of passages 7, 8, and 9, employing type 1- or type 2-specific oligonucleotide probes, revealed the presence of P2/Lansing but not P1/Mahoney sequences (Fig. 5). This result confirms that R1, which was derived from P1/Mahoney, was not present in these passages, and that no other subgenomic replicons or defective interfering particles were generated during the persistent infection. Furthermore no poliovirus-specific RNA was detected in passage 10, confirming that at this point cells were free of poliovirus.

Analysis of viral replication in SOFIA and SOFIA N2 cell

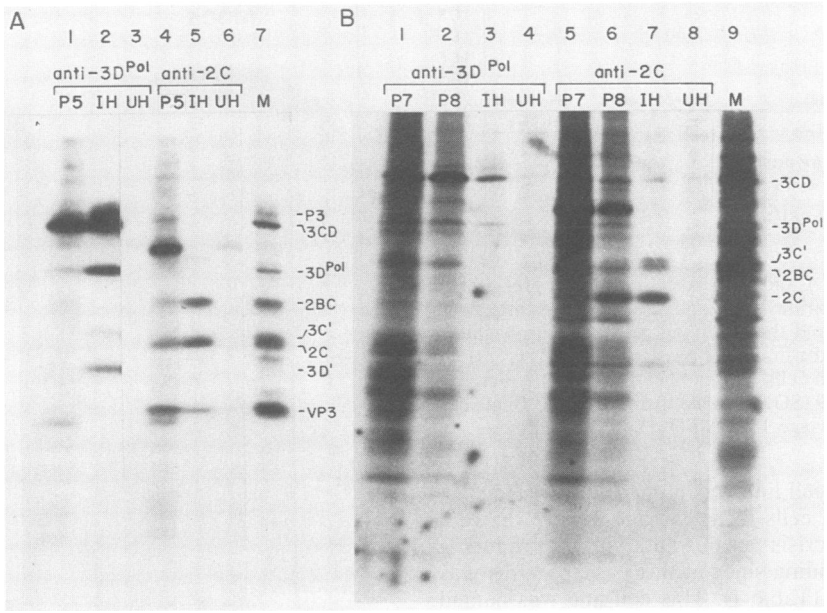

FIG. 3. Analysis of viral polypeptides in persistently infected cells. Cells from passages 5, 7, and 8 (P5, P7, and P8) and HeLa cells infected with P2/Lansing (IH) or mock infected (UH), were labeled with [^{35}S]methionine. Cytoplasmic extracts were prepared and immunoprecipitated with anti-3D^{pol} or anti-2C antisera and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cytoplasmic extracts from infected cells were used as molecular weight markers (M). (A) Analysis of persistently infected cells from passage 5. (B) Analysis of persistently infected cells from passages 7 and 8.

lines. Viral replication in SOFIA and SOFIA N2 cell lines was studied in one-step growth experiments (Fig. 6). No detectable CPE and very low levels of virus production (4 $\log_{10}$ fewer PFU than in HeLa cells) was observed in SOFIA cells infected with P1/Mahoney, and the virus did not form plaques on SOFIA monolayers. Infected SOFIA N2 cells showed a delay in the appearance and development of CPE but eventually produced as much virus as did HeLa cells. The efficiency of plating of P1/Mahoney on SOFIA N2 and HeLa cells was similar, except that plaques were smaller in SOFIA N2 cells (data not shown).

To determine whether the altered pattern of replication in SOFIA cells was due to early events in the viral life cycle, the processes of adsorption, penetration, and uncoating were bypassed by transfecting cells with poliovirion RNA (Fig. 7). Production of virus in transfected SOFIA and SOFIA N2 cells was markedly slower than in HeLa cells. However, SOFIA N2 cells produced nearly as much virus as HeLa cells, as found previously for virus infection. In contrast, transfected SOFIA cells produced nearly 6 $\log_{10}$ PFU/ml compared with 4.5 $\log_{10}$ PFU/ml when infected (Fig. 6), although this level was still lower than that observed for HeLa cells. These results suggest that both cell lines contain an intracellular block to poliovirus replication; in addition SOFIA cells contain a block to virus entry.

Virus binding studies were performed to further define the block to virus entry in SOFIA cells. Monolayers were

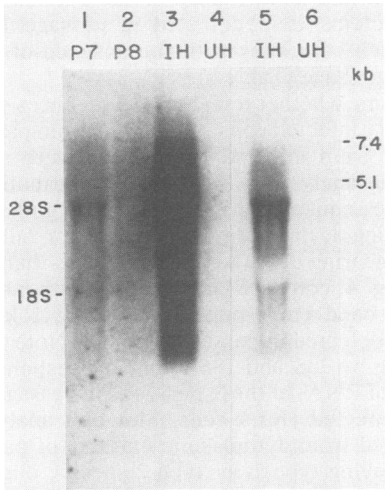

FIG. 4. Northern blot analysis of poliovirus RNA in persistently infected cells. Total cellular RNA was prepared from cells of passages 7 (P7) and 8 (P8) and from HeLa cells infected with P2/Lansing (IH) or mock infected (UH). RNAs were fractionated in a 1% agarose–formaldehyde gel, blotted onto nitrocellulose, and hybridized with a double-stranded ^{32}P-labeled cDNA probe representing the 3′ end of the P1/Mahoney genome. Lanes 1 to 4 are from a 24-h exposure; lanes 5 and 6 are the same as 3 and 4 but were exposed for 3 h.

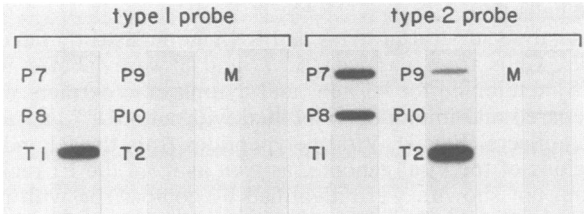

FIG. 5. Slot blot analysis of poliovirus RNA in persistently infected cells. Cytoplasmic extracts of cells from passages 7 to 10 (P7 to P10) and from HeLa cells infected with P1/Mahoney (T1) or P2/Lansing (T2) or mock infected (M) were denatured with formaldehyde-SSC, slot blotted on nitrocellulose paper, and hybridized with a 5′-end-labeled oligonucleotide specific for P1/Mahoney (type 1 probe) or for P2/Lansing (type 2 probe).

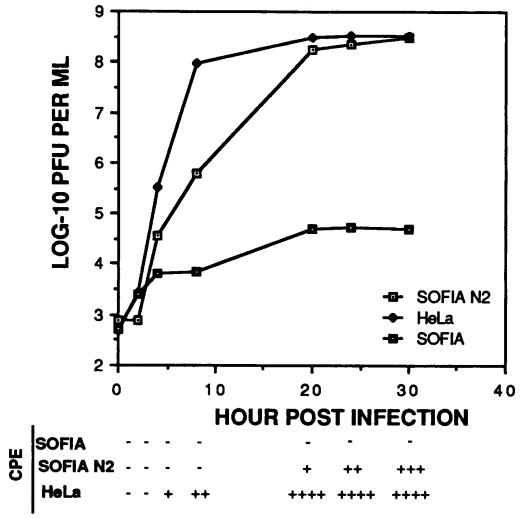

FIG. 6. Growth of poliovirus in SOFIA and SOFIA N2 cells. Monolayers of SOFIA, SOFIA N2, and HeLa cells were infected with P1/Mahoney at a multiplicity of infection of 20. Cell supernatants were sampled at the times indicated, and virus titers were determined by plaque assay in HeLa cells. CPE was estimated at each time point: −, no change; ++++, total CPE.

incubated with small amounts of virus (10^4 PFU), and the unbound virus was quantitated (Table 2). SOFIA cells bound similar amounts of virus (6%) as L cells, a mouse cell line known to lack poliovirus receptors. In contrast, SOFIA N2 and HeLa cells bound >70% of input virus. These results were confirmed with an in situ rosette assay with monoclonal antibody D171, directed against the HeLa cell receptor for poliovirus (20). Beads coated with anti-mouse antiserum adhered to D171-treated SOFIA N2 and HeLa cells but not to SOFIA cells (Fig. 8). Lack of receptors in SOFIA cells was also demonstrated by an ELISA with D171 (Fig. 9). It therefore appears that the absence of functional receptors plays a role in the resistance of SOFIA cells to poliovirus infection.

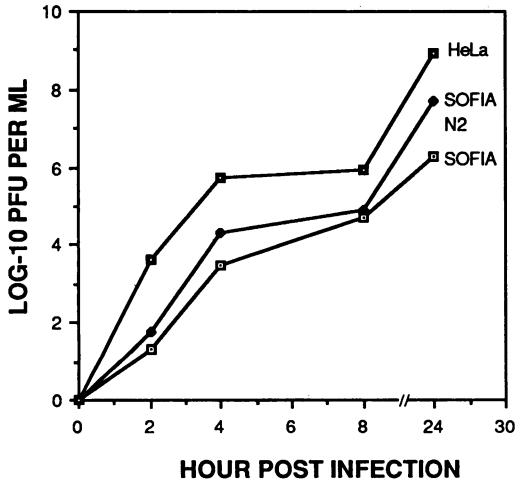

FIG. 7. Growth of poliovirus after RNA transfection of SOFIA and SOFIA N2 cells. SOFIA, SOFIA N2, and HeLa cells were transfected with 500 ng of P1/Mahoney poliovirion RNA. Cell supernatants were sampled at the times indicated, and virus titers were determined by plaque assay in HeLa cells.

TABLE 2. Poliovirus binding assay

Cells	% of virus bound to cells[a]	
	1 h	2 h
SOFIA	3 ± 5	6 ± 11
SOFIA N2	70 ± 4	79 ± 11
HeLa	74 ± 3	83 ± 2
L	3 ± 5	4 ± 7

[a] Mean of five independent assays and standard deviation.

Characterization of clones derived from SOFIA cells. The results shown in Fig. 6 raised the possibility that the SOFIA cell line was a mixture of cells that differed in susceptibility to poliovirus infection. To address this possibility, 13 cell lines were isolated by single-cell cloning. One of these clones, 1C6, was susceptible to poliovirus infection but produced low virus yields—3×10^5 PFU/ml, compared with 3×10^8 PFU/ml for the same number of HeLa cells—in the absence of CPE (data not shown). In addition, an infectious center assay revealed that only 0.05% of 1C6 cells produced virus after infection at a multiplicity of infection of 10. When transfected with poliovirion RNA all clones displayed reduced viral replication efficiency, as observed for the parental SOFIA cells (Fig. 7).

None of the SOFIA clones reacted with antireceptor antibody D171 in the in situ rosette assay or the ELISA, including clone 1C6, which appeared to be slightly susceptible to infection. A protection assay was performed to determine whether infection of 1C6 cells was mediated by a receptor recognized by D171. Monolayers of 1C6, HeLa, and L cells were treated with antibody D171 for 30 min and then infected with poliovirus. The inoculum was removed, and medium was added; samples were taken at different times postinfection, and titers were determined in HeLa cells. At 24 h postinfection HeLa cell monolayers treated with antibody D171 produced 2 $\log_{10}$ less virus than did monolayers treated with immunoglobulin G or not treated at all (Fig. 10). 1C6 cells were completely protected by D171, since treatment reduced virus yield to the background levels observed in L cells. Therefore poliovirus entry of 1C6 cells is mediated by the cellular receptor recognized by antibody D171.

To determine whether SOFIA cell lines contained major membrane alterations that also affected binding of other viruses, the cells were infected with two viruses that employ different receptors: echovirus 6, an enterovirus; and VSV, a member of the family *Rhabdoviridae* (Fig. 11). In contrast to poliovirus, echovirus formed plaques on uncloned SOFIA cells and all SOFIA clones but one, 2H2. However, plaques were smaller than those formed on HeLa cells, suggesting that these cells contain an intracellular block to replication of both poliovirus and echovirus. All cell clones were susceptible to VSV infection, and the resulting plaque size was similar to that observed in HeLa cells. Therefore VSV is not affected by the receptor alteration or the internal blocks found in SOFIA and its cloned derivatives.

DISCUSSION

Several carrier-state and steady-state types of persistent infections involving picornaviruses have been reported (4, 7–10, 12, 19, 26, 27, 29). Although poliovirus is highly cytocidal in HeLa cells, persistent infections have been established by supplying antipoliovirus antisera (1, 30) or by washing monolayers extensively (22). Poliovirus-resistant HeLa cells that

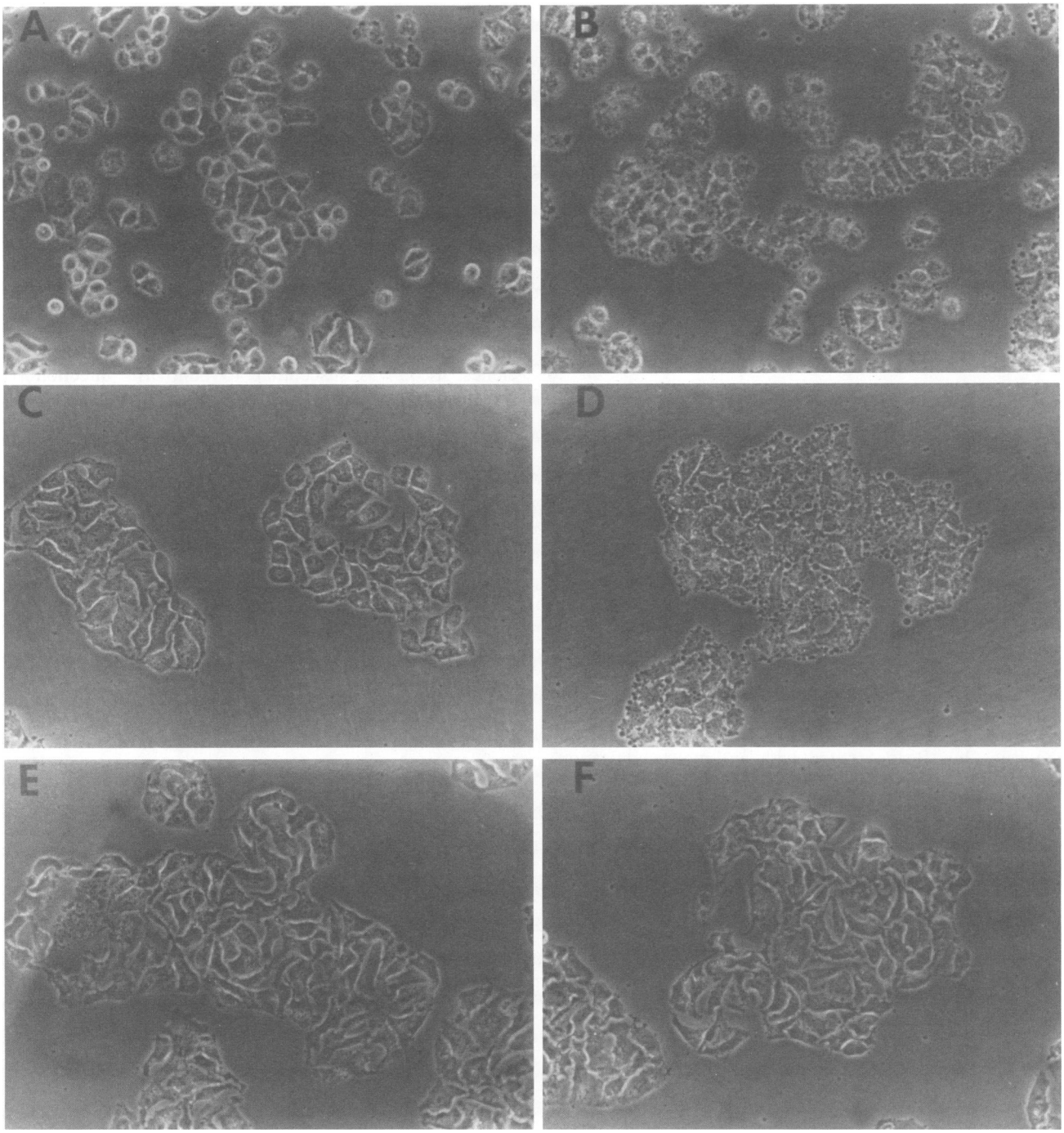

FIG. 8. In situ rosette assay for detection of cellular receptor for poliovirus. HeLa (A, B), SOFIA N2 (C, D), and SOFIA (E, F) cells were treated with monoclonal antibody D171 directed against the cellular receptor for poliovirus (B, D, F), or not treated with antibody (A, C, E). Beads coupled to anti-mouse antibodies were used to detect D171 on cell surfaces.

could not bind poliovirus were isolated from cells that survived infection with P1/Sabin (6). Persistent poliovirus infections have been established with cells of different origin (1, 5, 11, 21, 22, 30, 31), and susceptibility of human blood cell lines to poliovirus and the establishment of persistent infections has been linked to the cellular differentiation stage (11, 15, 21, 31).

Here we report that cotransfection of an in vitro-synthesized poliovirus subgenomic replicon and poliovirion RNA leads to the establishment of persistently infected HeLa cells, which then stabilize to yield cell lines containing different blocks to poliovirus replication. Defective interfering particles have been associated with the establishment of persistent infections (13), and it was recently reported that the subgenomic replicons used in this study may interfere with poliovirus replication (14). However the role of the subgenomic replicon R1 in the establishment of persistently infected cells is not known. Since no HeLa cells survived

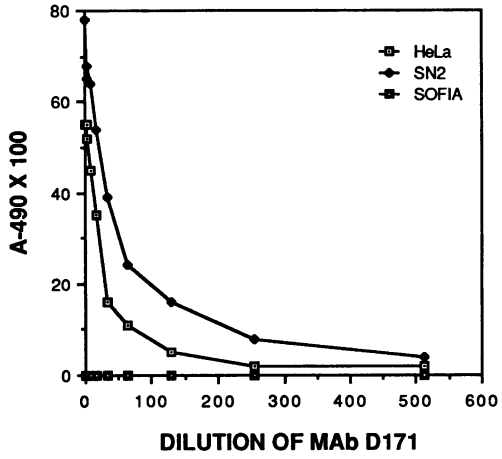

FIG. 9. ELISA for detection of cellular receptor for poliovirus. SOFIA, SOFIA N2 (SN2), and HeLa cell monolayers in 96-well plates were treated with twofold dilutions of monoclonal antibody D171, directed against the cellular receptor for poliovirus. After 2 h of incubation at room temperature, monolayers were washed three times with PBS–1% bovine serum albumin and incubated for 2 h with peroxidase-conjugated anti-mouse antibody. *O*-Phenylenediamine dihydrochloride was used as substrate, and the A_{490} was read.

after transfection of poliovirion RNA alone, it is possible that R1 either caused an interference state which allowed cells to survive viral infection or permitted, by complementation, the replication of defective poliovirus RNAs which otherwise would not have replicated and which established the persistent infection. However, it is unlikely that the persistence of virus was due solely to the replication of defective viral genomes in normal cells, because (i) no HeLa cell survived infection with supernatants from persistently infected cells (data not shown), and (ii) subgenomic replicons were not detected in persistently infected cells. Thus, although R1 may have played a role in the initiation of the persistent infection, it was not present as the cells evolved. This situation is in contrast to that observed in cells persistently infected with foot-and-mouth disease virus, in which naturally occurring subgenomic replicons of decreasing length were generated upon cell passage (8–10).

It is not likely that SOFIA cells existed in the original HeLa cell population; rather these cells were probably

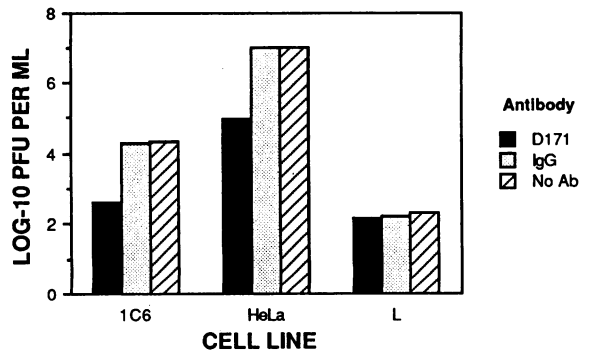

FIG. 10. Protection of 1C6 cells from poliovirus infection with D171 antibody. Monolayers of HeLa, L, and 1C6 cells were treated with monoclonal antibody D171 for 30 min and then infected with P1/Mahoney at a multiplicity of infection of 10. After 30 min of adsorption, monolayers were washed extensively. Supernatant samples were taken at 24 h postinfection, and titers were determined in HeLa cell monolayers.

selected during the evolution of the persistent infection. First, if SOFIA cells were present in HeLa cell stocks, then it would be expected that a fraction of cells would always survive transfection with poliovirus RNA. However we found that no cells survived poliovirus RNA transfection unless R1 was present. Second, the persistently infected cells underwent 6 months of crisis before the establishment of a stable line. If SOFIA cells existed in the HeLa cell population, they should be able to grow immediately without entering the crisis phase.

How did passage of the persistently infected cells for 6 months result in a stable cell line? The answer to this question is not known, but probably a combination of viral and cellular evolution was involved. Coevolution of virus and cells has been observed in persistent infection of BHK cells with foot-and-mouth disease virus (9), of L cells with reovirus (2), and of L cells with lymphotropic minute virus of mice (25). Furthermore crisis periods have also been reported in other viral systems in which coevolution of cells and virus took place (2, 9, 10). The emergence of viral mutants with a small plaque phenotype just before the loss of infectious virus suggests that replication-deficient viral mutants were selected, which perhaps could not efficiently kill cells (33). Analysis of the stabilized cell lines SOFIA and SOFIA N2 indicated that both cell lines contain intracellular blocks to viral replication, whereas the former also lack functional viral receptors. SOFIA N2 cells do not appear to contain a block to virus binding, and it is not likely that these cells are deficient in aspects of penetration and uncoating, since they produced numbers of infectious centers similar to those produced by HeLa cells (data not shown). Thus a combination of viral and cell evolution toward resistance to infection, perhaps initiated by R1-mediated interference, resulted in the stabilized SOFIA cell line.

It is not clear how freezing of the persistently infected cells resulted in the elimination of infectious virus and stabilization of the cells. Perhaps dimethyl sulfoxide, which was employed as an antifreezing agent, protected cells from infection. It has been shown that treatment of the human blood cell line HL-60 with dimethyl sulfoxide induces both differentiation and loss of susceptibility to poliovirus, suggesting that this drug might regulate cellular genes involved in poliovirus replication (15).

Infection of SOFIA cells resulted in the production of a very small amount of virus in the absence of CPE, suggesting that SOFIA cells might be a mixture of virus-resistant and -susceptible cells, which was confirmed by studying cell lines isolated by single cell cloning. One clone (1C6) out of 13 produced poliovirus, yielding 4 $\log_{10}$ fewer virus than HeLa cells. None of these clones contained the D171 epitope as determined by the in situ rosette assay or the ELISA (data not shown). However virus production in clone 1C6 was abolished by treatment of cells with monoclonal antibody D171, suggesting that these cells express low levels of the cellular receptor.

Echovirus 6 formed small plaques in uncloned SOFIA cells and in 12 of the 13 cloned cell lines. Since echovirus employs a different receptor than poliovirus, this result indicates that SOFIA cells do not contain gross membrane alterations that prevent poliovirus infection. However, the fact that echovirus 6 formed small plaques on these cells indicated that it could not bypass the internal block to replication in SOFIA cells. VSV, a negative-stranded RNA virus of a different family, replicated normally in SOFIA cell lines and in all clones, suggesting that the internal block(s) in these cell lines may be specific for enteroviruses.

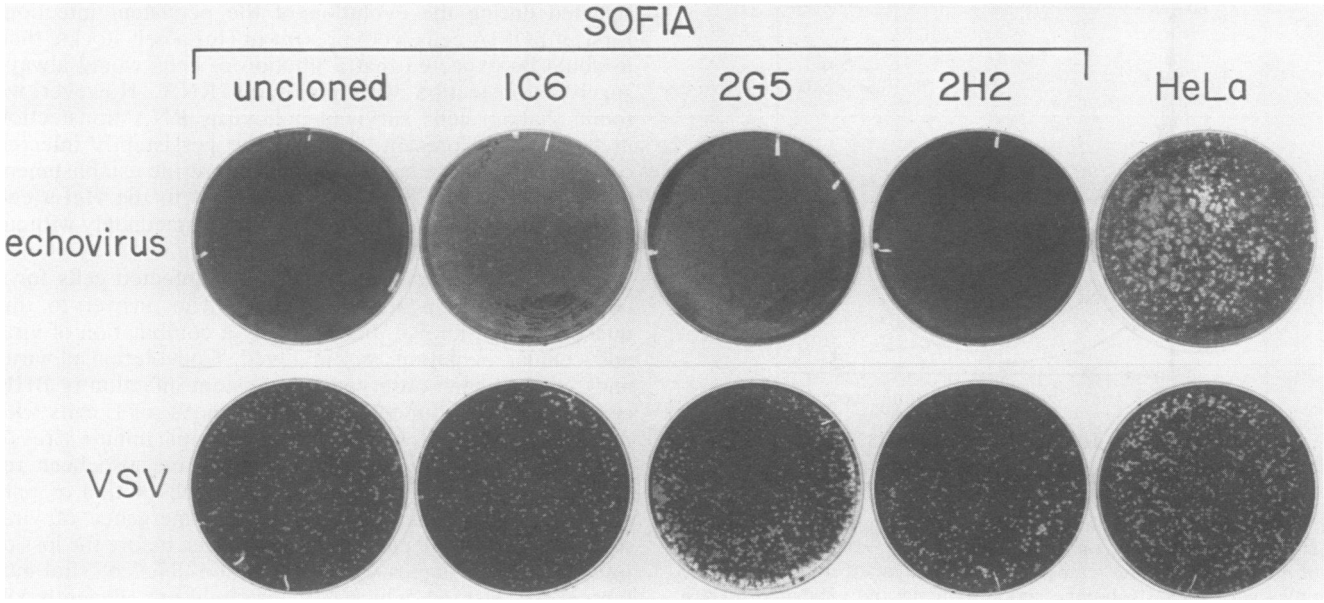

FIG. 11. Susceptibility of SOFIA cells to echovirus and VSV. Monolayers of uncloned SOFIA cells and cloned lines 1C6, 2G5 and 2H2, and HeLa cells were infected with echovirus 6 or VSV Indiana strain and incubated under a solid overlay. After 48 h at 37°C, the overlay was removed, and monolayers were stained with crystal violet.

The cell lines generated in this study will be of interest for elucidating different aspects of poliovirus replication. It will be of interest to determine why poliovirus receptors are absent on SOFIA cells, and a study of this question may provide information on the regulation of expression of the receptor gene. Furthermore an unravelling of the basis for the intracellular inhibition of poliovirus replication in SOFIA cells may identify host factors required for poliovirus replication.

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