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NOTES

Persistence of Echovirus 6 in Cloned Human Cells

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Establishment of a persistent infection by echovirus 6 in cloned human WISH cells (PI) was demonstrated. The cloned human WISH cells were maintained for 3.0 years (over 125 passages) and released virus continuously without cellular destruction. Neither temperature-sensitive virus mutants nor interferon appears to play a role in either establishment or maintenance of viral persistence. The majority of the virus produced by cloned human WISH cells is defective (2×10^6 particles per PFU) and differs from standard virus in its polypeptide profile and its inability to attach to parental WISH cells.

Many viruses produce persistent infections in humans and animals (20). Although human enteroviruses are usually cytolytic, they can establish persistent infections in vivo (4, 17–19) as well as in vitro (1, 3, 9, 13–15). Persistence of certain echoviruses and polioviruses in the central nervous system leads to development of progressive encephalitis in some individuals (4, 17, 18). The mechanisms involved in persistence are poorly understood. Viral persistence has been ascribed to (i) the presence of defective virus particles, (ii) the evolution of virus mutants, (iii) the presence of interferon or antiviral antibody, (iv) phenotypic or genotypic alterations of the cell, or (v) some combination of these events (5, 6, 16, 20). In vitro systems provide a useful approach to the identification and study of factors that regulate viral persistence.

Persistent in vitro virus infections have been categorized into two major groups in which either all of the cells are infected or only a small fraction is infected (6, 16). In this paper, we demonstrate the establishment of a steady-state infection of cloned human cells by echovirus 6 and describe the properties of this persistence.

Persistent infection of the WISH cell line (ATCC CCL-25) was produced by the inoculation of cell monolayers with plaque-purified echovirus 6 (2) at a high multiplicity of infection (10 to 30 PFU per cell). Most of the cultured cells were destroyed in 24 h at 37°C. Surviving cells were grown for 8 days, challenged with a similar dose of virus, and cloned by the limiting dilution method (10). The recovered clones that survived a third exposure to echovirus 6 (14 of 24) were passaged serially three to eight times and tested for virus production in WISH cells. Cytolytic virus was recovered from the medium of all 14 clones. Neutralization of viral infectivity by specific echovirus 6 antiserum (D'Amori) identified the isolated viruses as echovirus 6. Plaque titers of the supernatant fluid of the clones ranged from 0.3×10^6 to 2.5×10^6 PFU per ml. The cell clone, designated PI-P, was selected for further studies. PI-P cells were indistinguishable from uninfected WISH cells with respect to morphology and growth pattern. However, PI-P cells released virus (2×10^6 to 3×10^6 PFU per ml) and remained resistant to cytolysis by superinfecting doses of echovirus 6 (30 PFU per cell). Antiviral antibodies were not required for the establishment of this persistent infection.

The PI-P cells were cultured in monolayers for ca. 3 years (over 125 passages) in the absence of viral antiserum. Supernatants of the cultures were collected before each passage and examined for viral infectivity. The PI-P cells continuously synthesized and released virus without cellular destruction. Infectious virus was recoverable from the culture medium within 2 days of passage. Peak virus titers (1.6×10^6 to 2.5×10^6 PFU per ml) were attained after cultivation of monolayers for 3 to 4 days in the same medium. Comparable virus titers (2×10^6 PFU per ml), as measured on WISH cells, were obtained before and after storage of the PI-P cells at passage number 2 for 1 year at −160°C. A subline of these cells (PI-NP) was recovered from another batch of cells frozen at passage 7 for 2 years. These cells did not produce detectable infectious virus and were maintained in culture for 1 year (ca. 30 passages).

The number of persistently infected cells was estimated by indirect immunofluorescence with monkey anti-echovirus 6 (D'Amori) serum as the primary antibody and fluorescein-conjugated goat anti-monkey immunoglobulin G (Cappel Laboratories, Cochranville, Pa.) as the secondary antibody. The PI-P cells were examined at passages 40 and 67, whereas the PI-NP cells were examined at passage 13. Mock-infected WISH cells inoculated with maintenance medium and acutely infected WISH cells inoculated with echovirus 6 at a multiplicity of infection of 16 PFU per cell served as negative and positive controls, respectively. Inoculated cells were incubated for 8 h at 37°C. The virus-infected parental WISH cells as well as the PI-P cells displayed cytoplasmic and perinuclear fluorescence, which was absent in uninfected cells (Fig. 1). The virus inoculum for WISH cells was sufficient to infect 100% of the cells, and 94% of the examined cells (6,007 of 6,382) exhibited fluorescence and cytopathology. At the passages 40 and 67, 86% (899 of 1,064 and 3,921 of 4,528, respectively) of the PI-P cells were scored as positive for echovirus 6. Although not shown, viral antigens were also expressed by most of the PI-NP cells (1,269 of 2,041).

The proportion of PI-P cells that produced lytic virus was estimated by infectious center assays. PI-P cells and uninfected and acutely infected (36 PFU per cell) WISH cells were incubated for 3 h at 37°C. Unattached virus was

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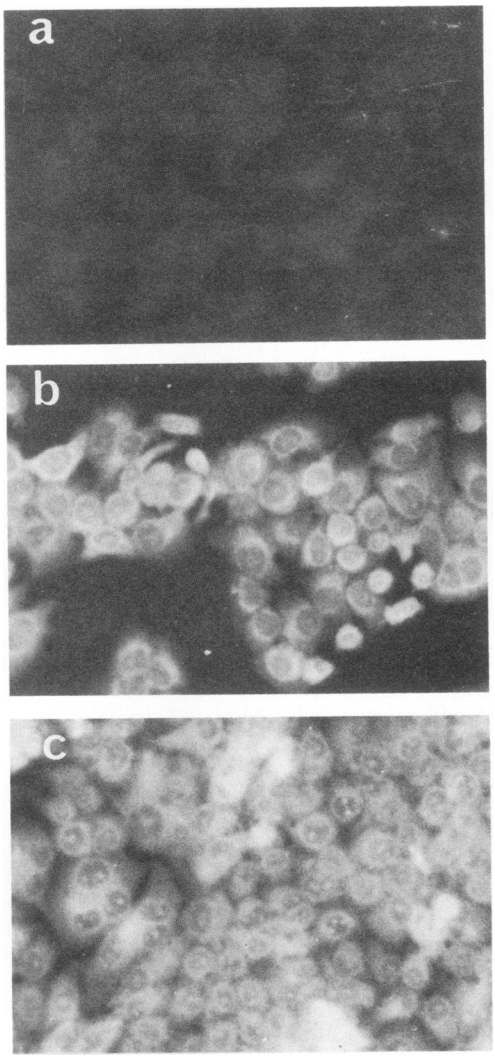

FIG. 1. Indirect immunofluorescent staining of uninfected (a) and acutely infected (b) WISH cells and persistently infected (c) PI-P cells. Cells were inoculated with either Eagle minimum essential medium containing 5% calf serum or echovirus 6 (16 PFU per cell) and incubated for 8 h at 37°C before fixing with acetone (at 4°C) and staining.

removed by washing monolayers five times with phosphate-buffered saline, and residual virus was neutralized with echovirus 6 antiserum before harvest of cells with trypsin (0.25%) and EDTA (0.02%). Inocula containing 50, 40, 20, and 10 PI-P cells or acutely infected WISH cells were each seeded on four replicate monolayers of WISH cells. Recovery of infectious centers from the acutely infected WISH cells was high; an average of 93% of the cells produced plaques, representing 85 to 100% recovery of infected cells (Table 1). In contrast, only 37% of the recovered PI-P cells produced infectious centers, with a variance of 2.5% over the fivefold difference in cell numbers plated. Virus yields from WISH cells were calculated to be ca. 300 to 500 PFU per cell, whereas the average yield of lytic virus from PI-P cells was 7 PFU per cell.

The effect of incubation temperature on virus persistence in PI-P cells was examined. Monolayers of PI-P cells that were grown and maintained at 37°C were subjected to lower or higher temperatures at different passage levels and then returned to 37°C. When PI-P cell monolayers at passages 26, 42, and 67 were incubated for 3 days at 40°C, they did not exhibit morphological changes and continued to produce virus (10^5 to 10^6 TCD_{50}/ml). Incubation of PI-P passage-26 and -35 monolayers at 34°C for 3 days and passage-42 monolayers at 33°C for 14 days did not alter cellular integrity, morphology, or the ability of cells to produce virus. The virus titers recovered from all of the described cultures after incubation at 37°C for 2 to 3 days ranged from 1.8×10^6 to 2.5×10^6 PFU per ml. Thus, the persistence of echovirus 6 in PI-P cells was not dependent upon the temperature of incubation.

Although antiviral antibodies were not required for maintenance of persistent infection in PI-P cells, the possibility that viral antiserum might cure the persistent infection of these cells was examined. PI-P cells at passages 6 and 50 were grown and maintained for 23 and 18 days, respectively, in the presence of an excess of echovirus 6 antiserum. The cultures underwent at least eight generations during the period of treatment. Untreated PI-P cells were handled in parallel as controls. The cells were fed every 2 to 3 days with fresh medium supplemented with either preimmune or virus-immune serum. Culture supernatants were screened for virus during and after antibody treatment. The cell monolayers remained intact throughout the experimental periods, and there was no detectable change in cell size or morphology as a result of treatment with viral antiserum. Virus was not detected in the culture supernatants from cells exposed to antiviral antibodies. When the cells were washed and incubated in medium lacking antiviral antibodies, virus was detected in the medium within 2 days. The recovered virus titers (2×10^6 PFU per ml) were similar to those recovered from PI-P cells that never received antibody treatment (1.6×10^6 to 2.5×10^6 PFU per ml).

The susceptibility of PI-P cells to superinfection by related and unrelated viruses was examined. Echovirus 6, the LSc 2ab strain of poliovirus I, and vesicular stomatitis virus (Indiana) (VSV) were used for these experiments. PI-P and WISH cell monolayers were inoculated with virus at a high multiplicity of infection (3 to 40 PFU per cell). The inocula were removed after incubation (for 1 h at 37°C), and the cells were washed five times with phosphate-buffered saline to remove unbound virus and reincubated. Uninoculated PI-P cells were harvested for determination of yields of virus (PI-echovirus 6) under the same conditions. Cells were sonicated in their culture medium and assayed for virus yields (PFU per cell). The yields of PI-echovirus 6 in the

TABLE 1. Infectious centers in cultures acutely or persistently infected by echovirus 6[a]

Cells	PFU per plate[b]		% Recovery
	Recovered	Expected	
WISH	56	50	112
	39	40	98
	15	20	75
	8	10	80
PI-P	19	50	38
	15	40	38
	8	20	38
	4	10	35

[a] Acutely infected WISH cell monolayers were inoculated with 36 PFU per cell, and persistently infected PI-P cells were harvested from monolayers at passage 81.

[b] Each number represents the average of four replicate plates.

presence of either poliovirus or VSV were determined by pretreatment of the cellular extracts with the appropriate neutralizing viral antiserum. Neutralization of echovirus 6 by specific antiserum allowed quantitation of the yield of the heterologous viruses from PI-P cells. Superinfection of PI-P cells with 40 PFU of echovirus 6 per cell did not alter significantly the average echovirus yield of 4.0×10^4 PFU from 10^5 cells. This viral infectivity was neutralized completely by the concentration of echovirus 6 antiserum used for assay of heterologous viruses. Although cytopathology was delayed by 2 to 3 days, both heterologous viruses were cytolytic for PI-P cells. The yield of VSV from PI-P and WISH cells was the same (ca. 136 PFU per cell). In contrast, the poliovirus yield from PI-P cells (1.4 PFU per cell) was only 1.0% of the virus yield obtained from WISH cells (110 PFU per cell). This reduced yield of poliovirus remains to be explained, and it may be due to virus- or host-induced modifications of PI-P cells which prevent efficient production of related picornaviruses.

The susceptibility of PI-P cells to superinfection by heterologous virus suggests that echovirus 6 persistence in these cells is not dependent upon interferon production. To explore this possibility further, supernatant fluids of PI-P cells were examined for their antiviral activity. Virus was removed from the culture supernatants of cells at passages 47, 50, and 51 by centrifugation (110,000 × *g* for 3 h). Vero cell (ATCC CCL-81) monolayers were treated (for 16 h at 37°C) with undiluted supernatants before challenge with VSV. Untreated monolayers served as controls. Average plaque titers of 112, 113, and 108 PFU per monolayer, obtained after treatment with the supernatants from cell passages 47, 50, and 51, respectively, did not differ from the titers obtained with untreated cells (113 PFU per ml). Thus, supernatant fluids from PI-P cells did not exhibit interferon-like antiviral activity.

The possibility that the resistance of PI-P cells to superinfection by echovirus 6 may be due to the inability of this virus to attach to PI-P cells was examined. Radioactively labeled purified echovirus 6 was prepared by incubating monolayers for 7 h in amino acid-deficient medium with either 62.5 μCi of [^{3}H]leucine (58 Ci/mmol) per ml or 10 μCi of [^{35}S]methionine (1,450 Ci/mmol) per ml at 2.5 to 3.0 h after virus inoculation (6 to 25 PFU per cell). Virus produced at 7 h after inoculation was purified in CsCl density gradients (11). The purified radiolabeled virus was added to PI-P monolayers for 1 h at 0 and 24°C. WISH cells and nonpermissive mouse L929 cells (ATCC CCL-1) were inoculated and incubated under the same conditions as positive and negative controls, respectively. Virus attachment was calculated from the radioactivity (trichloroacetic acid precipitable) that remained associated with the cells after repeated washing (Table 2). Viral attachment did not occur at 0°C in any of the cells tested. Echovirus 6 attached equally well to PI-P and WISH cells at 24°C (37 and 43%, respectively). Similar results were obtained with PI-P cells at early (passage 10) and late (passage 70) passages (data not shown).

TABLE 2. Attachment of [^{3}H]leucine-labeled echovirus 6 to standard and persistently infected WISH cells and nonpermissive mouse cells[a]

Cells	Counts per minute attached	% Attachment
WISH	3,652	42.6
PI-P	3,145	36.7
L929	688	8.0

[a] Monolayers of cells were inoculated with virus at 8 PFU per cell (8,563 counts per minute) and incubated for 60 min at 24°C, and attachment was estimated by determining the amount of trichloroacetic acid-precipitable radioactivity remaining with the cells after repeated washes with phosphate-buffered saline.

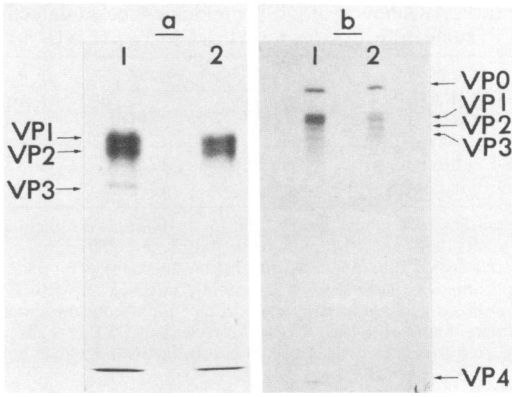

FIG. 2. Polypeptide profiles (VP0, VP1, VP2, VP3, VP4) of purified preparations of standard echovirus 6 and PI-echovirus 6. Panel a, silver stain of polypeptides after sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis of ca. 4×10^{10} disrupted particles of standard echovirus 6 (lane 1) and PI-echovirus 6 (lane 2). Disruption buffer consisted of 4 M urea, 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 10 mM Tris, 1 mM EDTA, 10% glycerol, and 0.05% bromphenol blue. Panel b, fluorograph of [^{35}S]methionine-labeled standard echovirus 6 before (lane 1) and after (lane 2) immunoprecipitation with polyclonal anti-echovirus 6 serum. ^{35}S-labeled virus preparations (125,000 counts per minute) were taken to 0.4 ml with immunoprecipitate buffer (140 mM NaCl, 20 mM Tris-HCl (pH 7.8), 1% Nonidet P-40, 0.4% sodium deoxycholate, 1 mg ovalbumin per ml, 0.5 mM phenylmethylsulfonyl fluoride). Anti-echovirus 6 serum was added, and the mixture was incubated (for 16 to 18 h at 4°C) before addition of a 40% suspension of protein A-Sepharose CL-4B beads (Sigma Chemical Co., St. Louis, Mo.). The washed immunoprecipitates were electrophoresed in a sodium dodecyl sulfate-polyacrylamide (15%) slab gel. The gel was treated with Fluoro-Hance (Research Products International Corp., Elk Grove Village, Ill.), dried, and exposed to Kodak X-Omat AR film for 42 h. Prestained protein markers were used for estimation of molecular weights in silver-stained gels.

The properties of viruses recovered from PI-P and PI-NP cells were examined. Infectious and noninfectious viruses were recovered in a band in CsCl at the same density (1.33 g/ml) as standard echovirus 6. Repeated centrifugation of this virus fraction in linear CsCl gradients did not result in separation of the particles. Virus preparations from PI-P or PI-NP cells contained the same number of virus particles (4×10^{12}/ml to 6×10^{12}/ml) as a standard virus preparation, as estimated from UV absorption at 260 nm (12). However, the virus preparation from PI-P cells had a much higher particle-to-PFU ratio (2×10^6) than standard echovirus 6 (1.2×10^3 particles per PFU).

Differences were observed between the polypeptide profiles of the PI-echovirus 6 and standard echovirus 6 (Fig. 2). Polyacrylamide (10 or 15%) slab gels were loaded with ca. 4×10^{10} disrupted particles of purified standard and PI-echovirus 6 preparations. After electrophoresis by the Laemmli system (7), the polypeptides were visualized by the silver stain (8). The polypeptides of standard echovirus 6 preparations have apparent molecular weights of 27,000, 25,750, 21,200, and 9,500 and are designated as VP1, VP2, VP3, and VP4, respectively (Fig. 2a, lane 1). VP1 usually appeared as

TABLE 3. Attachment of [^{35}S]methionine-labeled defective and standard echovirus 6 particles to WISH cells[a]

Virus particles	Counts per minute		% Attachment
	Attached to cells	Total	
Standard echovirus 6	6,758	12,896	52.4
Defective virus	1,035	11,130	9.3

[a] Suspensions (0.4 ml) of single cells (5×10^6/ml) were incubated with purified virus (10 μl) at 23°C for 90 min in phosphate-buffered saline (pH 7.2). Cells were washed four times before they were counted for radioactivity. Defective virus was purified from persistently infected cells (PI-NP), which did not produce detectable virus even after 50-fold concentration of virus preparations. Standard virus was recovered from WISH cells and was infectious. No attachment was detected in cells incubated with either virus at 0°C.

a doublet, whereas the VP3 band was not as intense as expected. These results are probably due to incomplete cleavage of VP1 and VP3 from their common precursor (12). This pattern was not altered by the presence of urea (3 M) in the gel (data not shown). An additional band that was detected in other virus preparations had an apparent molecular weight of 31,500 and was designated as VP0. These polypeptides were viral in origin, since they migrated in sodium dodecyl sulfate gels to the same positions as polypeptides of purified virus preparations which were immunoprecipitated by polyclonal anti-echovirus 6 serum (Fig. 2b, lane 2) but not by preimmune serum (data not shown). The PI-echovirus 6 preparation contained polypeptides with similar concentrations and apparent molecular weights of VP1, VP2, and VP4. However, VP3 was not detected in the PI-echovirus 6 preparation (Fig. 2a, lane 2). With more concentrated preparations of PI-echovirus 6 (ca. 10^{12} particles), a light protein band was seen that migrated to a similar position as VP3 and may have been due to the small fraction of infectious virus in these preparations. Virus particles radiolabeled with [^{35}S]methionine were recovered from PI-NP cells, purified, and electrophoresed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis slabs. The VP3 polypeptide was missing, whereas the other three capsid polypeptides migrated as the capsid polypeptides of standard virus. The absence of the VP3 band was probably not due to degradation during virus isolation or electrophoresis. The same profile was obtained in the presence of a protease inhibitor (0.5 mM phenylmethylsulfonyl fluoride). Furthermore, no VP3 was precipitated by antiviral antibodies which recognize VP3 in standard virus preparations that were handled in the same manner as the defective virus. Since the PI-echovirus 6 had the same density as standard virus (even after two runs on linear CsCl density gradients), there cannot be a major difference in protein content. The absence of a VP3 band in echovirus 6 preparations for PI-NP and PI-P cells may be due to (i) an alteration of the polypeptide so that it migrates with another protein, (ii) a difference in posttranslational processing, or (iii) a small deletion in the viral RNA genome.

Although the standard virus and PI-echovirus 6 preparations contained the same number of virus particles, the average titer of PI-echovirus 6 (2×10^6 PFU per ml) was only 1.0% of the average titer of standard echovirus 6 (2×10^8 PFU per ml). The possibility that the defective particles of PI-echovirus 6 interfere with standard echovirus 6 was examined. Duplicate WISH cell monolayers (ca. 10^5 cells per monolayer) were inoculated with 0.5×10^5, 1.0×10^5, or 2.0×10^5 particles of PI-echovirus 6 per cell. Washed cell monolayers were challenged after 1 h at 37°C with standard echovirus 6 (1.0 PFU per cell). Controls consisted of cells that were infected with either PI-echovirus 6 alone (1.0 PFU per cell, which is equivalent to 2×10^6 particles per cell) or standard echovirus 6 (1.0 PFU per cell, which is equivalent to 10^3 particles per cell). Virus yields were determined from sonicated cell lysates (1.0 ml) after incubation for 12 h at 37°C. The yields after infection with standard virus and PI-echovirus 6 were 6.6×10^6 and 1.0×10^7 PFU per ml, respectively. Pretreatment of the cells with PI-echovirus 6 before addition of standard echovirus 6 did not alter the virus yields. Titers of 6.5×10^6, 7.6×10^6, and 6.5×10^6 PFU per ml were recovered from cells pretreated with 0.5×10^5, 1.0×10^5, and 2.0×10^5 particles per cell, respectively. The possibility that PI-echovirus 6 did not attach to WISH cells was examined. Purified, ^{35}S-radiolabeled virus particles from PI-NP cells were added to suspensions of WISH cells. An insignificant fraction (9%) of the particles attached to cells under conditions in which 52% of the standard echovirus 6 attached to uninfected cells (Table 3). These results indicate that the noninfectious viruses do not interfere with infection of WISH cells by standard virus, because most of the PI-echoviruses are defective and cannot attach to and enter WISH cells.

As indicated above, a fraction of viruses recovered from PI-P cells was lytic for WISH but not for PI-P or PI-NP cells. The lytic virus from PI-P cells produced plaques of the same size (6.0 mm) and turbidity as those produced by standard echovirus 6. Single growth cycles of the two viruses were compared in WISH cells after inoculation of 4.4 PFU per cell (Fig. 3). There was no detectable difference in the time of appearance of newly produced virus or in the final virus titers. The yield of either virus was ca. 345 PFU per cell. Also, both viruses were neutralized equally well by echovirus 6 (D'Amori) antiserum, which had a neutralization titer for both viruses of ca. 4×10^5 for 150 PFU. Incubation temperatures (31 to 37°C) did not alter the extent of cytolysis or yield of PI-echovirus 6 and standard echovirus 6 preparations. Also, both viruses were protected by divalent cations Mg^{2+} and Ca^{2+} to the same extent (1%) when exposed at 50°C for 1 h. Thus, the infectious virus from PI-P cells was not distinguishable from standard echovirus 6.

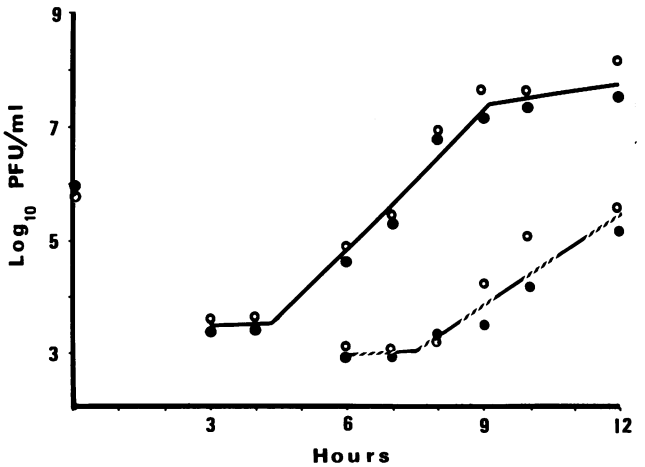

FIG. 3. Single growth cycle of standard echovirus 6 (closed circles) and PI-echovirus 6 (open circles) in WISH cells at 37°C after an input multiplicity of infection of 4.4 PFU per cell. Total virus titers (solid line) recovered from 10^5 cells in 1.0 ml of medium and virus titers released into 1 ml of medium (broken line) are shown.

The results demonstrate that we established a steady-state infection of cloned, cultured human cells by echovirus 6. The infection was maintained for 3 years (over 125 passages). Essentially all of the cells were infected and expressed viral antigens (Fig. 1). The only other enterovirus that produces a similar prolonged infection is hepatitis A virus (14). Other reported persistent enterovirus infections (1, 3, 9, 13, 15) are carrier-state infections in which only a small fraction of the cells is infected, and the cells that are infected are destroyed. Furthermore, viral antibody is either necessary for maintenance of viral persistence (1, 3, 15) or can be used to cure the infection (9, 13). In contrast, immune serum is not required for either establishment or maintenance of the echovirus 6 persistence. In addition, this persistent infection cannot be cured by treatment with excess antibody.

Temperature-sensitive virus mutants did not play a role in virus persistence, since neither the virus used initially nor the infectious virus recovered from persistently infected cells was temperature sensitive. Also, interferon did not appear to function significantly in virus persistence. PI-P cells were susceptible to superinfection and lysis by related (poliovirus) and unrelated (VSV) viruses, and supernatants of persistently infected PIP cells did not exhibit interferon-like activity against VSV. However, defective virus particles were produced in excess during the persistence of echovirus 6 in PI-P cells (2×10^6 particles per PFU) and in the absence of detectable infectious virus in PI-NP cells. It remains to be determined whether viral genotypic alterations occur during persistence and whether the defective particles are essential for maintenance of virus persistence or are nonfunctional by-products of the initial infection.

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