JOURNAL OF VIROLOGY, Nov. 1977, p. 580-589
Vol. 24, No. 2

Printed in U.S.A.

Persistent Infection of Cultured Mammalian Cells by Japanese Encephalitis Virus

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Received for publication 13 June 1977

Persistent infections were established by serial undiluted passage of flavivirus Japanese encephalitis virus in a line of rabbit kidney cells (MA-111). The persistently infected cells resembled uninfected cells in most respects. Low levels of infectious virions were released from a small percentage of cells, and a larger and more variable percentage was shown to possess viral antigen by fluorescent-antibody staining. Released viruses were shown to interfere with replication of wild-type Japanese encephalitis virus. Persistently infected MA-111 cells could not be superinfected with homologous wild-type Japanese encephalitis virus but could be superinfected with two heterologous viruses. Transfer of cell culture medium from persistently infected MA-111 cells to a line of African green monkey kidney cells (Vero) resulted in similar persistent infections in the latter cells. Temperature sensitivity and host-cell interferon production were not involved in establishment or maintenance of persistence. Determination of ratios of physical particles to infectious particles revealed that many defective, noninfectious viruses were present, suggesting that defective interfering particles may be responsible for persistency.

Several mechanisms for establishment and maintenance of persistent infections by normally cytocidal viruses have been hypothesized. Huang and Baltimore (8) suggested that defective interfering (DI) particles may play an important role in establishing persistent infections. Holland and Villarreal (6, 7) found that DI particles of vesicular stomatitis virus (VSV) were essential for persistent infection of BHK cells. Preble and Youngner (14-16), however, showed that the requirement for DI particles to establish persistence of VSV in L cells could be bypassed if infection were instead initiated with a low multiplicity of infection (MOI) of a temperature-sensitive (ts) mutant. They also suggested that selection of ts mutants was involved with persistency of Newcastle disease virus in several cell lines. Holland et al. (7) demonstrated slight temperature sensitivity of virus released from cells persistently infected with rabies virus, but they postulated that, although the ts mutants may be a stabilizing factor, they were not essential for establishment or maintenance of persistence.

Host-cell physiological factors are implicated in persistence by several authors. Cole and Hetrich (3) found that conjunctiva cells persistently infected with parainfluenza virus 3 maintained noncytocidal infection when kept in growth medium but quickly developed a cytopathic effect (CPE) and cell destruction when transferred to a maintenance medium. ter Meulen and Martin (13) noted an increase in both the number of cells showing positive fluorescence as well as the amount of infectious virus released by canine distemper virus persistently infected Vero cells, after two consecutive changes of the growth medium.

The role of interferon production by persistently infected cells is unclear. Inglot et al. (9) reported that persistence of Sindbis virus in mouse cells required the presence of interferon. Low levels of interferon were also found in Newcastle disease virus-infected L cells (20). Kawai et al., however, were unable to demonstrate interferon production in persistent infections of rabies virus in BHK cells (12).

Understanding the mechanisms of establishment and maintenance of viral persistence is extremely important. Several serious illnesses are now recognized results of chronic viral infections. Subacute sclerosing panencephalitis is definitely linked to the persistence of measles virus in the brains of afflicted children. Viruses are also being sought as causative agents of multiple sclerosis. Other diseases of unknown etiology may also have a link with persistent viral infections. Many vaccines are presently produced with attenuated viruses. The possible relationships between these defective viruses and their hosts have not been fully explored.

This report describes establishment of persistent infections by the flavivirus Japanese enceph-

alitis virus (JEV) in two mammalian continuous cell lines and some characteristics of the persistently infected cells and the viruses released by them.

MATERIALS AND METHODS

Virus, cells, and medium. The Nakayama strain of JEV was supplied by D. W. Trent, Center for Disease Control, Fort Collins, Colo. Wild-type (wt) stock consisted of twice plaque-cloned virus propagated in PS-38 cells after low MOI infection. Continuous lines of rabbit kidney cells (MA-111), African green monkey kidney cells (Vero), rhesus monkey kidney cells (LLC-MK-2), and porcine kidney cells (PS-38) were used. Growth medium was M199 plus 10% calf serum (CS). The growth and assay of JEV were according to the techniques of Inoue et al. (10, 11).

Solutions. Saline A consisted of 0.8% NaCl–0.04% KCl–0.035% $NaHCO_3$–0.1% glucose (wt/vol). Polyethylene glycol (PEG) (molecular weight, 5,700 to 6,700) was prepared as a 40% (wt/vol) solution in Hanks balanced salts. Scintillation cocktail was 2 liters of toluene, 1 liter of Triton-X-100, 12 g of 2,5-diphenyloxazole, and 0.3 g of 1,4-bis-(5-phenyloxazolyl)benzene.

Serial undiluted passage and establishment of persistent infections. After initial low MOI of MA-111 cells with wt stock JEV, the virus was passaged every 24 h by infecting fresh MA-111 cells with two-thirds of the medium from the previous passage. The remaining one-third was saved for assay. Most passages resulted in total monolayer destruction; however, after undergoing extensive CPEs and cell monolayer destruction, a few surviving cells in serial undiluted passages 31, 34, and 41 eventually grew back to persistently infected confluent monolayers and were passed by trypsinization. These were termed M31, M34, and M41 carrier cells. Separate cultures of Vero cells were infected with undiluted cell culture fluid from each of the established persistently infected MA-111 cultures. These also underwent extensive CPEs before establishment of persistent infection (see Table 4). They were labeled V31, V34, and V41 carrier cells according to the carrier culture fluid used for establishment.

Infectious center assay. Infected cells were suspended by trypsinization, washed twice in cold physiological saline, and counted in a hemacytometer. The cells were diluted in saline and allowed to adsorb to confluent monolayers of PS-38 for 4 h. An overlay consisting of M199 and 1% agar was then added. Plaques were observed 5 days later by application of 1:1,500 neutral red.

Fluorescent-antibody technique. An indirect fluorescent-antibody procedure was used with anti-JEV mouse ascites fluid provided by N. Karabatsos, Center for Disease Control, and fluorescein-conjugated goat anti-mouse serum. Cells were grown on glass cover slips, fixed in cold (−20°C) acetone, stained with antibody, and examined in a Zeiss microscope.

Interferon assay. A plaque reduction assay for interferon production was described by Wagner (23). Medium from persistently infected cultures was collected and the pH adjusted to 2 with 1 N HCl, left at 4°C for 24 h to destroy viral infectivity, and the pH returned to 7 with 1 N NaOH. The undiluted medium was then placed on confluent monolayers of homologous cells overnight and subsequently was removed, and the monolayer was washed twice with saline A. The cells were challenged with 100 PFU of VSV. Positive controls consisted of interferon induced by infection of normal MA-111 or Vero cells with Venezuelan equine encephalitis virus (VEE). Fresh growth medium served as a negative control. Both the positive and negative controls were pH adjusted as described.

Hemagglutination. The method of Clark and Casals (2) was used for hemagglutination. Virus for hemagglutination was concentrated with PEG as described by Della-Porta and Westaway (4). Goose erythrocytes were supplied by C. Calisher and D. Muth, Center for Disease Control.

Interference assay. Cell culture fluid was removed from persistent infections and made 8% (wt/vol) with PEG. After 2 h, the precipitate was collected by centrifugation at 10,000 rpm for 30 min in a Sorvall refrigerated centrifuge. The precipitate was suspended to 0.01 the original volume. Precipitation of culture medium served as a control. Twenty-five-cm^2 tissue culture flasks containing confluent monolayers of MA-111 or Vero cells were infected with one of the following: 0.1 ml (4×10^4 PFU/ml) of concentrated virus only, 0.1 ml of concentrated and 0.1 ml of (1×10^5 PFU/ml) wt stock, 1 ml of concentrated and 0.1 ml of wt virus, 0.1 ml of wt virus only, or 1 ml of control precipitate and 0.1 ml of wt virus. After 1-h adsorption of concentrated virus, cells were washed twice with saline A before infection with wt virus.

Radioactivity as an indicator of total virus particles. For incorporation of [^{3}H]uridine into virion RNA, normal confluent MA-111 cells were infected with JEV (MOI, 1), and 24 h later, the media from both confluent persistently infected and wt-infected cells were replaced with M199 containing 0.5% bovine serum albumin and 5 μCi of [5-^{3}H]uridine per ml (27.75 Ci/mmol). After 48 h, the media were removed, and the virus was concentrated with PEG as described above and then centrifuged through a continuous sucrose gradient (20 to 60%) for 12 h at 27,000 rpm at 5°C in a Beckman SW 40 rotor. One-half-milliliter fractions were collected, and specific gravities and radioactivity were determined.

RESULTS

Establishment of persistent infections. Four days after infection with serially undiluted

FIG. 1. *(A) and (B) MA-111 cells were infected with wt stock JEV, fixed in cold acetone (−20°C), and stained according to an indirect fluorescent-antibody procedure. Antigen is distributed evenly throughout the entire cell cytoplasm, with maximum fluorescence surrounding the nucleus. (C) and (D) M-34 carrier cells were fixed and stained as described. Fluorescence often appeared as cytoplasmic patches (C) but could also resemble wt-infected cells (D).*

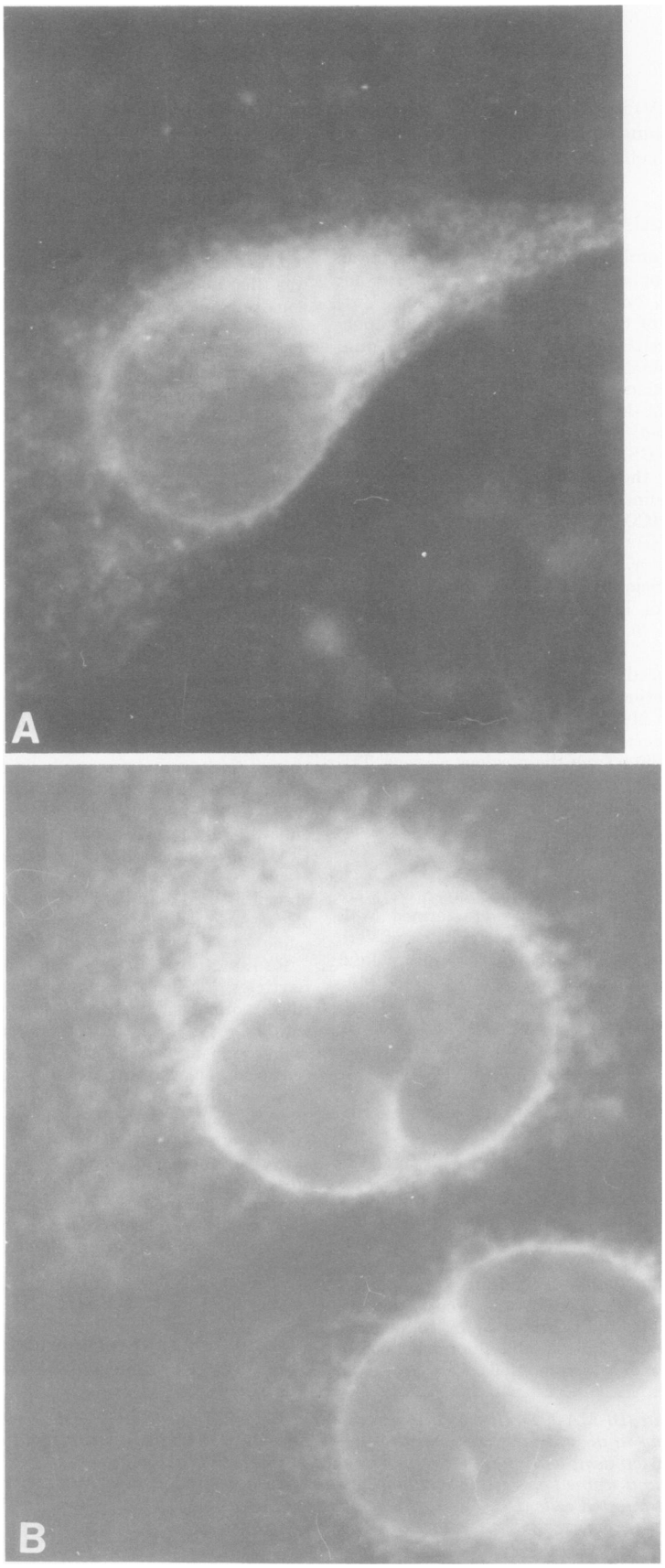

FIG. 1A and B

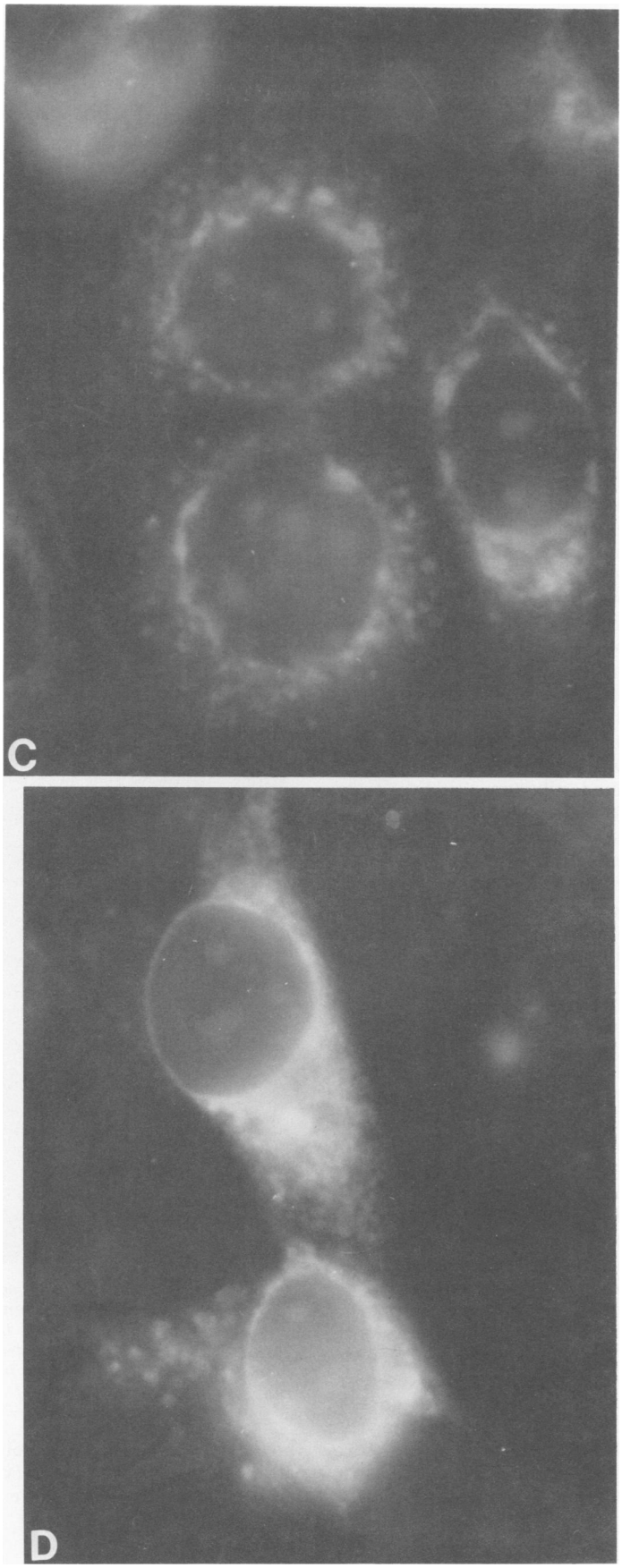

FIG. 1C and D

JEV, most MA-111 cells developed CPE and died. At certain passage levels (see above), a few cells remained and subsequently developed into confluent monolayers. Early in the establishment of persistence, the cells periodically underwent "crisis" stages, as described by Youngner and Quagliana (26). During crises, the cultures developed severe CPE, and many or all of the cells died. Those cells that survived eventually regained confluency and were shown both to have retained viral antigen by immunofluorescence and also to be releasing infectious virions. After approximately 4 months, the cultures stabilized and no longer underwent crises. The cultures have been passed at weekly intervals since. Fluorescent-antibody studies revealed few differences between persistently infected and wt-infected cells. Persistently infected cells often had a patchy, uneven distribution of antigen compared to a more uniform distribution in wt JEV-infected cells (Fig. 1). Phase-contrast microscopy of persistently infected and uninfected cells is shown in Fig. 2. A greater incidence of multinucleation was seen in persistently infected cells than in uninfected cells. Few other differences were noted.

Persistently infected cell growth requirements. Cell growth curves of persistently infected and uninfected cells were used to compare the rates of growth and survival of the two cell types. The persistently infected cells reached confluency at similar rates to uninfected cells, but, when the stationary phase was reached, growth abruptly ceased, and the cells began to exhibit CPE and die (Fig. 3). The number of cells present during the stationary phase always was less than in uninfected cells. One report showed that enrichment or depletion of the growth medium on persistently infected cells can cause changes in characteristics of cell growth and virus release (13). To determine the extent to which persistence is dependent on host-cell metabolism, persistently infected cells were either maintained on a minimal medium (MEM plus 2% CS) or were given frequent changes of growth medium (M199 plus 10% CS). No changes in titer of released virus, appearance of cultures, or number of cells expressing viral antigen as determined by fluorescent-antibody studies could be detected (data not shown).

Infectious center assay. The number of cells releasing infectious virions in a persistently infected population was determined as described. Results were correlated with the number of cells showing positive fluorescence in these same cultures. Table 1 shows data from assays of three different cultures. Little correspondence between the number of cells releasing infectious virus and the number of cells showing positive fluorescence was noted. Furthermore, although the number of infectious centers from all persistent populations remained relatively

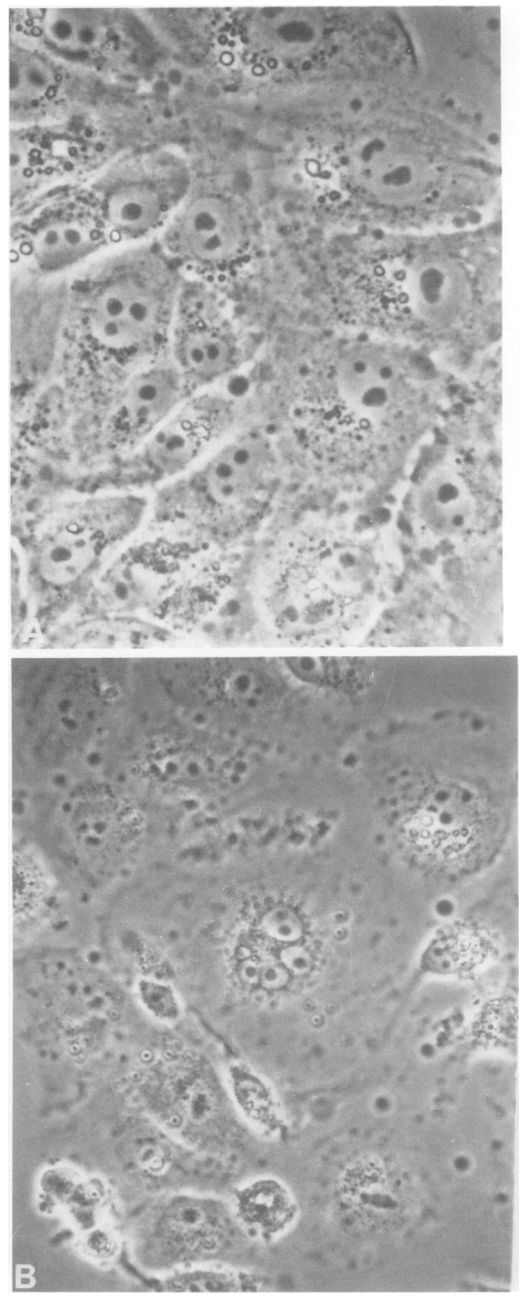

FIG. 2. *Phase-contrast microscopy reveals few differences between cultures of uninfected MA-111 cells and persistently infected cells. (A) Uninfected MA-111 (×200); (B) M-34. Multinucleated cells were seen more frequently in persistently infected cell cultures than in uninfected cells.*

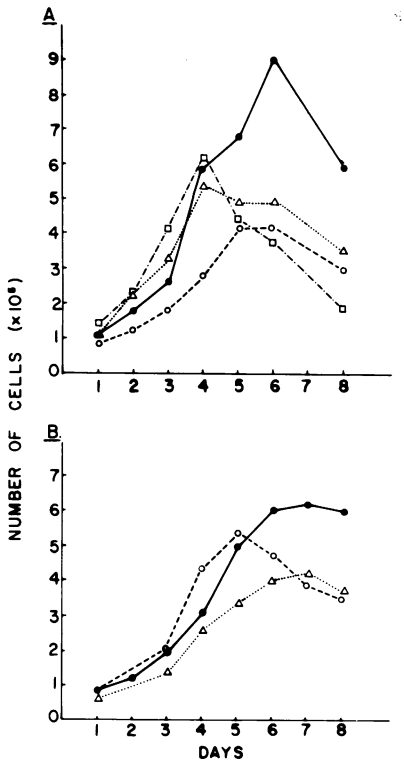

FIG. 3. *Persistently infected cells and normal MA-111 and Vero cells were removed by trypsin, counted, and seeded in 35-mm petri plates at 1 × 10⁵ cells per plate. Each day for 8 days thereafter cells on two plates were removed by trypsinization and counted. (A) ●, Vero control; △, V31; ○, V34; □, V41. (B) ●, MA-111 control; ○, M31; △, M34.*

TABLE 1. *Infectious center assay of persistently infected cells*[a]

Carrier cells	Infectious centers (%)	Cells fluorescing (%)
M-31	9	35
M-34	6	85
V-41	5	20

[a] The number of cells in a persistently infected population releasing infectious virus was determined by plating suspensions of persistently infected cells on confluent monolayers of PS-38 cells. The percentage of cells showing positive fluorescence was estimated by a differential microscopic count. Normal cells infected at an MOI of 25 and assayed 16 h p.i. showed 95 to 120% infectious centers.

stable (between 1 and 10%), the proportion exhibiting positive fluorescence varied with passage from about 1 to 90% (data not shown).

Interference by virus released from persistently infected cells. Persistently infected cells were shown by Inglot et al. (9) to be immune to superinfection with homologous virus but susceptible to heterologous virus. Confluent monolayers of persistently infected cells were infected with JEV, VEE, or VSV, as described for Table 2. Persistently infected MA-111 cells and Vero cells were completely resistant to superinfection by homologous JEV. Persistently infected MA-111 cells appeared to be slightly resistant to superinfection with the heterologous viruses VEE and VSV, but persistently infected Vero cells showed no resistance to these viruses. Viruses released from persistent infections also interfered with infection of normal cells by wt JEV. Medium was collected from persistently infected cells, and the virus was concentrated to achieve a sufficiently high MOI. Table 3 demonstrates that infection of normal MA-111 cells with a mixture of concentrated persistent virus and wt JEV resulted in a decrease in the titer of virus released after 48 h as compared to cells infected with wt JEV alone. Furthermore, the

TABLE 2. *Superinfection of persistently infected cells with JEV, VEE, or VSV*[a]

Carrier cells	Superinfecting virus	Days p.i.	PFU[b] test	PFU[c] control	PFU[d] wt
M-34	JEV	2	6×10^2	2×10^2	2×10^6
M-31	JEV	9	6×10^3	1×10^3	2×10^5
V-41	JEV	9	3×10^3	3×10^3	2×10^6
M-31	VEE	2	7×10^5	2×10^3	2×10^7
V-31	VEE	2	1×10^8	2×10^3	1×10^8
M-31	VSV	2	8×10^5	8×10^1	3×10^7
V-31	VSV	2	2×10^7	7×10^3	2×10^7

[a] Confluent monolayers of persistently infected cells in 25-cm² plastic flasks were infected with wt JEV, VEE, or VSV, each at an MOI of 1. Cell culture medium was collected and assayed for the presence of these viruses. VSV and VEE plaques were discernible 2 days p.i., whereas JEV plaques could not be seen until 5 days p.i.

[b] Medium from mixed infections collected and assayed on PS-38 cells.

[c] Control cultures were identical to test cultures but were not superinfected. Therefore, control titers indicate the background levels of infectious virus released by persistently infected cells.

[d] The wt titer indicates the PFU of JEV, VEE, or VSV per milliliter released 48 h after infection of normal MA-111 or Vero cells with the same wt stock.

TABLE 3. *Interference of persistent virus with replication of wt JEV in normal MA-111 cells*[a]

Persistent virus		wt virus		Virus released after 48 h (PFU/ml)
ml	PFU	ml	PFU	
0.1	4×10^3			2.5×10^5
		0.1	1×10^4	3.5×10^6
0.1	4×10^3	0.1	1×10^4	3.0×10^5
1.0	4×10^4	0.1	1×10^4	3.0×10^4

[a] Tissue culture medium was removed from persistently infected cells and concentrated with PEG. Concentrated persistent virus was adsorbed to cell monolayers for 1 h and then removed by two washes with saline A before infection with wt virus.

degree of reduction of total virus yield was proportional to the amount of persistent virus added.

Interferon production. Vero cells are reported incapable of producing interferon (5). Therefore, if interferon were necessary for establishment or maintenance of persistence in this system, carrier cultures would not arise in Vero cells. Media from four different persistently infected MA-111 cell cultures were used to infect Vero cells (MOI, ca. 0.01). Ten days postinfection (p.i.), all cells showed CPE. By 25 days p.i., all cultures began to regain confluency, and, at 30 days p.i., the cultures were passed. Throughout this time, medium was changed every 5 days, and titers of released infectious virus were determined. Data from assays during the establishment of two persistently infected Vero cultures are shown in Table 4.

At 40 days p.i., all Vero cell cultures were determined to be persistently infected by positive fluorescent antibody and by release of infectious virus. The persistent state was maintained in Vero cells for 10 months. The lack of interferon production by persistently infected cells was confirmed by direct assay for interferon by plaque reduction, as described by Wagner (23). Results demonstrate that Vero cells do not produce interferon, but that wt-infected MA-111 cells do (Table 5). Neither persistently infected MA-111 cells nor persistently infected Vero cells produce large amounts of interferon.

Temperature sensitivity. Individual plaques formed by wt JEV or virus released from persistently infected cultures were picked and tested for temperature sensitivity. Youngner et al. have suggested that ts mutants may be necessary for establishing persistent infections (25). To determine if ts mutants were required early in establishment of persistence in this system, plaques were also picked from medium collected 20 to 25 days after infection of Vero cells with virus released from persistently infected MA-111 cells (Table 6). ts mutants appear neither to be essential for successful establishment of persistently infected cultures, nor to be selectively released after the cultures have stabilized.

Further evidence for the lack of temperature sensitivity in virus from persistent infections was demonstrated by growing duplicate persistently infected cultures, which had been routinely incubated at 37°C, at a permissive temperature of 33°C and a nonpermissive temperature of 40°C. At 7-day intervals, after examination for evidence of CPE and collection of medium for assay, the cultures were passed, and incubation was continued at the same temperature. No significant differences in amount of virus released or in CPE were detected at the two temperatures during 5 weeks (data not shown).

Hemagglutination. To determine if persistently infected cells produced a higher ratio of physical particles to infectious particles than wt-infected cells, the following ratio of hemagglutinating (HA) units to PFU was devised: HA/PFU = [reciprocal of last dilution showing complete HA/(PFU/ml)] × 10^4 (a 10^4 unit was arbitrarily multiplied to avoid small numbers). Medium

TABLE 5. *Interferon production by newly infected and persistently infected cells*[a]

Interferon source	No. of VSV plaques
V-31 carrier cells	84
Vero cells induced with VEE[b]	80
Control[c]	102
M-34 carrier cells[b]	90
MA-111 cells induced with VEE[b]	0

[a] Cells were challenged with 100 PFU of VSV after treatment with interferon obtained from newly or persistently infected cells.

[b] Medium collected from persistent infections or from normal cells infected with VEE.

[c] pH-adjusted medium.

TABLE 4. *Establishment of JEV persistently infected vero cells*

Days p.i.	31[a]			34[a]		
	CPE	Titer (PFU/ml)	Confluent (%)	CPE	Titer (PFU/ml)	Confluent (%)
0	0	3×10^{3}[b]		0	7×10^{2}[b]	
5	0	4×10^{4}		0	8×10^{3}	
10	2+	1.5×10^{6}		2+	2×10^{5}	
15	2+	1.5×10^{5}		4+	2×10^{4}	
20	2+	1×10^{4}		4+	4×10^{3}	
25		1.5×10^{3}	80		8×10^{3}	30
30		1×10^{4}	80		2×10^{4}	95
35		1×10^{5}	100		2×10^{4}	100

[a] Media transferred from persistently infected M-31 and M-34 cultures to Vero cells.

[b] At day 0, medium from persistently infected M-31 and M-34 cultures was assayed prior to infection of Vero cells.

TABLE 6. *Temperature sensitivity of plaques picked from persistently infected cultures and wt JEV-infected cells*[a]

Virus and cells	No. of plaques tested	No. of plaques showing temperature sensitivity
M-34 carrier cells	100	0
M-41 carrier cells	60	1
wt JEV/normal MA-111 cells	75	0
V-34 carrier cells (20 to 25 days p.i.)	110	2

[a] Medium from persistently infected cultures was collected at different times after the establishment of persistence, and virus titer was established by plaque assay on monolayers of PS-38 at 33°C. Plaques were picked and immediately placed onto confluent monolayers of cells in 17-mm wells containing 1 ml of medium, which were incubated at 33°C until 4+ CPE was observed. Medium from each well was inoculated into duplicate microtiter plates seeded with PS-38 and incubated at 33 or 40°C. If, after 5 days, 4+ CPE developed at 33°C and, after 3 days, 0 or 1+ CPE was seen at 40°C, the plaque was determined to possess ts characteristics.

collected from persistently infected cells showed a 400-fold increase in HA/PFU compared to medium collected from wt-infected cells. This suggests a much higher number of noninfectious particles than is found in wt populations.

Incorporation of [^{3}H]uridine. An estimate of the proportion of physical particles to infectious units was also determined by incorporation of [^{3}H]uridine into cell-released virus. Sucrose density gradients were prepared, fractions were collected, and radioactivity and density were determined as described. Three peaks of radioactivity could be discerned on each gradient. A ratio of radioactivity to infectivity was determined for each fraction according to the following formula: radioactivity/infectivity = counts per minute (cpm) in a peak/PFU put on gradient. Table 7 illustrates the relationship between cpm/PFU for both the persistent and wt infections. Plaque assays of each peak localized the highest titer of infectivity in the peak with approximate density of 1.19 from both gradients (data not shown). These data suggest that the persistent infection had a higher proportion of particles incorporating [^{3}H]uridine in relation to infectious particles than did the wt-infected culture and that the presence of large numbers of defective particles did not produce noticeable change in density of peaks.

DISCUSSION

This study describes persistent infections of MA-111 cells with the normally cytolytic flavivirus JEV. The infections were initiated with virus that was previously passed undiluted 30 to 40 times in MA-111 cells. Persistence has been maintained through 40 cell passages over a period of 14 months. Distribution of viral antigen as measured by immunofluorescence is often patchy and uneven in persistent cultures, whereas wt infections have a smooth, even appearance. The number of persistently infected cells possessing viral antigen can vary considerably. Even when most cells show positive fluorescence, however, only a small percentage of cells release infectious virions. One explanation for this observation may be that defective viruses and viral components that do not mature to infectious forms are present within and released from cells. Appearance of persistently infected cultures closely resembles uninfected cells, although more multinucleated MA-111 cells are seen in infected cultures. Growth characteristics of persistently infected cells parallel uninfected cells until a stationary phase is reached, at which point the infected cells begin to die.

Interferon production and temperature sensitivity were excluded as possible mechanisms for establishment or maintenance of persistently infected MA-111 cells. Persistence could easily be transferred from MA-111 persistent infections to Vero cells, a line incapable of interferon production (5). Plaque reduction assays also demonstrated that little or no interferon was produced by persistently infected MA-111 or Vero cells. Temperature sensitivity was studied by growing virus at permissive and nonpermissive temperatures. Virus released from persistent infections was not ts, either early in the establishment of persistence or later after cultures had stabilized.

We have shown that persistently infected MA-111 cells cannot be superinfected with wt JEV and that virus released from persistent infections can interfere with wt infection of normal MA-

TABLE 7. *Comparison of incorporation of [^{3}H]uridine to infectivity in wt JEV-infected cells and M-34 carrier cells*[a]

Peak	Density in peak fraction (g/cm^3)	wt JEV infection (cpm/PFU)	M-34 carrier culture (cpm/PFU)
1	1.19	2.0	143
2	1.21	3.8	168
3	1.23	4.0	65

[a] Labeled virus was banded on a continuous sucrose gradient (20 to 60%) for 12 h at 27,000 rpm in a Beckman SW 40 rotor. Fractions were collected, and densities were determined. Radioactivity was determined by liquid scintillation counting of portions of each fraction.

111 cells. Slight interference with replication of heterologous viruses in persistently infected MA-111 cells may be due to interferon below our level of detection. Ratios of physical particles to PFU demonstrated that large amounts of noninfectious particles are present in medium of persistent infections. These results suggest that DI particles may be the causative agent of these persistent infections. DI were suggested as possible agents of persistence by Huang and Baltimore (8) and were described by Holland and Villarreal (6, 7). Although some DI viruses can be separated from wt on the basis of density (6, 7), the presence of large amounts of noninfectious particles does not alter sucrose density patterns of virus released from persistently infected cells as compared to wt virus. These results are not unexpected, since studies with the alphaviruses Sindbis and Semliki Forest have shown that it is difficult to separate DI particles from standard virus by the use of density gradients (1, 18, 24). Studies are currently under way to determine the nature of virus-specified RNA in virions and in persistently infected cultures as compared to wt infections.

Kinetics of cell growth suggested that an unstable equilibrium may be established between DI and wt viruses in persistent infections. As cells reach high density, competent virions may be passed from cell to cell at an increasing rate, resulting in CPE and cell destruction. Evidence for this idea was demonstrated by lower numbers of cells present in persistent infections at stationary phase compared with uninfected cells and in the rapid decline in infected cell numbers thereafter. The titer of infectious virus in cell culture medium did not change when cells were in stationary phase nor when persistently infected cells were grown in an enriched or depleted medium, suggesting that host-cell growth is unrelated to virus production.

Host-cell repair, however, could be involved in maintaining persistence. Cells capable of synthetic and metabolic activity might favorably compete with viruses for necessary replication and repair mechanisms. But, if cell processes were impaired due to contact inhibition or limitation of some factors necessary for repair, the viruses might then be able to generate cell destruction faster than cells could repair it. Maintenance medium may slow cell activity without inhibiting it sufficiently to result in the cell death observed as density increased.

ter Meulen and Martin (13) recently suggested the presence of a virus-induced protein that is produced in greater quantity during persistent than during lytic infections and inhibits the replication of lytic canine distemper virus. They felt that this protein was continually produced but was labile. Although we have not looked for such a protein, our results are inconsistent with the presence of this sort of inhibitory agent, because, unlike that of ter Meulen and Martin, our system did not exhibit increased CPE or infectious virus titer after removal of cell culture medium and replacement with fresh medium.

Determination of factors responsible for establishment and maintenance of persistent infections in vitro by viruses that normally are cytolytic is essential for understanding serious illnesses that can result from chronic viral infections in vivo. Many mechanisms may be involved in establishment and maintenance of persistence. Our results seem to be most consistent with a model involving DI viruses, although final proof awaits characterization of virion RNA.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant AI 12660 from the National Institute of Allergy and Infectious Diseases.

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