Inapparent Viral Infection of Cells In Vitro

III. Manifestations of Infection of L Mouse Cells by Japanese Encephalitis Virus¹

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ABSTRACT

DUBBS, D. R. (University of Minnesota, Minneapolis), AND W. F. SCHERER. Inapparent viral infection of cells in vitro. III. Manifestations of infection of L mouse cells by Japanese encephalitis virus. *J. Bacteriol.* 91:2349-2355. 1966.—Nine strains of Japanese encephalitis (JE) virus were propagated serially in cultures of L cells reaching titers of $10^{3.5}$ to $10^{6.3}$. Although cytopathic effects were not seen in cultures of contiguous L cells after infection with JE virus, cell growth was inhibited. Moreover, cell destruction was readily apparent in infected cultures of sparse, noncontiguous L cells. Differences in the size of cell population of infected and noninfected cultures (i) occurred despite only 0.2 to 3.5% of the cells in infected cultures being associated with infectious virus, (ii) were greater in actively growing cultures than in those kept in maintenance media, and (iii) were probably in part related to an interferon produced in infected cultures.

Overt disease usually does not result from infection of man and animals with Japanese encephalitis (JE) virus unless the central nervous system is invaded and encephalitis develops (20, 23, 28, 30). Viremia probably results from viral replication in non-neural tissues, yet the absence of disease suggests that non-neural cells escape injury. In an attempt to understand the relationship between non-neural cells and JE virus in vivo, studies were undertaken with strain L mouse fibroblasts in vitro as a model cell derived originally from a susceptible host and easily cultivated in vitro (5, 7). This report describes two methods of detecting JE virus in L-cell cultures which ordinarily show no cytopathology: (i) by measurement of virus-induced inhibition of cell growth in cultures of contiguous L cells, and (ii) by infection of sparse populations of widely separated L cells which, in contrast to contiguous cells, are destroyed by JE virus.

MATERIALS AND METHODS

Viruses. Eight strains of JE virus from Japan were employed after three to seven mouse brain passages

from the following: (i) *Culex tritaeniorhynchus* mosquitoes [strains M5/596, M5/572, and M5/560b (24, 26)]; (ii) blood of Black-crowned Night Heron nestlings [strain 281776, 281777, 281801 (26)]; and (iii) brains of fatal human cases of encephalitis [strains 55-1735 and 55-1456 (26)]. The ninth strain, Nakayama, was isolated in 1935 from an encephalitis patient in Japan (1, 26), and underwent more than 70 mouse passages prior to use. Virus inocula for L cell cultures were 10% (w/v) suspensions of mouse brains harvested 3 to 7 days after intracranial inoculation of either weanling (3 to 4 weeks old) or suckling (1 to 5 days old) Swiss albino mice. Brains were triturated in a mixture of rabbit serum, 2 parts, and Hank's (10) balanced salt solution (BSS), 3 parts, and centrifuged at $175 \times g$ for 10 min; supernatant fluid was stored in sealed glass ampoules on Dry Ice.

A strain of western encephalitis (WE) virus adapted to destroy L cells (6) was used for interferon assays; the virus was in fluid from the 40th passage in L-cell cultures.

Cell cultures. L strain mouse fibroblasts were grown in a mixture of 200 parts horse serum (HS), 1 part yeast extract (YE; Difco), and 799 parts Hanks' BSS (HS-20, YE-0.1, BSS-79.9). Tube cultures were prepared by inoculating approximately 10^6 cells in 1 ml of growth medium per test tube (16 by 125 mm), and incubating at 37 C for 3 to 7 days. Just prior to inoculation with virus, medium was replaced with 0.9 ml of one of three mixtures: (i) 200 parts HS, 1 part YE, and 799 parts BSS (HS-20, YE-0.1, BSS-79.9); (ii) 10 parts chicken serum and 90 parts maintenance solution (21) modified to omit purines and pyrimidines (CS-10, MS-90); and (iii) 5 parts HS and

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95 parts MS (HS-5, MS-95). Horse and chicken sera were free from JE viral antibody by mouse neutralization test (14), and were heated at 56 C for 30 min before storage at 5 or -20 C until used.

Primary cultures of chicken embryos were prepared as described elsewhere (14); during interferon assays, HS-5, MS-95 was used.

Virus assays. JE virus was usually assayed in weanling mice or in cultures of L cells; for interferon experiments, JE and WE viruses were assayed in primary cultures of chicken embryo cells (CEC) with a solid medium, as described elsewhere (14). Five Swiss albino mice, 3 to 4 weeks of age, were inoculated intracranially with 0.03 ml of each 10-fold dilution of virus made in rabbit serum-5, BSS-95. After 14 days of observation, weanling mouse, intracranial 50% lethal doses of virus ($WM_{ic} LD_{50}$) were calculated (18) from deaths occurring between 2 and 14 days. Alternatively, three to five tube cultures of L cells containing 0.9 ml of HS-20, YE-0.1, BSS-79.9 were inoculated with 0.1-ml samples of each virus dilution, and incubated at 37 C for 6 to 10 days. Cytonecrosis did not occur, but the numbers of L-cell cultures infected and noninfected for each virus dilution were determined by inoculating supernatant fluid from each culture intracranially into weanling mice or into cultures of a continuous strain of porcine kidney cells (PK-2a), in which cell destruction from JE virus infection occurred (12). [PK-2a, clone 13 cells (19) were propagated in HS-10, YE-0.1, BSS-89.9. The medium was replaced every 5 to 7 days, and cells were transferred to new culture bottles every 2 to 4 weeks. Cells were released from glass at room temperature with a solution containing 0.1% trypsin and 0.25% ethylenediamine tetraacetate (EDTA) in water containing the NaCl, KCl, and glucose concentrations of Hanks' (10) BSS. Cells were sedimented at $32 \times g$ for 5 min and resuspended in growth medium. Tube cultures were prepared as for L cells, and medium was replaced with 0.9 ml of HS-8, MS-92 before virus inoculation.] Cultures of L cells used for titrations of JE virus were scored as noninfected or infected (i.e., production of sufficient virus to kill mice or PK-2a cells after incubation at 37 C for 6 to 10 days), and thus 50% infectious doses (ID_{50}) of virus were calculated (18).

Serial passage of JE virus in L-cell cultures. Serial 10-fold dilutions of each of eight strains of virus were inoculated into three cultures of L cells containing CS-10, MS-90, and into five weanling mice. After 6 or 7 days at 37 C, supernatant fluids were harvested and pooled for each virus dilution; a sample was tested in mice for infective virus, and the remainder was stored in sealed glass ampoules on Dry Ice. For the next passage, newly prepared cultures were inoculated with fluid from cultures of the previous passage receiving the highest dilution of virus which had produced mouse infective virus. Serial passage of strain M5/596 was initiated by inoculation of $10^{8.9}$ $WM_{ic} LD_{50}$ into cultures of L cells. Undiluted fluid from infected cultures was used for inocula for subsequent passages of strain M5/596.

Measurement of percentage of cells associated with infective virus. Supernatant fluid was removed from

L-cell cultures which were infected 4 to 21 days previously with $10^{8.7}$ L-cell ID_{50} of JE virus strain M5/596. A 1-ml amount of a mixture of 2 parts JE viral horse antiserum and 3 parts BSS (log neutralization indices versus JE virus for the several antisera employed ranged from 2.9 to 3.5) was added to each tube. Cultures were incubated at 37 C for 2 hr and rinsed eight times with 1 ml of BSS at pH 6.8; the cells were removed from the glass with 0.2% trypsin at pH 7.4 to 7.6. Horse antiserum was added to the trypsinized cell suspension to yield a final concentration of 40% and the mixture was chilled in an ice bath for 30 min. Cells were sedimented at $32 \times g$ for 5 min at 5 C, and allowed to remain in contact with antiserum for an additional 30 min in an ice bath to neutralize virus released by rupture of cells during centrifugation. The supernatant fluid containing antiserum was removed, and cells were resuspended in 10 ml of chilled BSS at pH 7.4. This step diluted the antiserum at least 1:250. Cells were counted in a hemocytometer, and diluted in chilled BSS to yield appropriate cell concentrations between 1 and 500 cells per 0.1 ml. Cell-associated virus was assayed by inoculation of cell suspensions into L cultures or suckling mice; production of infective virus by each L-cell culture was determined 6 to 10 days after inoculation, by transfer of supernatant fluid to two porcine kidney cell cultures. Presence of extracellular virus which might have escaped neutralization by antiserum or dissociated from antibody upon dilution was ruled out by filtering cell suspensions through a Millipore membrane (porosity, 450 $m\mu$) and testing the filtrates for infectivity. Since these membranes were not shown to absorb an appreciable amount of JE virus, absence of infective virus in the filtrates indicated that all infective virus in cell suspensions was associated with nonfilterable particles, namely, cells. [One-half of a diluted mouse brain suspension of strain M5/596 JE virus was passed through a Millipore membrane (porosity, 450 $m\mu$). When assayed in cultures of CEC (14), the unfiltered virus suspension produced 1.5×10^4 plaques per milliliter, and the filtered suspension produced 1.8×10^4 plaques per milliliter.] The number of cells yielding 1 mouse LD_{50} or 1 cell culture ID_{50} was then calculated (18), and the numbers of cells required for 1 ID were derived by use of the Poisson equation (16).

Detection of interferon in fluid from L-cell cultures infected with JE virus. The supernatant fluid was harvested from 12 tube cultures of L cells in HS-5, MS-95, which had been inoculated with 4×10^5 plaque-forming units (PFU) and incubated at 37 C for 4 days. This fluid and 11 ml of fluid harvested from similar uninfected cultures were treated in parallel; they were centrifuged at $800 \times g$ for 10 min at 5 C, adjusted to pH 6.5 with 0.3 N HCl, mixed with 1 ml of goose erythrocytes (packed at $900 \times g$ for 5 min), and incubated at 25 C for 4.5 hr. The mixtures were then centrifuged at $800 \times g$ for 10 min to remove erythrocytes, and the fluids were acidified to pH 3.1 with less than 0.1 volume of 0.3 N HCl. After 5 min at 25 C, a similar volume of 0.3 N NaOH was added to bring the pH to 7.3. A 0.2-ml sample of each fluid was placed on monolayer tube cultures of L cells and CEC for 2 hr at 36 C. Then 0.1-ml samples of 10^{-2} through 10^{-8}

dilutions of WE virus were adsorbed to cells for 30 min at 37 C, and 1 ml of HS-5, MS-95 was added. Titration end points were determined after 4 days at 37 C. The WE virus used contained $10^{8.2}$ 50% tissue culture doses (TCID_{50}) per ml when measured in either L or CEC cultures with fluid medium, and 9×10^7 PFU/ml in CEC cultures with solid medium (14).

RESULTS

When these studies were begun in 1956, JE virus growth in L cells had not been reported, although the virus had been propagated in other cells in vitro (27). Thus, to begin these experiments, several strains of virus were inoculated into cultures of contiguous L cells, and serial passages of virus were attempted. After finding that virus multiplied in such cultures without production of overt cytonecrosis, the cell-virus system was examined further to determine (i) the percentage of cells associated with infectious virus, (ii) whether interferon was produced, (iii) whether cell population growth was affected, and (iv) whether virus infection of sparse cell populations would result in overt cell destruction.

Propagation of JE virus in populations of contiguous L cells without overt cytonecrosis. Nine strains of JE virus in mouse passage 3 or 4 from bird blood (281776, 281777, and 281801), mosquitoes (M5/560b, M5/572, and M5/596), or human brain (55-1456 and 55-1735), or in

mouse passage >70 from human spinal fluid (Nakayama), were passed serially three or four times in L-cell cultures over 18 to 39 days to result in cumulative dilutions of original mouse brain virus of 10^{13} to 10^{18} . The titers of virus inocula ranged from $10^{-0.4}$ to $10^{3.3}$ WM ic LD_{50} per ml, and of virus harvests 6 or 7 days later, from $10^{3.5}$ to $10^{6.3}$. Virus was demonstrated in harvests of each final passage by the death of five weanling mice inoculated intracranially with undiluted culture fluid. All nine strains of virus were identified as JE virus after serial passage in L-cell cultures by preparation of complement-fixing antigens from infected mouse brain, and fixation of complement with 8 units of specific JE antibody made in guinea pigs (1, 2).

During the serial passage of nine strains of JE virus in cultures of contiguous L cells, with the use of input multiplicities as high as 0.5 WM ic LD_{50} per cell, no overt cytonecrosis was seen.

Percentage of L cells associated with infectious JE virus. The absence of overt destruction of L cells despite growth of JE virus in cultures suggested that only a small percentage of cells might be producing infectious virus. When this percentage was measured, it was found that only 0.2 to 3.5% of cells were associated with JE virus (strain M5/596) infectious for L cells or suckling mice (Table 1). The percentage of cells

TABLE 1. Percentages of L cells associated with infective JE virus strain M5/596

Medium, virus passage ^a	Days after inoculation	Avg cell population ($\times 10^6$) in three cultures		Fraction of L-cell cultures (or mice) ^b becoming infected after inoculation with indicated numbers of L cells from other infected cultures							No. of cells from infected cultures yielding one L cell or suckling mouse		Per cent cells associated with infective virus
		Infected	Uninfected	500	200	100	50	10	5	1	ID_{50}	ID^c	
Growth, MB-6	5	312	NC ^d		5/6	4/6	1/6	0/6	0/6	0/6	85	123	0.8
Growth, MB-7	6	483	702	6/6	2/6	1/6	0/6	0/6	0/6	0/6	238	334	0.3
	10	376	941	6/6	2/6	0/6	0/6	0/6	0/6	0/6	302	436	0.2
Growth, MB-3, L-6	4	221	NC		6/6	4/6	2/6	0/6	0/6	0/6	71	102	1.0
					9/18 ^b	3/9	3/15	0/14	0/16	0/15	189	272	0.4
Maintenance, MB-7	6	330	368	6/6	6/6	6/6	1/6	0/6	0/6	0/6	66	95	1.1
	11	331	418		6/6	6/6	6/6	0/6	1/6	0/6	20	29	3.5
	16	260	491		6/6	6/6	4/6	1/6	1/6	0/6	23	33	3.0
	21	204	383		6/6	5/6	3/5	2/5	1/5	0/5	23	33	3.0

^a Growth medium = HS-20, YE-0.1, BSS-79; maintenance medium = HS-5, MS-95. Virus inoculum = $10^{5.7}$ L-cell ID_{50} per culture. MB = mouse brain; L = L-cell culture.

^b Assayed in suckling mice (1 to 5 days old) inoculated intracranially.

^c If it is assumed that one cell associated with infective virus is sufficient to infect an L-cell culture or kill a suckling mouse, then, by application of the Poisson equation, the concentration of cells which will infect 50% of the cultures or kill 50% of the mice will contain, on the average, 0.696 cells associated with infective virus per unit volume.

^d Not counted.

associated with infectious virus was slightly higher in cultures kept 11 to 21 days in maintenance medium (3.0 to 3.5% of cells) than in cultures examined after 4 to 10 days in growth or maintenance medium (0.2 to 1.1%).

It was interesting to note that suckling mice were no more susceptible to intracellular or cell-associated JE virus than L-cell cultures (Table 1). Upon initial consideration, this was unexpected because suckling mice inoculated intracranially are more sensitive to extracellular JE virus than weanling mice (8, 9), whereas weanlings and L-cell cultures are approximately equal in sensitivity. (Infectivity titers determined simultaneously in weanling mice and L-cell cultures for mouse passages 6 and 7 of strain M5/596 of JE virus were $10^{8.7}$ and $10^{8.5}$ LD₅₀ per ml in mice and $10^{8.7}$ and $10^{8.4}$ LD₅₀ per ml in L-cell cultures.) The failure of suckling mice inoculated intracranially to die from quantities of cell-associated virus smaller than those detectable in L-cell cultures suggested that suckling mice detected only cells containing preformed, fully infectious virus, whereas L-cell cultures supported viability of cells associated with virus long enough to permit some virus reproduction. In porcine kidney cell cultures, where L cells could be easily identified morphologically, the L cells persisted for at least 1 week. Thus, the amount of virus detected in cultures may have been greater than

that originally associated with the L cells under assay, so that the cultural detector system gained in sensitivity for cell-associated JE virus and became equal to that of suckling mice.

Production of an interferon. Since most L cells were not associated with infectious JE virus, studies were initiated to determine whether the cells were protected by an interferon. Fluids from normal and JE-infected L-cell cultures, treated as described in Materials and Methods, were tested on cultures of L cells and CEC by challenge with a strain of WE virus ordinarily destructive for these cells (6). Titers of WE virus in L-cell cultures, which had been pretreated for 2 hr at 37 C with "interferon" preparations from either normal or JE virus-infected L-cell cultures, were $10^{8.2}$ and $10^{8.5}$ TCD₅₀ per ml, respectively. In contrast, in cultures of CEC, WE virus titer was $10^{8.2}$ TCD₅₀ per ml in both sets of cultures. This demonstration of an acid-stable substance which protected murine, but not avian, cells from destruction by a heterologous virus provided evidence that an interferon was produced in L-cell cultures infected with JE virus.

Inhibition of cell growth during infection of L-cell cultures with JE virus. No overt cytonecrosis was seen upon careful microscopic examination under $\times 100$ magnification, and yet a few L cells in confluent sheets were associated with infectious JE virus. Therefore, growth rates

FIG. 1. Temporal relationships between growth inhibition of contiguous L cells and production of Japanese encephalitis virus.

FIG. 2. Decrease in sparse L-cell populations after infection and production of Japanese encephalitis virus.

of cells in infected and noninfected L-cell populations were examined to detect possible differences not readily discernible by microscopic observation. Media which permitted cell proliferation and media which only maintained cell populations were used.

After 10 to 14 days of incubation at 37 C in growth medium (HS-20, YE-0.1, BSS-79.9), L-cell populations reached higher levels in un-

infected cultures than in cultures infected with JE virus (Fig. 1). In cultures inoculated with either $10^{5.2}$ or $10^{1.2}$ L-cell ID₅₀ of virus (i.e., input multiplicities of 0.85 and 8.1×10^{-5}), L-cell populations reached only 300,000 to 600,000 cells per culture; whereas, in uninfected cultures, populations reached 800,000 to 900,000. Cell populations maintained at a relatively stable level in maintenance medium (HS-5, MS-95) showed a similar but less striking difference between uninfected cultures and those infected at a multiplicity of 0.17 (Fig. 1). This inhibition of L-cell population growth seemed to be related to virus reproduction, since it occurred 3 to 10 days after virus inoculation, at a time when levels of infectious virus reached 10^4 to 10^6 L-cell ID₅₀ per ml of cultural fluid. Moreover, it occurred sooner with larger than with smaller virus inocula (Fig. 1). Although cytonecrosis was the most likely direct effect of virus to reduce cell numbers, this was not seen in cultures containing contiguous cells. Therefore, populations of sparse, noncontiguous cells were studied since individual cells could be more easily examined, and lower concentrations of interferon were probably produced.

Propagation of JE virus in sparse populations of L cells with overt cytonecrosis. When noncontiguous cell cultures were inoculated with $10^{2.5}$ L-cell ID₅₀ of strain M5/596 of JE virus, virus increased in titer in cultural fluids within 2 days, and persisted for at least 8 days at 36 C (Fig. 2). Cell populations fell from 60,000 cells per tube to essentially none by 8 days after inoculation of virus (Fig. 2), and cell destruction was obvious by microscopic examination within 6

FIG. 3. Unstained L cells in growth medium 6 days after inoculation of a culture of sparsely populated cells with $10^{2.5}$ L-cell ID₅₀ of Japanese encephalitis of virus strain M5/596. $\times 75$.

FIG. 4. Normal L cells in an uninoculated control culture photographed on the same day as Fig. 5. $\times 75$.

days of inoculation (Fig. 3). In contrast, populations of control, uninfected cultures increased to levels of 100,000 to 300,000 cells per tube within 6 to 8 days of incubation (Fig. 2), and cells became contiguous (Fig. 4). Sparse cell populations declined more rapidly when input multiplicities of 4 and 5 (strain M5/596) or 12 (strain 281801) were used; in such experiments, by 2 days after inoculation of virus, cell populations of infected cultures were distinctly lower than those of uninfected cultures.

DISCUSSION

These studies revealed that JE virus represented by strains isolated from mosquitoes, birds, and man grew in cultures of contiguous L mouse fibroblastic cells without cytopathic effects. The inappreciability of this virus-cell interaction resulted from production of an interferon and association of only a few cells (0.2 to 3.5%) with infectious virus. These small percentages of cells associated with infective virus were similar to those reported for other cell-virus systems (4, 29). However, L cells grew less rapidly in infected than in uninfected cultures, providing a method for detecting JE virus infection of cultured L cells in the absence of visible cytopathic effects. Virus infection of cells was also apparent in cultures of widely separated L cells. Cytopathic effects were easily seen, probably because individual cells could be examined microscopically, interferon was in low concentrations, and cells were associated with more infectious JE virus than in cultures of contiguous cells.

The mechanisms causing inhibition of L-cell population growth in cultures infected with JE virus are not completely understood. Differences in cell populations between infected and uninfected cultures could be partially explained by cytonecrosis caused by virus, but other factors seemed to be involved. Uninfected cells grew from 702,000 to 941,000 over a 4-day period in growth medium (an average increase of 60,000 cells or 8.5% per day) (Table 1). Yet, in infected cultures at any one time, only 0.3% of the cells were associated with infective virus. If inhibition of cell population growth were due entirely to destruction of cells associated with infective virus, then the cell populations in infected cultures containing growth medium would have increased in size by 8.2% (8.5 - 0.3) per day. Yet the cell population actually decreased 6% per day (from 483,000 to 376,000 over 4 days). Thus, additional factors must have been involved in inhibition of growth of L-cell populations infected by JE virus. One such factor probably was interferon, which has been shown to depress L-cell growth (17), but other unknown mechanisms could also have been operative.

Growth of JE virus in L mouse cells derived originally from subcutaneous connective tissue with the destruction of only a few cells is consistent with the lack of pathology in non-neural connective tissues of infected humans and animals (15, 31). The fact that a variety of vertebrate tissues support growth of JE virus in vitro (26), and that asymptomatic viremia frequently occurs in animals (3, 13, 22, 24, 25), suggests that this virus grows in non-neural tissues in vivo without producing sufficient cytonecrosis to cause disease. Whether interferon is produced or cell replication is inhibited in vivo, as has been demonstrated in vitro, is speculative at this time, but certainly such phenomena might occur. Since interferon occurs in brain tissues and affects multiplication of arboviruses in mouse brains (11), production of encephalitis by JE virus is evidently not due to an inherent failure of brain tissues to make or to respond to interferon. Perhaps the usual absence of encephalitis in primary infection of man and animals with arthropod-borne encephalitis viruses is due to such protective mechanisms operating in brain as well as in non-neural tissues. The occasional case of encephalitis might then be related primarily to failure of protective mechanisms, perhaps overwhelmed by large quantities of virus or by unusually virulent strains. Additional studies of these problems are obviously needed to clarify further the pathogenesis of arthropod-borne viral encephalitides.

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