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Persistence of West Nile Virus in L-929 Mouse Fibroblasts* (33385)

RUTH V. JARMAN, PAUL N. MORGAN, AND CARL E. DUFFY

Department of Microbiology, University of Arkansas Medical Center, Little Rock, Arkansas 72201; and Veteran's Administration Hospital, Little Rock, Arkansas 72206

It has been demonstrated that under certain conditions a variety of mammalian cell cultures can be chronically infected with viruses and that both cells and infecting virus can replicate simultaneously (1–3). Chronic infections have been induced in the presence of both specific antiviral antibodies and nonspecific antiviral substances as well as by nutritional deficiencies (3). In only a few instances have chronically-infected cell cultures been established when routine cultural methods were employed (3). The present paper reports the establishment of a chronic infection in L-929 mouse fibroblasts (4) with a virulent group B arbovirus, West Nile virus (5), using routine cultural methods.

Materials and Methods. Cell cultures. The L-929 mouse fibroblasts were obtained from Microbiological Associates, Inc. Stock cell cultures and infected cell cultures were grown in a modified Eagle's minimum essential medium (6)[1] prepared in Hanks' balanced salt solution[1] and supplemented with 1 m*M* sodium pyruvate,[1] 0.1 m*M* nonessential amino acids,[1] 50 units of penicillin/ml, 100 μg of streptomycin/ml and 5% calf serum[1] (H-MEM). All cell cultures were grown in 12 ml of medium in tightly sealed 250-ml square glass bottles incubated at 36°. Medium was routinely replaced at 3- or 4-day intervals. Subcultures were initiated by replacement of H-MEM with 0.2% trypsin.[2] After cells were released from the glass, they were centrifuged, trypsin was decanted, and cells were resuspended in fresh H-MEM.

Virus. The West Nile virus (WNV) used in this study was obtained from American Type Culture Collection as a 10% mouse brain suspension (twenty-fifth passage) in normal mouse serum. Stock virus [Arkansas Mouse Passage 8 (AMP 8)] was prepared as supernatant fluid of infected mouse brain homogenate in sufficient 50% normal rabbit serum in saline to make a 10% brain suspension (w/v). Sealed glass ampuls of AMP 8 were shell frozen and stored in a CO_2 box. The titer of WNV in AMP 8 was determined by intracerebral inoculation of 3-week-old Swiss mice. Tenfold dilutions were made in H-MEM and 0.03 ml of each dilution was injected into each of 5 mice. The LD_{50} titers were calculated by the method of Reed and Muench (7) and ranged from $10^{-7.2}$ to $10^{-7.7}$ when titrated five times over a period of 14 months.

Initial infection of cell cultures with WNV. After removal of medium from normal

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[1] Microbiological Associates, Inc.

[2] Difco certified, 1:250.

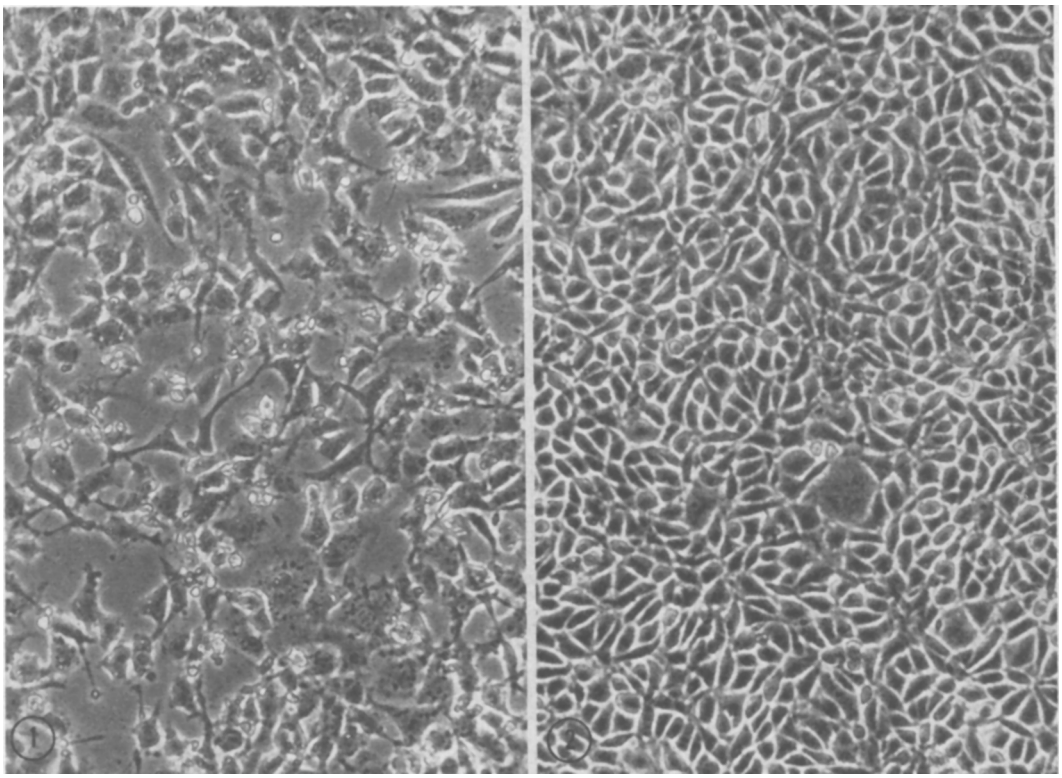

FIG. 1. L-929 mouse fibroblasts 5 days after infection with mouse brain suspension of West Nile virus; unstained, phase contrast, 125×.

FIG. 2. L-929 mouse fibroblasts 5 days after addition of normal mouse brain suspension; unstained, phase contrast, 125×.

cultures, cells were infected with WNV by the addition of 2 ml of a 1:10 dilution of AMP 8. After 30 min at 36°, the inoculum was removed and cell monolayers were rinsed with medium. Infected cultures were then incubated at 36° in 12 ml of fresh H-MEM.

Assay of infectious virus from infected cell cultures. In each experiment, equal amounts of growth medium from two or more infected cell cultures were removed, pooled, and considered as a 10^{-1} dilution. Tenfold dilutions were prepared as described previously and were injected intracerebrally into young mice. The LD_{50} titers were determined in the same manner as described above for AMP 8. After taking samples, the remaining medium was discarded, monolayers were rinsed, and cultures were reincubated in fresh H-MEM.

Neutralization tests were done with WNV in AMP 8 and with virus obtained from infected cell cultures. Specific WNV rabbit antiserum was obtained from Dr. William McD. Hammon of the University of Pittsburgh. Equal volumes of antiserum or normal rabbit serum were added to tenfold dilutions of virus and the mixtures were placed in a 37° water bath for 2 hr before intracerebral inoculation into Swiss mice. The LD_{50} titers were calculated as described previously.

Results. Initial infection of L-929 mouse fibroblasts with WNV resulted in no marked cellular destruction; however, distinct and specific morphological alterations occurred which were readily recognized in subsequent cultures. Changes were most noticeable after 4 or 5 days and included increased cellular granularity, less distinct fibroblastic processes and clumps of cells separated by open areas in the monolayer as though the cells had been pulled together (Fig. 1). Control cell

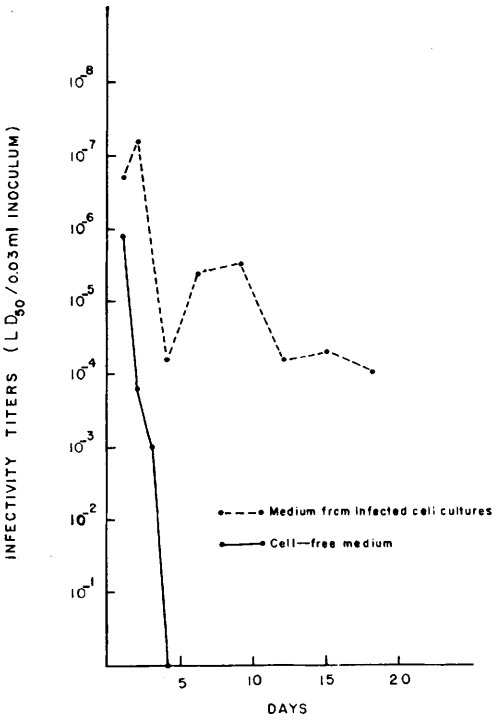

FIG. 3. Titers of West Nile virus in cell-free medium and in medium from infected cell cultures.

cultures which received the same amount of normal mouse brain suspension did not show these changes (Fig. 2).

After infection, these cultures were maintained for 3 weeks by changing the medium regularly. During this period, infectious virus was found to persist in the medium in rather high concentrations. In a control experiment, AMP 8 was added to growth medium under identical conditions except for the absence of cells. Figure 3 shows data from these experiments. In the absence of cells and without dilution by medium changes, infectious virus was completely inactivated after 4 days of incubation at 36°. In the presence of L-929 cells and after two complete medium changes, viral titers had decreased about 1000-fold (10^{-7} to 10^{-4}) during the same period of time; however, after 4 days, the titers remained rather constant even after regular changes of medium.

On day 21 after infection, subcultures of the viral-infected cells were made. No alterations in normal procedures were necessary. Fewer packed cells were obtained from the infected cultures but after subculture, the cells adhered to the glass surfaces, multiplied and continued to show the characteristic morphological changes described above. Additional subcultures of cells and titrations of medium for virus were done at varying intervals and data from some of these experiments are given in Table I. Total time from infection of the original cells, which were designated as Line A, to final titration of medium was 3 months and 3 days.

During this period, additional chronically-infected cell cultures were readily established and similar subcultures were made of them. Periodic assays for WNV in growth medium showed a significant amount of infectious virulent virus to be present whenever tested (Table I).

From these data, it appeared that the cells were chronically infected and were producing infectious virus at a constant rate. In an effort to demonstrate an increase in viral titer soon after subculture, infected cells of Line B (Table I) were harvested routinely but were washed 6 times in 5-ml volumes of H-MEM. After subculturing the washed cells, samples of medium were removed at various

TABLE I. West Nile Virus in Medium from Chronically-Infected L-929 Cell Cultures.

Subculture no.	Time (days) after subculture	Line A	Line B	Line C	Line D
1	6	4.0[a]	6.4	5.4	
	12	4.0			4.6
2	3	4.6			
	6	3.8	4.4		5.8
	19			4.3	
	49				3.2
3	6	5.2	4.2		
	9			4.5	
	15	4.5			
	35			4.4	
4	5	3.8	5.5		
	9			5.2	
5	1	3.4	5.4		
	30	2.0			

[a] Mouse infectivity titers expressed as positive $\log_{10}$ LD_{50}/0.03 ml of inoculum.

TABLE II. West Nile Virus from Chronically-Infected L-929 Cells of Line B.

Item	Titers
Medium from fourth subculture	5.5[a]
Supernate from washed cells, sixth wash	2.3
Medium from fifth subculture after (hr)	
2	3.8
24	5.4
48	5.6

[a] Mouse infectivity titers expressed as positive $\log_{10}$ LD_{50}/0.03 ml of inoculum.

intervals and assayed for infectious virus. Data from these experiments are presented in Table II. Prior to subculture, the viral titer of the medium was $10^{-5.5}$. After the cells were washed, the viral titer was reduced about 1000 times. Two hr after seeding the cells, the viral titer in the supernatant had increased to $10^{-3.8}$ and 24 and 48 hr after subculture, titers had returned to levels maintained prior to subculture.

The identification of viruses as WNV was confirmed by neutralization tests. Table III shows results of these experiments. The infectivity of the mouse brain-adapted virus (AMP 8) used as the original inoculum was reduced 10,000 times (from 10^{-8} to 10^{-4}) in the presence of WNV antiserum. The infectious virus present in growth medium from chronically-infected cultures (Line C) was completely neutralized by the same antiserum.

Discussion. These experiments showed that an unusual host cell–virus relationship developed when virulent West Nile virus, capable of producing fatal encephalitis in mice, was introduced into cultures of L-929 mouse fibroblasts. Morphological changes in cells became evident and infectious virus was produced consistently but the infected cultures were not completely destroyed. This cell–virus association was maintained by changing the growth medium regularly and it was continued after routine subculture of the infected cells. One of these chronically-infected cultures has been continued through nine subcultures for more than 9 months after initial infection and similar titers of infectious virus have been demonstrated in the growth medium whenever tested. It would appear that this association can be maintained indefinitely under normal conditions of growth.

Neutralization tests using specific WNV antiserum showed that the virus from infected cells was antigenically WNV and the same or closely related to the mouse brain virus which was used to initiate the infection.

Chambers (8) reported an example of cell–virus association with a group A arbovirus, Western equine encephalitis virus, in a strain of L-cells. In her report, the association was characterized by periodic enhanced cellular lysis without significant increase in viral titers. She also indicated that this carrier-culture was difficult to maintain and that virus could be lost without explanation. Subcultures of these cells were not done routinely but it was possible to maintain the association for several months.

The studies of Hearn and Brown (9) described a cell–virus relationship after Venezuelan equine encephalitis virus, another group A arbovirus, was grown in L-cells. The infected cultures were maintained for 9 months but their data do not indicate that the cells were routinely subcultured. It was, however, reported that infected cells could be induced to produce more virus by ultraviolet irradiation or by glutamine deficiency.

To our knowledge, the data reported here describe the first chronically-infected cell culture involving a group B arbovirus which apparently can be subcultured indefinitely. Dubbs and Scherer (10) have reported an inapparent infection of L-cells by Japanese

TABLE III. Neutralization Tests of WNV from Mouse Brain and Virus from Chronically-Infected L-929 Cells.

	Titers (rabbit serum)	
	Normal	Immune
Mouse brain WNV (AMP 8)	8.0[a]	4.0
Growth medium from infected cells	4.6	0

[a] Mouse infectivity titers expressed as positive $\log_{10}$ LD_{50}/0.03 ml of inoculum.

encephalitis virus, another group B arbovirus. In their studies, it was shown that the virus could persist in cell cultures for 21 days. However, the infected cells were not serially propagated and therefore could not be considered as chronically infected.

In this report, it was shown that no special procedures were necessary to maintain the balance between L-929 cellular multiplication and WNV replication. Morphological changes in the infected cells were seen in subcultures and infectious virus was consistently present in the culture medium.

Summary. When West Nile virus was added to cultures of L-929 mouse fibroblasts, characteristic morphological changes were produced. The infected cultures were not destroyed although infectious virus was consistently demonstrated in the growth medium. Infected cells were readily subcultured and the virus–cell relationship was continued without any alteration in cultural procedures.

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Increased Susceptibility to Murine Hepatitis Virus Infection by Treatment with Iron Salts* (33386)

JOEL WARREN, RACHEL J. MASON, LUIZ H. BARBOSA, KAREN L. GABBARD, AND MARVIN BUCY

Chas. Pfizer & Co., Inc., Department of Biologics Research, Terre Haute, Indiana 47808

This study was undertaken to determine whether susceptibility of mice to a viral hepatitis infection could be modified by the administration of iron salts. Ferric ammonium citrate (FAC) was selected since large doses are capable of inducing centrilobular hepatic necrosis (1), and because Bullen, *et al.* (2) cite their own observations as well as those of others (3) in support of the thesis that active and passive immunity to several bacterial infections in rodents can be abolished by the inoculation of iron salts. These reports suggest that this may be related to saturation of transferrin, a bacteriostatic iron-binding serum protein (4), and not to a direct effect of iron on the microorganisms. The present paper describes an enhanced susceptibility in mice to mouse hepatitis virus (MHV) following the inoculation of soluble iron and the protective effects of a chelating agent, EDTA, against this disease.

Materials and Methods. Virus. MHV-3 was obtained from Dr. M. Pollard in an infected primary explant of mouse embryo. It was passed intraperitoneally in day-old mice and their livers were harvested 5–7 days later when macroscopic lesions were prominent. A clarified 10% homogenate of this tissue in Eagle's basal medium + 10% fetal calf serum was frozen at —60° and served as a standard inoculum.

Mice. Swiss albino mice, originally of a hybrid NIH stock, were obtained from a local dealer. Because maturing animals are increasingly resistant to high doses of MHV, newborn litters 1–2 days of age were used exclusively. Randomized sucklings from

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