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Chikungunya Virus Infection of Cell Monolayers by Cell-to-Cell and Extracellular Transmission

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Both cell-to-cell and extracellular transmission of chikungunya virus infection was demonstrated in BHK21/C13 cell monolayers. The mode of virus infection may depend on the cell line. Virus transmission in L-929 and guinea pig lung cell lines was extracellular.

A preliminary study directed toward the development of an immunofluorescent assay of chikungunya virus based on the enumeration of individual infected cells in monolayer cultures was hampered by the appearance of foci containing several infected cells. These foci were noted even when inoculated cell monolayers were incubated in the presence of a potent antiviral serum overlay. That virus infection may proceed by cell-to-cell transmission was suggested by these observations. This mode of infection in cell cultures has been reported previously with herpes B (1) and respiratory syncytial viruses (4). In the present study, we offer evidence that both cell-to-cell and extracellular transmission of chikungunya virus infection occurs in cell monolayers.

The Banganike strain of chikungunya virus (6), supplied by William A. Hankins, Fort Detrick, Frederick, Md., was used. It was in the form of a 10% suckling mouse brain suspension and had a titer of $10^{8.9}$ LD_{50} per ml by intracerebral assay in this host. The two baby hamster kidney (BHK) cell lines used in this study, designated BHK21/C13 and BHK21, were obtained from the American Type Culture Collection, Rockville, Md., and from Microbiological Associates, Bethesda, Md., respectively. The BHK21/C13 line morphologically consists of elongated fibroblastic cells; the BHK21 line is mainly short fibroblastic cells. Nutrient medium for both cell lines consisted of Earle minimum essential medium supplemented with 1% glutamine (200 mM), 10% tryptose phosphate broth, and 10% fetal calf serum (FCS). Maintenance medium consisted of equal parts of nutrient medium and Earle minimum essential medium. Nutrient and maintenance media for L-929 (mouse fibroblast) cells were medium 199 plus 5% FCS. Nutrient medium for guinea pig lung cells was Eagle basal medium, 10% FCS, and 0.5% lactalbumin hydrolysate; cells were maintained in Eagle basal medium plus 5% FCS. All media contained 50 μg of streptomycin and 75 μg of kanamycin per ml. Infected cell monolayers were stained by the direct immunofluorescent method using hyperimmune rhesus monkey serum conjugated with fluorescein isothiocyanate (3) to observe the spread of virus and to enumerate foci of infection. Reagents, fluorescent equipment, and other technical procedures used to carry out the immunofluorescent method are described in detail elsewhere (2).

TABLE 1. *Evidence of cell-to-cell transmission of chikungunya virus infection in BHK21/C13 cell monolayers*

Test condition	IFU[a]/50 microscopic fields
Virus-inoculated cell monolayers[b]	21
Trypsinized infected cell monolayers, suspended in antiviral serum medium, inoculated onto uninfected cell monolayers	17
Supernatant medium from above, free of cells, inoculated onto uninfected cell monolayers	0

[a] Immunofluorescent focus units.
[b] Incubated at 35 C for 1 hr to ensure virus penetration into cells, and then incubated at 35 C for 20 hr in presence of antiviral serum medium.

To substantiate the preliminary indication of cell-to-cell transmission of chikungunya virus infection in BHK21/C13 cell monolayers, cover-slip cell cultures were inoculated with 0.2 ml of an appropriate dilution of virus suspension, and inoculum was attached as described previously with another arbovirus (2). Cover-slip cell cultures were then incubated at 35 C for 1 hr in the presence of maintenance medium to allow for vi-

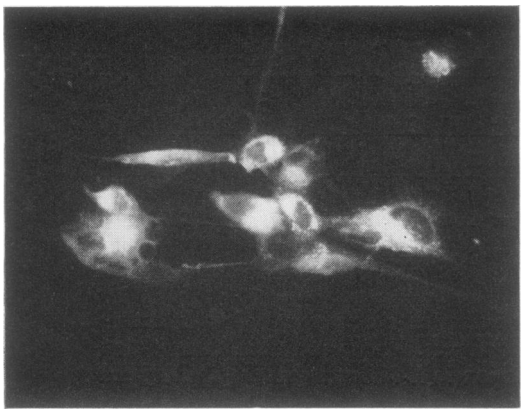

FIG. 1. *Fluorescent focus of chikungunya virus infection in BHK21/C13 cell monolayer in the presence of antiviral serum.* ×225.

rus penetration. A group of these inoculated cell monolayers was then incubated at 35 C for 20 hr in the presence of a 1:10 dilution of antiviral serum in maintenance medium. Another group of inoculated cell monolayers was dispersed with trypsin and suspended in maintenance medium containing antiviral serum. The cells were then sedimented onto uninfected cell monolayers by centrifugal force (500 × *g*, 10 min). The residual inoculum, free of cells, was pooled and introduced onto uninfected cell monolayers. All cell cultures were then incubated at 35 C for 20 hr and subsequently stained. Foci of immunofluorescent cells appeared in the presence of antiviral serum (Table 1) and also with trypsin-dispersed cells that had been sedimented onto uninfected cell monolayers (Fig. 1). Recovery of infected cells dispersed with trypsin was 80% efficient. No infectious virus was detected in cell-free inoculum. The ability of cell-associated virus (trypsin-dispersed cells) to initiate foci of infected cells in the presence of antiviral serum is direct evidence of cell-to-cell transfer of virus infection.

To determine whether chikungunya virus infection by cell-to-cell transfer is limited to the BHK21/C13 cell line, cover-slip cell cultures of different cell lines were infected with virus and incubated at 35 C for 20 hr either in the presence of medium containing antiviral serum or maintenance medium. Foci of infected cells occurred in both BHK21/C13 and BHK21 cell lines in the presence of antiviral serum (Table 2). Large numbers of both foci and individual infected cells were noted in the absence of antiviral serum, which indicates that extracellular infection had occurred. The detection of virus in harvested medium from these latter cell cultures is additional evidence for extracellular transfer of infection. No virus was found in medium harvested from cell cultures incubated earlier with antiviral serum.

TABLE 2. *Cell-to-cell and extracellular transmission of chikungunya virus infection in different cell monolayers*

Cell line	Antiviral serum medium	Cell maintenance medium
BHK21/C13	2.4×10^8 IFU[a]/ml	TNTC[c]
BHK21	2.6×10^7 IFU/ml	TNTC
L-929	5.8×10^5 CIU[b]/ml	5.3×10^5 IFU/ml
Guinea pig lung	2.1×10^5 CIU/ml	1.5×10^5 IFU/ml

[a] Immunofluorescent focus units consisting of 3 to 15 infected cells.

[b] Cell-infecting units; individual infected cells.

[c] Too numerous to count; cell monolayers contained both fluorescent foci and individual infected cells.

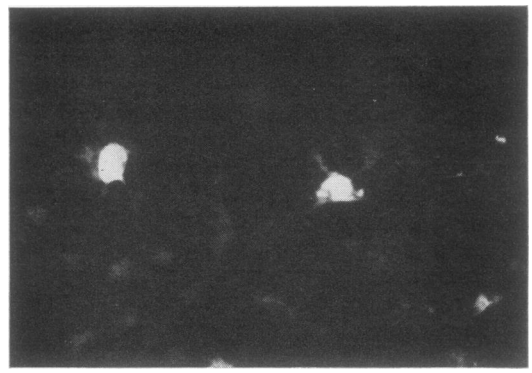

FIG. 2. *Individual guinea pig cells infected with chikungunya virus in the presence of antiviral serum.* ×225.

In contrast, only individual infected cells were noted in the L-929 and guinea pig lung cell cultures that had been incubated with antiviral serum (Fig. 2). This indicates an extracellular mode of virus infection. Of the cell lines tested, the BHK21/C13 cells appeared to be the most susceptible to chikungunya virus infection. The findings of this study show that chikungunya virus infection of BHK cell monolayers may take place by both cell-to-cell and extracellular transmission and that the mode of virus transfer may depend on the cell line. In comparable studies with Venezuelan equine encephalomyelitis virus in the

BHK21/C13 cell line and other cell lines (L-929, McCoy, guinea pig lung), similar results were obtained (Hahon, *unpublished experiments*). The observations reported here, together with those on the neoplastic transformation of BHK cells and its derivatives by viruses (5), suggest that these cells possess some unique biological membrane structure or physiology that is conducive for cell-to-cell transmission of virus particles.

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