

Characteristics of Serially Propagated Monkey Kidney Cell Cultures with Persistent Rubella Infection

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ABSTRACT

MAASSAB, H. F. (University of Michigan, Ann Arbor), AND J. A. VERONELLI. Characteristics of serially propagated monkey kidney cell cultures with persistent rubella infection. *J. Bacteriol.* **91**:436-441. 1966.—A persistent infection of LLC-MK₂ cells with rubella virus has been established and maintained for over 3 years. This "carrier culture," designated as LLC-MK₂-RAL, possesses distinct morphological and biological characteristics when compared with the original uninfected LLC-MK₂ line. The mechanism of viral persistence has not been entirely elucidated, but available data suggest a regulated infection with transmission of the virus directly from cell to cell or through cell division. Interferon was isolated from RAL (rubella-associated line) culture, which explains partly the wide spectrum of resistance to superinfecting viruses. Amantadine, although inhibiting cultures of LLC-MK₂ cells infected with rubella virus, failed to cure the "carrier culture."

Rubella virus was originally demonstrated in tissue culture by means of its interfering effect (11) and its cytopathogenic action in primary human amnion tissue culture (20). Isolation and growth of the virus in a continuous rhesus monkey kidney cell line (LLC-MK₂) has been reported previously (8, 15). Rubella infection in this line produces morphological alterations which are distinct and specific, but not extensive (14). Since these changes do not lead to a rapid destruction of infected cells, it has been possible to establish and maintain a persistently infected cell line in our laboratory for 35 passages over a period of 3 years. Some characteristics of this continuously infected cell line at the 22nd passage level will be presented here. It is noteworthy to state that the same type of results were also obtained at an earlier passage level of this line (10th passage).

MATERIALS AND METHODS

Control line. The cells used were a continuous line of rhesus monkey kidney (LLC-MK₂) (7). They were grown and maintained in medium 199 with 1% horse serum. To obtain monolayers, a standard suspension of cells containing approximately 75,000 cells per milliliter was used to seed tissue culture flasks of different sizes with various amounts, depending upon the type of experiment. A uniform monolayer was usually obtained in 5 to 7 days, with one feeding interval.

Establishment of the "carrier state." The LLC-MK₂

line of cells was infected with a high multiplicity of WM strain of rubella isolated in our laboratory. The line, which will be designated throughout this study as RAL (rubella-associated line) culture, is subcultured every 4 weeks by use of approximately 75,000 to 90,000 cells per milliliter. When subculturing, 0.25% of 1:250 trypsin (Difco) was used. The trypsin was left in contact with the cells for 10 min at 36 C, which was sufficient for dispersing the monolayers into individual cells. A medium of the same composition as that employed for the control culture was used for growing and maintaining the cells. The feeding schedule consists of changing 50% of medium every 3rd day. The RAL and control cultures were handled in a similar manner during this study by use of approximately the same number of cells when subculturing and by following the same feeding schedule.

Infectivity titrations. Cell sheets of LLC-MK₂ line were formed in 5 days in tissue culture tubes seeded with 75,000 cells per milliliter. Sets of four tubes were inoculated with 1 ml of serial 10-fold dilutions of virus in media and were fed every 3 days. On the 12th day, 100 TCID₅₀ per milliliter of echovirus 11 was added in 1 ml. Readings were taken when the echovirus 11 cytopathic effect (CPE) was complete in control tubes. Titters were recorded as the reciprocal of the log₁₀ dilution showing 50% interference (Int₅₀).

RESULTS

Morphological characteristics. Distinct differences in appearance exist between the two cultures by the 2nd day after seeding. The RAL

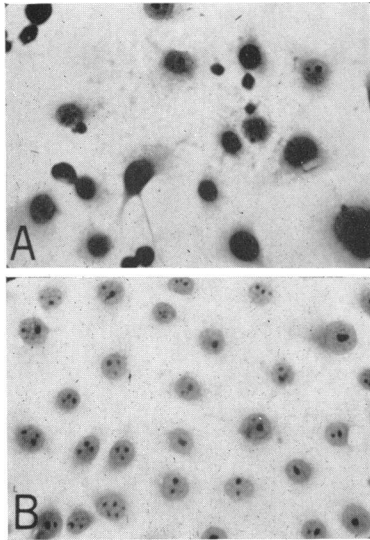


FIG. 1. Morphological characteristics of the two cultures on the 2nd day after seeding. (A) RAL cells. (B) Control cells. Giemsa stain. $\times 400$.

culture exhibits sparsity of cells, with the majority of cells being rounded; also, the cell sheet is not as uniform as the control. Figure 1 compares the two lines at higher magnification. In the RAL culture (Fig. 1a) small rounded cells (with no apparent cytoplasm) are evident the 2nd day after seeding. A more dramatic morphological differentiation between the two cultures is apparent the 5th day after seeding (Fig. 2). In the RAL culture (Fig. 2a, b, c), distinguishing features can be seen, as compared with the control cells (Fig. 2d): intracytoplasmic inclusions which are eosinophilic, irregular-shaped nucleoli which are always prominent and enlarged. The morphological characteristics of the "carrier culture" were not aggravated by mechanical manipulations.

Biological characteristics. The data presented in Fig. 3 show the rates of increase in numbers of cells of RAL and control cultures during a 21-day interval. It is evident that the RAL culture grows more slowly, and that there is a longer lag period prior to the first increase in number of cells. Nevertheless, the number of cells is reached in the two cultures on the 21st day after seeding, but the RAL cultures always exhibit a lower number of cells than the control, the difference ranging between a 1.0 and 1.5 \log_{10} . Figure 4 shows the total infectious yield (i.e., the combined titer of the cells plus the medium) of rubella virus from the RAL culture. It is evident that rubella virus titers start to rise between zero hours and the 2nd day after subculturing. The yield increases,

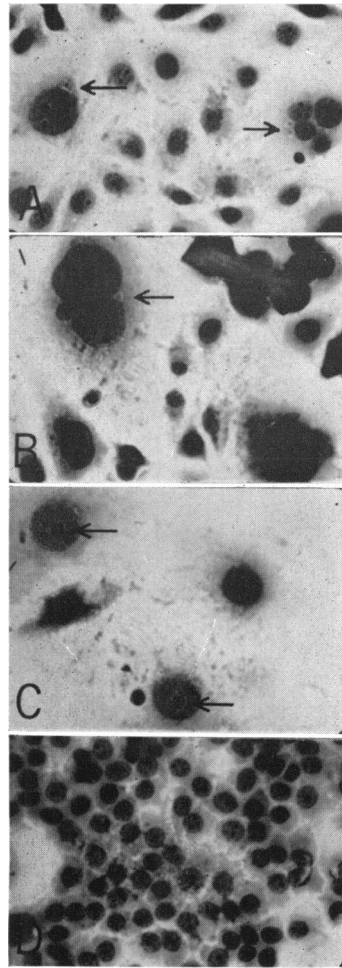


FIG. 2. Morphological characteristics of the two cultures on the 5th day after seeding. Three representative fields (A, B, C) of RAL cells, with arrows showing intracytoplasmic eosinophilic inclusions and enlarged prominent nucleoli. Control cells are shown at (D). Giemsa stain. $\times 400$.

although no increment in cell number occurs (Fig. 3). The maximal titer of rubella virus is reached 4 to 8 days after subculturing the cells. The virus yield and shape of growth curve in RAL cells is similar to that obtained in LLC-MK₂ cells infected with rubella virus at multiplicities comparable with those used in starting the "carrier state" (8).

Another characteristic of the RAL culture is its resistance at all times to superinfection by either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) viruses. Figure 5 shows the behavior of echovirus 11 in both control and chronically infected cells. The cells from both

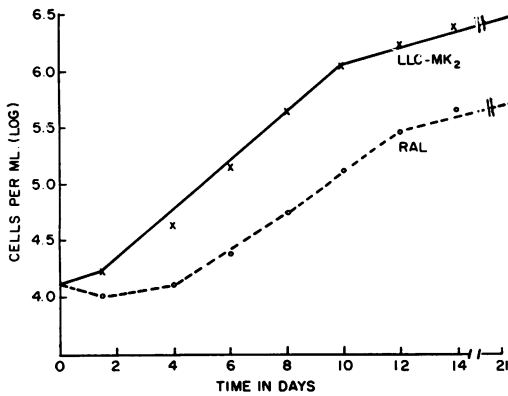


FIG. 3. Growth pattern of the two cultures.

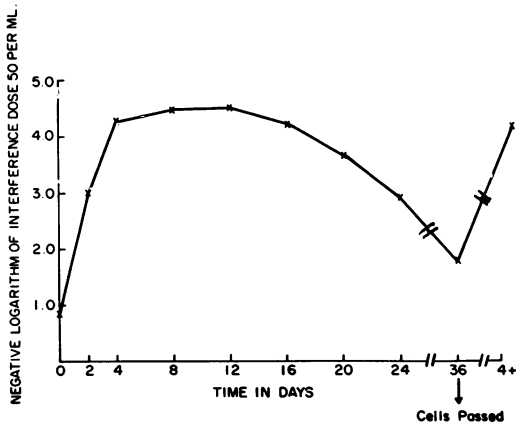


FIG. 4. Total infectious yield of rubella virus from RAL culture.

cultures were infected with 1,000 infectious doses of echovirus 11 right after trypsinization and before seeding. Samples were taken at the designated intervals and were titrated in LLC-MK₂ cells for the infectious yield of echovirus 11. The RAL culture showed progressive loss of titer to echovirus 11, and no CPE was seen with prolonged incubation, although the control culture showed increase in titer accompanied by typical cellular destruction. Similar results have been observed with vaccinia (DNA virus), poliovirus, coxsackie A₉, and parainfluenza 3.

The RAL culture is resistant to the action of amantadine (a highly stable compound *in vitro*). Upon treatment of five successive passages of RAL cells with amantadine, infectious virus titer was approximately $10^{-3.5}$ Int₅₀ per milliliter for both treated and untreated cultures (Table 1). Amantadine was present throughout the experimental period for the entire five passages of the culture. It was previously reported that amanta-

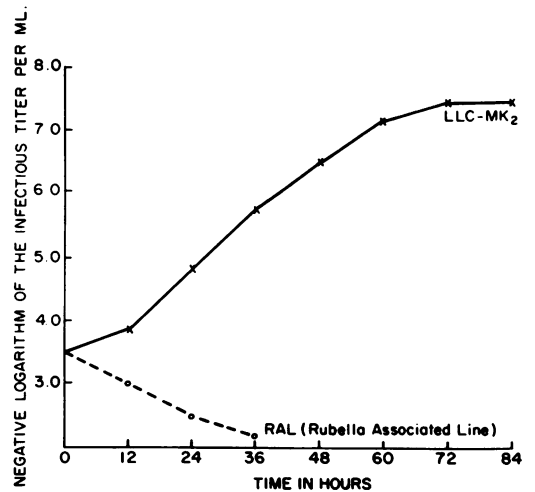


FIG. 5. Growth of echovirus 11 in the two cultures.

TABLE 1. Effect of amantadine on growth of rubella virus in the two cultures

Type of culture	Amantadine concn μg/ml	Infectivity titer at fifth passage*
RAL (chronic)	—	3.5
	31.5	3.7
LLC-MK ₂ (acute) †	—	4.7
	31.5	0.0

* Titer = negative log of dilution showing 50% interference.

† LLC-MK₂ culture infected with WM strain of rubella virus in the presence of amantadine.

dine inhibits rubella virus multiplication (9). This was found to be the case in LLC-MK₂ cells (infected at zero hours in presence of amantadine) where there was complete suppression of rubella virus growth. Amantadine has been shown to act at an early phase of virus cycle (3, 9; Cochran et al., Ann. N.Y. Acad. Sci., *in press*), and it was postulated that it interferes with penetration of viruses into susceptible cells. The fact that amantadine does not inhibit rubella virus growth in RAL cells suggests that the virus is intracellular and thereby is not influenced by its action. The implication is that virus in RAL cells appears to be transmitted from cell to cell directly and not from the extracellular phase to other susceptible cells. The RAL culture cannot be cured by high titer γ -globulin. The "carrier culture" and LLC-MK₂ cells infected with rubella virus were treated for three successive passages with high titer (1:2,048)

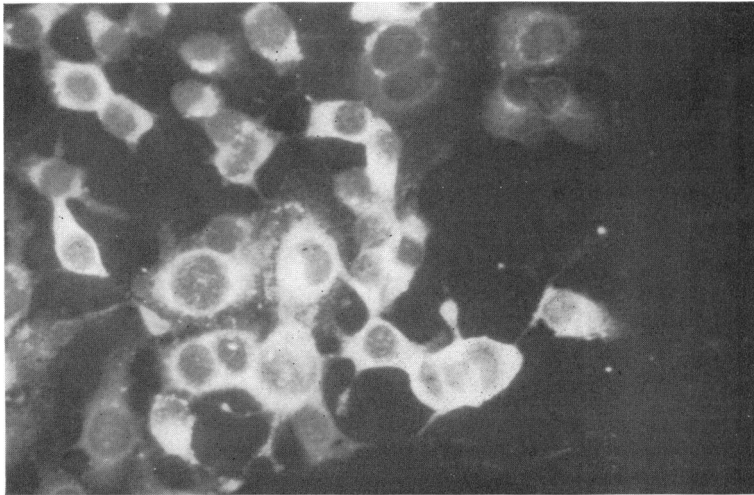


FIG. 6. Culture of RAL cells 5 days after seeding. Indirect immunofluorescent technique (1:8 dilution of human convalescent serum). $\times 180$.

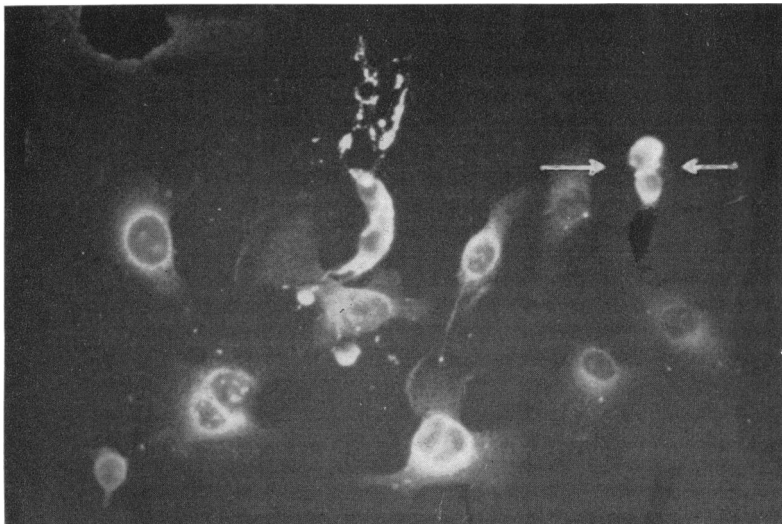


FIG. 7. RAL cells stained with fluorescein-labeled antibody to human γ -globulin by indirect technique, after 1:8 dilution of human serum from patient convalescing from rubella infection. Arrows indicate a cell undergoing mitosis with intense peripheral fluorescence. $\times 180$.

neutralizing γ -globulin. Once the γ -globulin is removed, it was not possible to recover rubella virus from the infected LLC-MK₂ culture. However, infectious virus with a titer of $10^{-4.0}$ Int₅₀ per milliliter was recovered from "carrier culture" upon removal of the γ -globulin.

The RAL culture has also been used for the development of a rapid and specific test for rubella antibodies by the indirect method of fluorescent microscopy (1), and, in addition, immunofluorescence was used to determine the

percentage of infected cells. Figure 6 illustrates the reaction of a serum from a convalescent patient with infected cells 5 days after subculturing the cells. When the culture is freshly seeded, dividing cells may be seen with intense peripheral fluorescence (Fig. 7); the same type of cells was followed for a period of 5 hr in the microscope and was eventually divided into two distinct cells with intense fluorescence. The RAL culture has also been used recently as a source for rubella complement-fixing antigen (13).

DISCUSSION

The persistent viral infection of tissue cultures, allowing both viral and cell multiplication, has been of considerable interest to investigators since Feller, Enders, and Weller (4) demonstrated more than 20 years ago the possibility of long-term cultivation of vaccinia-infected cells. Many other systems have been described since that time (5, 17). In some, the carrier system is dependent on the replacement of cells undergoing cytopathic change from infection by rapid cell proliferation or on the addition of viral inhibitors or specific antibodies. The RAL cultures do not necessitate such factors. The data available seem to indicate that one or both of the mechanisms discussed below are in operation.

Henle and co-workers (6) demonstrated a persistent infection in L (MCN) cells infected with Newcastle disease virus which is dependent on an equilibrium between the protective action of interferon (elicited by infectious and noninfectious virus) and the number of unprotected cells allowing complete viral replication. This type of relationship has previously been postulated by Wagner (1960).

Production of interferon in rubella-infected cultures has been demonstrated in primary human amnion (10) and in RAL culture (Maassab and Veronelli, *unpublished data*). Hence, the broad spectrum of viral resistance exhibited by LLC-MK₂ cells infected with rubella and by the RAL line leaves little doubt that interferon plays a role in the resistance of these cultures to superinfection.

The curves of cell multiplication (Fig. 3) and virus yield (Fig. 4) can be interpreted in relation to the equilibrium hypothesis. Viral replication, with its maximum at approximately the 4th day after infection, would be responsible for the production of interferon directly or through the action of incomplete or thermoinactivated rubella virus with a half life of 2 hr and 15 min at a temperature of 36 to 37 C (14). Cells protected by interferon with a maximal rate of multiplication between days 6 and 12 could then populate the culture. These cells carry the antigen and can divide, but are not producing active virus. It is also apparent from Fig. 3 and 4 that, when cells are not dividing, they are actively producing infectious rubella virus. The maximal viral yield is produced before cells start to increase in number. When cells start multiplying, the infectious yield does not change appreciably and eventually decreases.

Some of the data previously analyzed suggest, however, a similarity between RAL cultures and the carrier systems of mumps-human conjunctiva

cultures (18, 19) or measles in HeLa cells (12). In these systems, the infected cells retain the ability to divide and thus perpetuate the infection by direct transmission to the daughter cells. However, virus multiplication and release are reduced, as compared with the cytocidal cycle of the same agent in other cells or under different conditions, which suggests that these steps are under some kind of intracellular regulation or control [hence the name of "regulated infection" proposed by Walker (17)]. Recently, it has been shown by Chang, Goldhaber, and Dunnebacke (2), with reference to lipovirus and by use of time-lapse cinematography, that infected cells can still divide, thereby transmitting the infection to daughter cells.

Failure to "cure" the RAL cells with amantadine (at concentrations capable of entirely aborting the infection of original LLC-MK₂ cultures) or by addition of high neutralizing titer γ -globulin is evidence in favor of perpetuation of infection in RAL cultures either through cell division or by cell-to-cell spread.

The data obtained by the use of the indirect immunofluorescent technique also favor this point of view. Besides the occasional recognition of antigen-bearing cells apparently undergoing division (Fig. 7), it was observed that the number of stainable cells increases during the period of active cell multiplication and represents almost 100% of the population by the 10th day. Since at the same time the infectious virus yield is in a declining phase (Fig. 4), this would imply that the ability to produce specific antigens can be carried along during cell division without a parallel increase in virus yield. It is conceivable that the alteration of conditions represented by passage offsets some regulatory mechanism and permits the release of infectious virus, as seen during the first 2 weeks after reseeding (Fig. 4).

The mechanisms discussed are not mutually exclusive, and further research is being directed to the elucidation of their relative importance in maintaining this persistent infection.

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