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A Latent Infection of Baby Hamster Kidney-21 Cells with Mumps Virus (39959)

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Introduction. We have recently reported the emergence of temperature-sensitive variants from a persistent infection of HeLa cells ($HeLa_{pi}$) with measles virus (1) and from a persistent infection of baby hamster kidney-21 cells (BHKpi) with mumps virus (2). The usefulness of *in vitro* persistent infections as correlates for *in vivo* disease processes is well recognized (3-5). The need for further study of mumps virus is indicated since the agent may be associated with clinical disorders such as diabetes (6), arthritis (7), primary endocardial fibroelastosis (8), Hodgkin's disease (9), and a number of other organ involvements (10-12). Moreover, the possibility exists that a persistent viral infection may be initiated by the use of attenuated strains of mumps virus in vaccine preparations.

Our previous studies describing the characteristics of BHKpi revealed that at least two different populations of cell clones could be isolated (2). The first type consisted of normal BHK-21 cells lacking any detectable mumps virus components, as determined by hemadsorption, immunofluorescence, or released virus analysis. These uninfected cells were shown to be as susceptible to mumps virus as the original BHK-21 cells used in initiating the persistence. The second type consisted of BHK-21 cells demonstrating mumps virus antigens by hemadsorption (Had) and immunofluorescence (ImF); however, no detectable released virus was demonstrable by assay in either primary chicken embryo or HeLa cell monolayers.

This report describes a tissue culture system (BHK_{LI}), derived from cell cloning of BHKpi, which is infected with a latent-inducible mumps virus.

Methods. Cells. BHK-21 cells, clone 13, and HeLa cells were obtained from the American Type Culture Collection (Rockville, Maryland). Primary chicken embryo fibroblasts were prepared from 10- to 11-day-old embryonated eggs (Northwest Farms, Portland, Oregon). Cells were grown in Eagle's minimal essential medium (MEM) with Hank's salts, 10% heat-inactivated fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, New York), 0.1% $NaHCO_3$, 100 units/ml of penicillin G (potassium), and 100 μg/ml of streptomycin sulfate. Cultures were maintained in the above media with 2% FCS. Cultures were incubated at either 33, 37, or 39° in a 5% CO_2 atmosphere. Once monolayers were confluent, or after virus infection, the cells were maintained in the above media with 2% FCS. Cell cultures were tested for mycoplasma contamination in complete mycoplasma media as previously described (13, 14).

Viruses. The wild-type mumps virus used in these studies (MuVo) was obtained from the Communicable Disease Center (Atlanta, Georgia). The sample was a clinical isolate from a throat washing. The persistent infection was established as described previously (2) by addition of a 1:10 dilution of the clinical isolate without further passage. Wild-type mumps virus stocks were grown by allantoic inoculation of 7-day-old embryonated chicken eggs (Northwest Farms, Portland, Oregon) at 37°, and harvested 4 to 6 days later. Virus stocks were concentrated in an Amicon ultrafiltration unit (Lexington, Massachusetts) with an XM300 membrane. Newcastle disease virus, NDV (Herts strain), and vesicular stomatitis virus, VSV (Indiana strain), were kindly supplied by J. S. Youngner (University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania).

Solutions. Calcium and magnesium-free phosphate-buffered saline (CMF-PBS) used for cell washings and as diluent for antisera, and calcium and magnesium-containing PBS, used as diluent for erythrocytes, were

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filtered through a 0.22-μm membrane (Millipore Corporation, Bedford, Massachusetts).

Immunofluorescence (ImF). Guinea pig anti-mumps virus serum (Enders, egg, allantoic) was obtained from Flow Laboratories (Rockville, Maryland) and stored at −20°. Sheep anti-guinea pig serum conjugated to fluorescein isothiocynanate (FITC) was obtained from Sylvana Corporation (Millburn, New Jersey). A 1% solution of sodium azide was prepared and used at a final concentration of 0.1% in antiserum and conjugate. The specificity of the antiserum and conjugate, and the fluorescent focus assay used to assay mumps virus, have been previously reported in detail (2).

Microscopy. Specimens were observed on a Zeiss GF microscope fitted with a dark-field condenser, type 50 barrier filter, and auxiliary lens type 1. A Reichert illumination unit was used with an Osram HBO 220W mercury vapor bulb as the light source and a BG-12 excitation filter. A Zeiss 35-mm camera was used with Kodak Daylight High Speed Ektachrome (ASA 160). Optimal exposure ranged from 60 to 120 sec.

Infectious centers. Cell monolayer cultures were rinsed with CMF-PBS, scraped with a rubber policeman, and rinsed in maintenance medium. Cells were then counted, adsorbed to HeLa cell monolayers, and assayed by the fluorescent focus forming assay (2).

Hemadsorption (Had). Hemadsorption tests were performed by the method of Henle (15) using chicken erythrocytes.

Homologous and heterologous viral challenge. Cell monolayers of BHK and BHKpi cell clones were grown to confluency and the number of cells per dish was determined. The cultures were inoculated with MuVo, vesicular stomatitis virus (VSV), Indiana strain, or Newcastle disease virus (NDV), Herts strain, at a multiplicity of infection (moi) of 1. NDV and VSV were adsorbed for 1 hr and MuVo for 2 hr at 37°, after which the plates were rinsed with CMF-PBS and incubated in MEM plus 2% FCS. Culture fluids were harvested 24 hr postinfection for NDV and VSV, and 48 hr for MuVo. NDV and VSV were assayed by plaque formation in chicken embryo monolayer cultures and mumps by the fluorescent focus assay.

Cell growth. Cell doubling times of BHK and BHKpi cell clones were determined by plating cells in 10 × 35-mm plastic tissue culture dishes at a density of 2.0×10^4 cells/plate in MEM supplemented with 10% FCS. Duplicate samples were harvested and counted every 24 hr to 120 hr, and fresh MEM with 10% FCS was added every 48 hr.

Results. Preliminary observations. Cloning studies of a persistent infection of BHK-21 cells with mumps virus (BHKpi) were carried out to determine the mechanism(s) of maintenance of the carrier state *in vitro*. As previously reported (2), two types of clones could be isolated from the parent BHKpi population. One type of clone (exemplified by clone 6′) demonstrated the lack of mumps virus antigens by Had, ImF, and released virus analysis. A second type of clone (exemplified by clones 3′ and 15′) exhibited mumps virus involvement by both Had and ImF through cell passage 30; however, no released mumps virus could be detected.

If the cell clones (3′, 6′, 15′) were shifted from an incubation temperature of 37 to 33° for 2–3 days, the following was observed. Cell clone 6′ did not demonstrate mumps virus involvement by either Had, ImF, or released virus analysis. However, with the same treatment clone 3′ demonstrated 6.8×10^4 FFU/ml of mumps virus released into the culture medium, and clone 15′ released 3.4×10^4 FFU/ml. No released virus could be obtained from clone 3′, 6′, or 15′ when the cultures were cocultivated with primary chicken embryo fibroblasts at 37°, or if cultures were frozen and thawed.

Cell clone 3′ was then analyzed for the kinetics of induction of MuV_{LI} when shifted from 37 to 33°. Cultures were grown to confluency at 37°. At time zero, cultures were shifted to 33°, while control cultures were kept at 37° for comparison. Aliquots were collected at intervals following the temperature downshift and assayed by the fluorescent focus assay in HeLa cells as described elsewhere (2). The results are contained in Fig. 1. At 10 min following

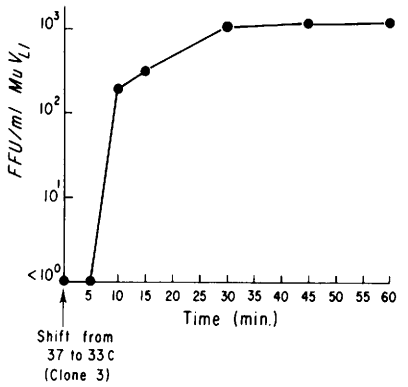

FIG. 1. Time sequence of MuV_{LI} induction at 33°.

downshift, infectious mumps virus was observed in the supernatant fluids. The peak released virus titer observed was 10^3–10^4 FFU/ml. Control cultures which were kept at 37° continued to be nonproductive for mumps virus. If, however, cultures which were induced to release virus were returned to 37° (from 33°) after thorough washing, MuV_{LI} continued to be released 24 and 48 hr later. The titer of cultures kept at 33°, following a temperature downshift, remained at the 10^3 FFU/ml level.

In an attempt to determine if transcription or translation of macromolecular synthesis was in some way responsible for the induction of MuV_{LI}, actinomycin D (AMD), cycloheximide (CHA), and puromycin (P) were used to study this effect. Briefly, AMD, CHA, or P (50 μg/ml) were added to clone 3′ cultures 1 hr prior to shifting from 37 to 33°. Samples were taken 1 and 24 hr after the downshift. AMD, CHA, or P did not significantly inhibit the release of MuV_{LI} from cultures incubated at 33°. Control cultures of clone 3′ BHK_{LI} cells were treated identically with AMD, CHA, and P, and kept at 37°. Samples taken at times corresponding to 1 and 24 hr in the downshifted cultures showed no released virus.

MuV_{LI} was completely neutralized with mumps virus antiserum (guinea pig anti-mumps virus, Flow Laboratories, Rockville, Maryland). No inhibitory effect was observed when MuV_{LI} was incubated with pooled normal guinea pig serum (supplied by Dr. G. A. Leslie, University of Oregon Health Sciences Center). In addition, the size of fluorescent focus produced by MuV_{LI} (Fig. 2) was significantly smaller than that produced by MuVo or MuVpi.

Comparative growth rates were determined for clones 3′, 6′, and 15′, in addition to BHK-21 cells. However, no significant differences in generation time were observed.

Homologous and heterologous viral challenge. To determine if the cell clones were resistant to superinfection with homologous or heterologous viruses, MuVo, NDV, or VSV was adsorbed to BHK-21 cells, and cell clones 3′, 6′, and 15′ at a moi of 1. The results are given in Table I, and show an inhibition of homologous virus; no significant inhibition of heterologous viral challenge was observed.

Infectious centers. Cell cultures (clones 3′, 6′, and 15′) were analyzed for the percentage of cells releasing infectious mumps virus. Since an incubation temperature of 33° was found to be different from 37° with regard to properties associated with BHK_{LI}, clones were (i) grown at 33° and assayed at 33°, (ii) grown at 37° and assayed at 33°, and (iii) grown at 37° and assayed at 37°. The results of these experiments are contained in Table II. No significant differences were observed between 33 and 37° in the percentage of cells forming infectious centers.

Temperature sensitivity analysis. The non-temperature-sensitive nature of MuV_{LI} was determined in two ways. Initially, MuV_{LI} was tested for its efficiency of plating at 33 and 39°. The results of these experiments are contained in Table III. No significant difference was observed in the relative plating efficiency of MuV_{LI} at any of the passages tested. In addition, MuV_{LI} was tested for its temperature sensitivity of growth at 33 and 39°. Samples were adsorbed to HeLa cell monolayers at 33 or 39° for 1 hr. Virus was removed and the cells were rinsed twice with phosphate-buffered saline. Maintenance medium was added and cultures were incubated at their respective temperatures. MuV_{LI} was harvested at Day 4 postinfection by one cycle of freeze–thawing, and assayed at 37° by the fluorescent focus assay. No significant difference in replicative efficiency was observed with any of the MuV_{LI} populations tested.

Discussion. Previous reports from this

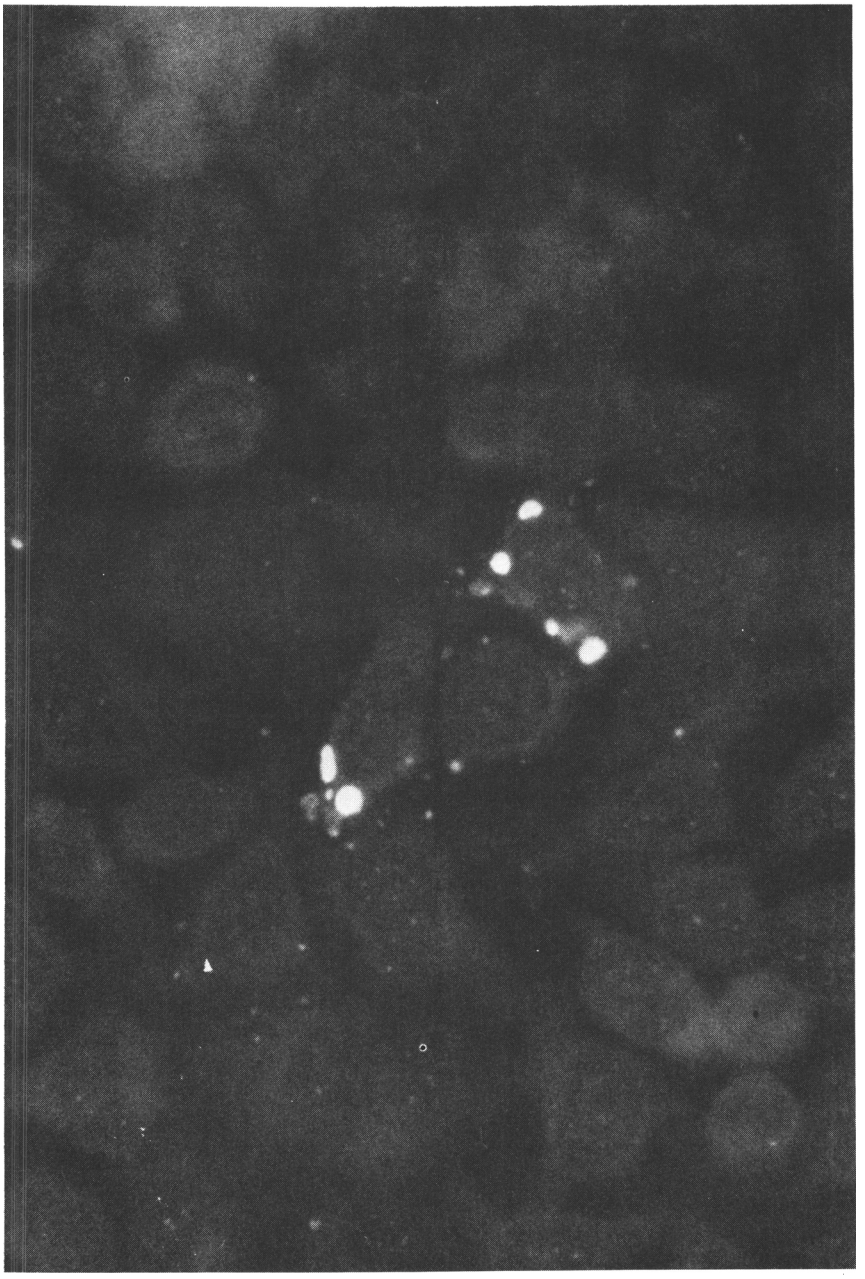

FIG. 2. MuV_{LI} fluorescent focus (FFU) in HeLa cell monolayer. 1120×.

laboratory have demonstrated that persistently infected BHK cells (BHKpi) produced infectious, temperature-sensitive mumps virus (MuVpi) which is less cytopathic than wild-type mumps virus (MuVo). In this report we have shown that cell clones have been isolated from the parent BHKpi population (BHK_{LI}) which were infected with a latent-inducible mumps virus (MuV_{LI}). The properties of this infection are summarized in Table IV.

At least two mechanisms may be functioning to maintain the BHKpi persistence. Temperature-sensitive (ts) virus may be selected for very early in the infection, most probably from preexisting spontaneous ts

TABLE I. HOMOLOGOUS AND HETEROLOGOUS CHALLENGE OF BHK AND BHKpi CELL CLONES WITH MuVo, NDV, OR VSV.

Virus	Cell line	Released virus titer/ml	Log reduction
VSV	BHK	1.5×10^7	0
	Clone 3′	5.0×10^6	0.47
	Clone 6′	1.1×10^7	0.13
	Clone 15′	4.0×10^7	0
NDV	BHK	3.8×10^4	0
	Clone 3′	2.7×10^4	0.15
	Clone 6′	2.8×10^4	0.14
	Clone 15′	8.0×10^4	0
MuVo	BHK	2.0×10^2	0
	Clone 3′	3.8×10^1	0.72
	Clone 6′	2.9×10^2	0
	Clone 15′	1.9×10^1	1.02

TABLE II. INFECTIOUS CENTER DETERMINATION OF CELL CLONES 3′,6′, AND 15′.

Cells assayed at	Percentage of infectious centers of cells grown at 33°		Percentage of infectious centers of cells grown at 37°		
	Clone 3′	Clone 15′	Clone 3′	Clone 6′	Clone 15′
33	22.8	4.2	30.4	0	5.3
37	N.D.[a]	N.D.	27.5	0	5.1

[a] Not determined.

TABLE III. EFFICIENCIES OF PLATING OF MuV_{LI} AT 33 AND 39°.

	Released virus at 33° (FFU/ml)	Released virus at 39° (FFU/ml)	Ratio of plating efficiencies 39°/33°
MuV_{LI}, p 10[a]	3.0×10^4	2.5×10^4	0.83
MuV_{LI}, p 20	1.5×10^4	2.0×10^4	1.30
MuV_{LI}, p 25	3.7×10^4	5.1×10^3	0.18
MuV_{LI}, p 30	2.0×10^4	5.1×10^3	0.26

[a] Cell passage number from original clonal isolation.

mutants. Although the virus released from the infection is relatively noncytopathic, it appears to be lethal to the host cells since no virus-producing clones could be isolated. Therefore one possible mechanism is that of an equilibrium type. That is, producer cells can release low levels of infectious MuVpi viruses which infect normal uninfected cells. The cell killing by MuVpi must approximately equal the rate of appearance of the susceptible normal cells. Since treatment of BHKpi cultures with mumps antiserum does not cure the cultures, but only eliminates virus production (unpublished observations), a second mechanism of maintenance is apparent.

This second mechanism is the latent mumps infection. Such latently infected cells do not produce infectious virus, are resistant to challenge with MuVo, and yet still elicit mumps virus antigens which do not dilute out with subsequent cell passage. Therefore, these antigens are continuously produced in the latently infected cells (BHK_{LI}).

If defective interfering particles (DIP) are responsible for maintaining the latent infection at 37°, it is difficult to understand the rapid appearance of MuV_{LI} in the culture fluids following incubation at 33° (10 min). Pretreatment of BHK_{LI} cultures with actinomycin D, cycloheximide, or puromycin did not block this rapid release. If sufficient DIPs were present at 37° to completely inhibit the appearance of MuV_{LI}, they must become nonfunctional in this respect within 10 min of the downshift. Furthermore, if the latent state were controlled by DIPs, it would be reasonable to expect that they would also be present in sufficient quantity to (a) inhibit the appearance of MuV_{PI} at 37° in the original BHKpi cultures, and (b) to prevent the isolation of uninfected cells containing no viral antigens. MuV_{PI} is released in the BHKpi cultures at 37° and normal BHK-21 cells can easily be isolated by cloning from the persistently infected cultures (2). These results are in contrast to

TABLE IV. SUMMARY OF PROPERTIES OF BHK_{LI} AND MuV_{LI}.

Properties of BHK_{LI}	Properties of MuV_{LI}
1. 50–80% express mumps virus antigens (Had or ImF).	1. Neutralized by MuVo antiserum.
2. At 37° do not release infectious mumps virus.	2. Fluorescent focus smaller than MuVo or MuVpi.
3. Release infectious mumps virus (10^3–10^4 FFU/ml) when downshifted to 33°.	3. Not temperature sensitive.
4. Interference with MuVo, but not with NDV or VSV.	
5. Clone 3′ formed approximately 25% infectious centers at 33 or 37°.	

those obtained by cloning experiments on cultures of a persistent infection of HeLa cells by measles virus. These experiments gave rise to cells which contained viral antigens and produced no infectious virus at either 33 or 37°; however, the viral antigens in these cultures completely disappeared by passage 5 of the cloned cells, suggesting that these cells had been infected with a nonreplicating form (DIP) of measles virus (1). No such cells were obtained upon cloning BHKpi cells. Further, if DIPs are produced in BHKpi cultures, they are of the same density as MuVpi or MuVo (1.19 g/ml) (2). However, these data do not eliminate the possibility that DIPs have a significant role in both the persistent and latent infection as described. Experiments are in progress in our laboratory to determine the size of the virion RNA in populations of MuVpi and MuV_{LI} isolated from their respective cultures.

The other factors that have been reported as having a role in the initiation and maintenance of persistent infections *in vitro* include interferon, specific antibodies, genetically resistant cells, temperature-sensitive mutants, and DNA intermediates. In the original BHKpi, neither interferon nor specific antibodies are involved (2). Since the cloning experiments give rise to uninfected cells which are susceptible to wild-type mumps virus (MuVo), genetically resistant cells can also be eliminated. The properties of the cell populations obtained in these cloning experiments suggest that DIPs do not contribute in a major way to the maintenance of this persistence. Thus, ts mutants and DNA intermediates remain as factors possibly moderating this persistence.

Temperature-sensitive mutants are clearly involved in the maintenance of the equilibrium mechanism described above. These mutants which can be isolated from the MuVo population are perhaps selected because of their slower rate of growth, a property which could cause a longer survival of cells infected by this mutant than by the wild type. This, plus their decreased yield per infected cell, permits a balance or equilibrium with uninfected cells.

However, in the latently infected cells, the state of the virus and the manner in which it is released are as yet unknown. Studies are in progress to determine if a DNA intermediate is involved in this latency.

It should be pointed out that the studies reported here of a persistent infection derived from a clinical sample of mumps virus have shown that a latent infection can be produced by this ubiquitous agent. The medical importance of this finding is as yet unknown.

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1. Armen, R. C., Evermann, J. F., Truant, A. L., Laughlin, C. A., and Hallum, J. V., Arch. Virol. **53,** 121-132 (1977).
2. Truant, A. L., and Hallum, J. V., J. Med. Virol. **1,** 49-67 (1977).
3. Johnson, K. P., and Norrby, E., Exp. Mol. Pathol. **21,** 166-178 (1974).
4. Holland, J. J., Villarreal, L. P., and Etchison, J. R., *in* "Mechanisms of Virus Disease. ICN-UCLA Symposia on Molecular and Cellular Biology" (W. S. Robinson and C. F. Fox, eds.), Vol. 1, pp 131-158. W. A. Benjamin, Menlo Park, Calif. (1974).
5. Ackermann, W. W., Ann. N. Y. Acad. Sci. **67,** 392-402 (1957).
6. Sultz, H. A., Hart, B. A., Zielezny, M., and Schlesinger, E. R., J. Pediatr. **86,** 654-656 (1975).
7. Smith, J. W., and Sanford, J. P., Ann. Int. Med. **67,** 651-659 (1967).
8. St. Geme, J. W., S. R. Noren, and Adams, P., N. Engl. J. Med. **275,** 339-347 (1966).
9. Truant, A. L., and Hallum, J. V., Oncology **33,** 241-245 (1976).
10. Nicolis, G. L., Sabetzhadam, R., Hsu, C. S. C., Sohval, A. R., and Gabrilova, J. L., J. Amer. Med. Assoc. **223,** 1032-1033 (1973).
11. Wood, C. B., Bradbrook, R. A., and Blumgart, L. H., Brit. J. Clin. Practice **28,** 67-69 (1974).
12. Gray, J. A., Brit. Med. J. **1,** 338-340 (1973).
13. Hayflick, L., Tex. Rep. Biol. Med. **23,** (Suppl. 1), 288-303 (1965).
14. Hayflick, L. and Stanbridge, E., Ann. N. Y. Acad. Sci. **143,** 608-621 (1967).
15. Henle, W., *in* "Diagnostic Procedures for Viral and Rickettsial Infections" (E. A. Lennette and N. J. Schmidt, eds.), 4th ed., pp 457-482. American Public Health Association, New York (1969).

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