BIKEN JOURNAL Vol. 23 15-23, 1980

PERSISTENT INFECTION OF MOUSE TUMOR CELLS WITH MUMPS VIRUS

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(Received October 13, 1979)

SUMMARY A mouse tumor cell line (MCT) persistently infected with mumps virus (strain Urabe) was obtained. No antibody or chemicals were required for establishment or maintenance of the infected cell line (M-MCT). No difference was observed by light microscopy between MCT and M-MCT cells, except for cytoplasmic inclusion bodies in 10–20% of the M-MCT cells. Hemadsorption of chicken erythrocytes, which did not necessarily coincide with inclusion positive cells was shown by 10–20% of the M-MCT cells. Immunofluorescence staining revealed that almost all M-MCT cells contained mumps virus antigens, which were seen mainly in the cytoplasm as fine granules, dots, or large clumps. Infectious mumps virus was consistently detected in the culture fluid and the released virus showed some temperature sensitivity when assayed at 34°C and 40°C. M-MCT was resistant to superinfection with homologous virus and showed some resistance to heterologous viruses. Thirty one clones of M-MCT were isolated by the soft agar method. Some, but not all, clones had viral antigen and all those with antigen released virus into the culture medium. The growth rates of MCT and M-MCT cells as monolayer cultures in vitro were similar, but the transplantability of M-MCT cells in syngeneic C57BL/6 mice was lower than that of MCT cells.

INTRODUCTION

Persistent infection in cell cultures has been reported in a variety of virus-cell systems. Various factors have been found to be involved in regulation of the persistent infection (Walker, 1964; Joklick, 1977); namely cellular permissiveness, viral cytopathogenicity, defective interfering particles, temperature-sensitive mutant, interferon and integration of the viral genome into that of the host cell. Information on the mechanism of persistent infection in vitro is useful in understanding the mechanisms of persistent infection in vivo and chronic viral infection.

Mumps virus is one of the most contagious pathogenic agents in children. Although usual cases of mumps virus infections are mild,

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various complications, such as meningoencephalitis and epidymo-orchitis, often develop. Mumps virus has also been suggested to be an etiological agent in some chronic diseases, such as arthritis and diabetes mellitus (Smith and Sanford, 1967; Sultz et al., 1975). These facts prompted us to investigate the virus-cell interaction in mumps virus infection. Persistent infections with mumps virus have been reported in Lung-to cells (Henle et al., 1958), in human conjunctiva cells (Walker and Hinze, 1962) and recently in BHK-21 cells (Truant and Hallum, 1977a; 1977b), but details of the mechanisms of establishment and maintenance of infection are still unknown.

This paper reports the establishment and some properties of mouse tumor cells persistently infected with mumps virus.

MATERIALS AND METHODS

1. *Cells*

The MC 57 G cell line (from a methylcholanthrene-induced tumor of a C57BL/6 mouse, kindly supplied by Dr. Y. Hosaka, Osaka University) was cultured in Eagle's MEM supplemented with 5% fetal calf serum (FCS), 0.07% $NaHCO_3$, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. The MCT cell line was established in our laboratory by injection of MC 57 G cells into a C57BL/6 mouse and culture of the tumor cells in vitro. The M-MCT cell line was obtained by infecting MCT cells with mumps virus, as described in the Results. MCT and M-MCT cells were cultured in the medium used for culture of MC 57 G cells. CV-1 cells and L 929 cells were maintained in medium 199 supplmented with 5% FCS, 0.07% $NaHCO_3$, 100 units/ml of penicillin, and 100 μg/ml of streptomycin.

2. *Virus*

The Urabe strain of mumps virus was originally isolated by Dr. K. Yamanishi, Osaka University. The virus has been passed four times in human embryonic kidney cells and twice in LLC-MK2 cells. The culture medium after low speed centrifugation (3,000 rpm, 30 min) was used as virus solution, and stored at -70°C before use. The virus was not cloned before experiment.

The Miyake strain of mumps virus, Seibert strain of herpes simplex virus type 1 (HSV-1) and Indiana strain of vesicular stomatitis virus (VSV) were also used.

3. *Cell growth*

Cell growth was examined by plating cells in 60×15 mm Falcon plastic tissue culture dishes at a density of 5×10^5 cells per dish in 5 ml of MEM supplemented with 5% FCS and 0.21% $NaHCO_3$. The dishes were incubated at 37°C in 5% CO_2 without changing the medium during the experiment. Samples were harvested at definite times, washed once with PBS and once with 0.02% EDTA, and then trypsinized. The resulting single cell suspension was stained with crystal violet and counted in a hemocytometer.

4. *Assay of infectious virus*

Infectious virus was assayed by the end point dilution technique by measuring hemadsorption of CV-1 cells in 96-well microtiter plates. The growth medium of the microtiter plates was removed and the plates were inoculated with 25 μl of 10-fold dilutions of the virus. The plates were stood for 2 hr at room temperature to allow adsorption and then 100 μl of medium 199 containing 3% FCS and 0.21% $NaHCO_3$ was added and the plates were incubated in 5% CO_2 in air at 37°C for 7 to 10 days. Then hemadsorption of chicken erythrocytes was measured. For this, the medium was decanted and 50 μl of 0.33% chicken erythrocytes was added. After incubation for 60 min at 4°C, the sedimentation pattern of erythrocytes was examined. Cultures were scored as infected when the erythrocytes did not form round clusters at the bottom.

5. *Indirect immunofluorescence test*

Cells on coverslips were washed three times with PBS, dried in air and then fixed in acetone at room temperature for 5 min. They were stained by the indirect method. The fixed cells on coverslips were exposed to mouse or guinea pig anti-mumps serum at 37°C for 30 min and washed three times with PBS. They were then exposed to fluorescein isothiocyanate (FITC) labeled goat anti-mouse or guinea pig serum at 37°C for 30 min and washed three times with PBS. Then the labeled cells were mounted with 90% glycerin in borate buffer (pH 9.0), and examined under ultraviolet light. The controls used were normal cells treated with anti-

mumps serum followed by FITC labeled serum, persistent infected cells treated with normal serum followed by FITC labeled serum, and normal and persistently infected cells treated with FITC labeled serum only.

6. *Hemadsorption*

Hemadsorption was examined using 0.33% chicken erythrocytes. Cells grown on glass bottles or coverslips were washed three times with PBS, covered with 0.33% chicken erythrocytes, incubated for 60 min at 4°C and then washed gently three times with cold PBS. Then they were examined by light microscopy immediately, or after fixation with 0.5% glutaraldehyde and staining with Giemsa, or after fixation with Bouin's fixative and staining with hematoxylin-esoin. Under the conditions used uninfected cells did not show hemadsorption.

7. *Staining of cultured cells*

Cells grown on coverslips were washed three times with PBS, fixed for 15 min in Bouin's fixative and stained with hematoxylin-eosin. The morphology of the cells and inclusion bodies in the stained cells were examined by light microscopy.

8. *Interferon assay*

The supernatant culture fluid was assayed for interferon in mouse L 929 cells with VSV by the plaque reduction method as described by Youngner et al. (1966). The culture media of MCT and M-MCT were centrifuged at 25,000 rpm for 60 min to remove the virus released from M-MCT, and then 2-fold dilution of the fluid (3.0 ml) were placed on L 929 cells in 60 mm petri dishes and incubated for 24 hr. Then the fluid was removed and the L 929 cells were challenged with about 50 PFU of VSV. Plaques were counted after 2 or 3 days.

9. *Transplantability of MCT and M-MCT in C57BL/6 mice*

MCT and M-MCT cells in monolayer cultures were trypsinized, counted in a hemocytometer and suspended in cultured medium. Various numbers of cells, as described in Table 4, were inoculated subcutaneously into syngeneic C57BL/6 mice.

10. *Colony formation in soft agar*

Colony formation in soft agar was examined by the method of Macpherson and Montagnier (1964). Concentrations of 0.33 to 0.35% agar over a basal layer of 0.5% agar were used. Clones of M-MCT were established by picking up colonies in soft agar with a pasteur pipette.

RESULTS

1. *Establishment and some properties of the persistent cell line*

MCT cells at the second passage in vitro were infected with mumps virus (strain Urabe) at an input multiplicity of about 5 $TCID_{50}$/cell and incubated at 37°C. Most of the cells showed cytopathic effects and became detached from the glass, but some cells survived and grew into a confluent monolayer. The cell line obtained in this way was named as

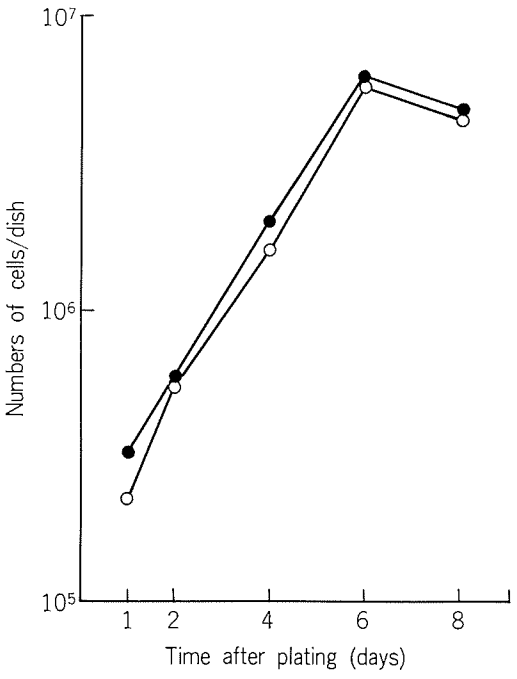

FIGURE 1. Comparison of multiplications of MCT and M-MCT. Cells were plated at a density of 5×10^5 cells in 5 ml of medium per 60 mm petri dish. Cells were counted at the indicated times after plating as described in the Materials and Methods. MCT and M-MCT were used at passage 28 and 24, respectively. Numbers of MCT cells (○——○); Numbers of M-MCT cells (●——●).

M-MCT. M-MCT has been passed about once a week, with 60 subcultures over a period of 12 months. No specific antibody or chemicals have been required at any time for either the establishment or maintenance of this line.

MCT and M-MCT cells grown on coverslips were fixed and stained with hematoxylin-eosin. Microscopic examination showed that 10–20% of the M-MCT cells contained cytoplasmic inclusion bodies, but otherwise the cells appeared similar as to MCT cells. The extent of hemadsorption (10–20% of the cells) varied from preparation to preparation, but usually it was low on the second day of plating and high in older cells. Inclusion bodies and hemadsorption were not always expressed on

TABLE 1. *Number of cells inoculated and plating efficiencies of MCT and M-MCT in soft agar*

Expt. No.	No. of cell inoculated	No. of colonies per dish (Average)		Plating efficiency (Average %)	
		MCT	M-MCT	MCT	M-MCT
Expt. 1.	400	37.5	10.5	9.4	2.6
MCT; P. 27	200	15.5	2.5	7.8	1.3
M-MCT; P. 20	100	7.0	1.0	7.0	1.0
	50	4.5	0	9.0	0
Expt. 2.	800	133.0	37.0	16.6	4.6
MCT; P. 38	400	64.3	17.0	16.1	4.3
M-MCT; P. 31	200	23.0	6.7	11.5	3.4
	100	8.0	3.0	8.0	3.0

Colonies were obtained by the method of Macpherson and Montagnier (1964). Values are means for two (Expt. 1.) or three (Expt. 2.) dishes.

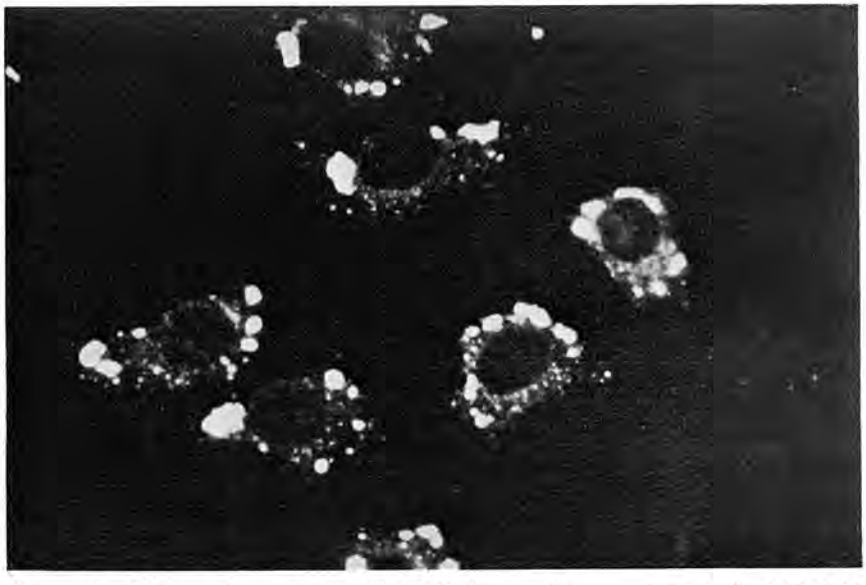

FIGURE 2. Mumps virus antigen in M-MCT (Immunofluorescent staining). M-MCT cells were stained by the indirect method as described in the Materials and Methods.

the same cells: hemadsorption was observed on cells with or without inclusion bodies.

The multiplications of MCT and M-MCT cells during on culture for 8 days were compared as described in the Materials and Methods. Figure 1 shows the growth curves of MCT (at the 28th passage) and M-MCT (at the 24th passage). There was no marked difference in the growths of the two cell lines in monolayer cultures, but the cloning efficiency of M-MCT cells in soft agar was lower than that of MCT cells (Table 1).

2. *Intracellular viral antigen in M-MCT*

Viral antigen was examined by indirect immunofluorescence staining of fixed cells. Almost all the cells contained mumps virus antigen (Fig. 2). The antigen appeared exclusively in the cytoplasm in the form of fine granules, dots or large clumps. Nuclear fluorescence was never seen without cytoplasmic fluorescence, though in some cells fine granules were distributed throughout the cell. When M-MCT cells were harvested one day to seven days after plating for examination of viral antigen, but no distinct differences were found between the different samples in the patterns or percentages of stained cells. Expression of the viral genome at 40°C was similar to that at 37°C, as shown by culture of cells at 40°C. Moreover when cells were shifted from 37°C to 40°C and passaged 7 times at 40°C, the pattern and percentage of fluorescent staining at the high temperature were similar to those at 37°C, and the cells grown at the two temperatures were morphologically indistinguishable by light microscopy (data not shown). These results show that incubation at high temperature did not cure the persistence of mumps virus in M-MCT.

3. *Virus release from M-MCT and temperature-sensitivity of the released virus*

The medium from M-MCT cultures was examined for infectious virus. Infectious mumps virus was always detected in the culture medium of M-MCT. The viral titer was assayed by the hemadsorption method as described in the Materials and Methods, and the results in one experiment are shown in Fig. 3. The viral titer fluctuated under the conditions used, but usually it was highest

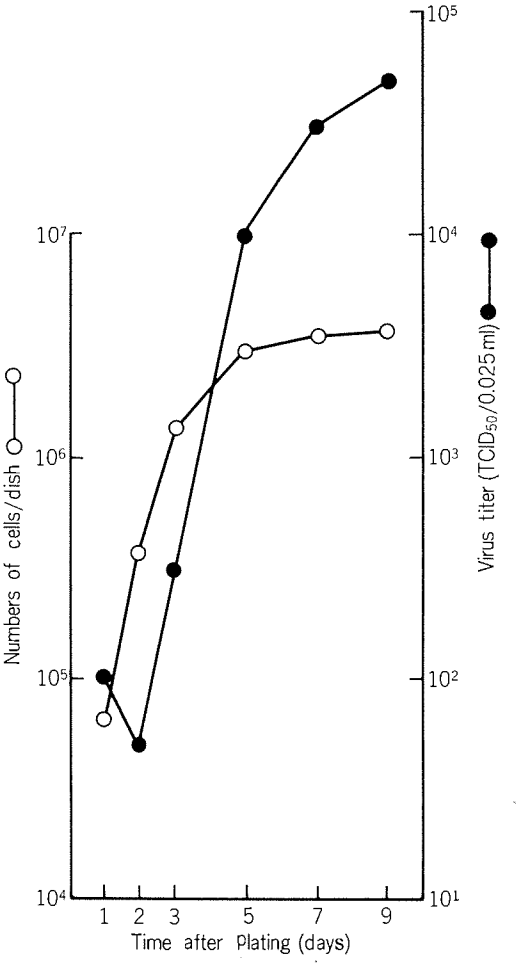

FIGURE 3. Number of M-MCT cells and titer of virus released from M-MCT after plating of cells. M-MCT cells (passage 17) were plated at a density of 2×10^5 cells per dish in 5 ml of medium. The number of cells and the titer of virus in the culture fluid were determined at the indicated times after plating as described in the Materials and Methods. Average number of cells (○——○); Average titer of accumulated virus (●——●).

TABLE 2. *Temperature sensitivity of released virus (MV_{pi}) and parental virus (MV_o) at 34°C and 40°C*

Virus	Virus titer[a] ($TCID_{50}$/0.025 ml)	
	at 34°C	at 40°C
MV_o	$10^{5.5}$	$10^{5.3}$
MV_o	$10^{6.3}$	$10^{6.3}$
MV_{pi}	$10^{5.5}$	$10^{5.0}$
MV_{pi}	$10^{4.3}$	$10^{3.5}$

[a] Virus titers were assayed by hemadsorption as described in the Materials and Methods using culture fluid after low speed centrifugation.

about 7 days after plating. The released virus was titrated at 34°C and 40°C (Table 2). Although the titer assayed at 40°C was lower than assayed at 34°C, the difference was not sufficient to indicate that the released virus (MV_{pi}) was a temperature-sensitive mutant.

4. *Replications of homologous and heterologous viruses in MCT and M-MCT cells, and the presence of interferon in the cultured fluid from M-MCT*

MCT and M-MCT were challenged with homologous virus. As shown in Fig. 4, the Urabe strain of mumps virus could not replicate in M-MCT, but could replicate in

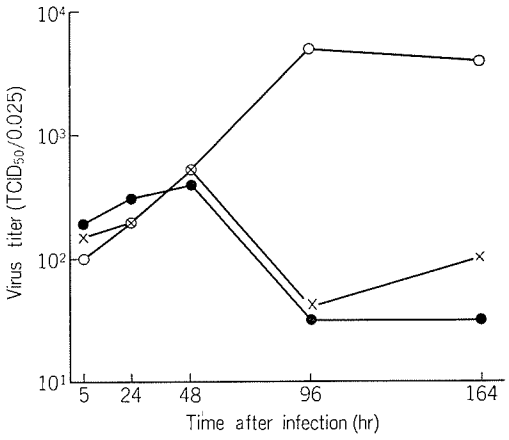

FIGURE 4. Replication of mumps virus (strain Urabe) in MCT (passage 22) and M-MCT (passage 15). Virus was infected at an input multiplicity of about 2 $TCID_{50}$/cells. Virus in the culture fluid was titrated at the indicated times after infection as described in the Materials and Methods. MCT infected with the Urabe strain (○——○); M-MCT infected with the Urabe strain (●——●); uninfected M-MCT (×——×).

TABLE 3. *Replication of herpes simplex virus type 1 (HSV-1) and vesicular stomatitis virus (VSV) in MCT and M-MCT cells*[a]

Virus	Time after infection	Virus titer (PFU/ml)		
		MCT	M-MCT	Ratio (M-MCT/MCT)
		(P. 28)[b]	(P. 26)	
HSV-1	2 hr	2.7×10^4	3.1×10^4	1.15
	17 hr	3.0×10^5	6.8×10^4	0.23
	40 hr	6.4×10^6	2.8×10^6	0.44
		(P. 61)	(P. 50)	
VSV	2 hr	4.0×10^4	4.2×10^4	1.05
	17 hr	2.8×10^7	6.2×10^6	0.22
	24 hr	1.6×10^7	3.4×10^6	0.21

[a] Cells were infected at an input multiplicity of about 2 PFU/cell. At the indicated times after infection, samples (infected cells and media) were frozen. Thawed samples were centrifuged at low speed and the supernatants were assayed on human embryonic lung cells and mouse L 929 cells for HSV-1 and VSV, respectively.

[b] Figures in parentheses are passage numbers of the cells used.

MCT. M-MCT cells were also resistant to superinfection with another strain of mumps virus (strain Miyake) (data not shown). Herpes simplex virus type 1 (HSV-1) replicated in both MCT and M-MCT cells, though replication was somewhat restricted in M-MCT cells (Table 3). Replication of VSV was also somewhat restricted in M-MCT cells (Table 3). The culture fluid from M-MCT cells was assayed for interferon in L 929 cells as described in the Materials and Methods. Two-fold diluted supernatant culture fluid from M-MCT cells decreased the number and size of VSV plaques by about half, but more diluted samples had no effect on VSV plaque formation. The supernatant culture fluid from MCT cells, which had been treated in the same manner as that from M-MCT cells, did not affect the number or size of VSV plaques. The factor in the supernatant of M-MCT cell cultures that affected the plaques was stable when kept overnight at pH 2.0 (data not shown). These findings suggest the presence of a low level of interferon in the cultured fluid from M-MCT cells.

5. *Isolation of M-MCT clones by soft agar culture*

A total of 31 clones were isolated from agar suspension cultures and tested for viral antigen, hemadsorption, intracytoplasmic inclusion bodies and viral release. Of these clones, 23 were isolated from M-MCT at the 13th passage and 8 from M-MCT at the 19th

TABLE 4. *Transplantability of MCT and M-MCT in C57BL/6 mice*

Expt. No.	Time after inoculation (Week)	No. of cells inoculated per mouse[a]	Development of Tumor	
			No. of mice with tumor	No. of mice inoculated
			MCT (P. 20)[b]	M-MCT (P. 14)
Expt. 1.	2	10^4	0/4	0/5
		10^5	1/5	0/5
		10^6	3/5	0/4
	6	10^4	0/4	0/5
		10^5	1/5	1/5
		10^6	4/5	1/4
			MCT (P. 26)	M-MCT (P. 17)
Expt. 2.	3	2×10^4	0/5	0/5
		2×10^5	3/5	0/5
		2×10^6	4/5	2/5
	6	2×10^4	1/5	0/5
		2×10^5	3/5	1/5
		2×10^6	5/5	2/5
			MCT (P. 23)	M-MCT (P. 28)
Expt. 3	3	5×10^6	4/4	0/5
	5	5×10^6	3/3[c]	0/5

[a] MCT and M-MCT cells were trypsinized, counted, suspended in medium and 0.1 ml (Expt. 1 and 3) or 0.2 ml (Expt. 2) of cell suspension was inoculated subcutaneously into syngeneic C57BL/6 mice.

[b] Figures in parentheses are passage numbers of the cells used.

[c] One mouse died in week 4.

passage. Viral antigens were detected in 22 clones by immunofluorescence staining, but 9 clones did not contain viral antigens. All the clones with viral antigen showed hemadsorption, intracytoplasmic inclusion bodies and release of infectious viruses into the medium, though their degrees of expression of these characters varied. None of the clones without viral antigen showed hemadsorption, intracytoplasmic inclusion bodies or viral release, even after 15 subcultures, or subculture at 34°C.

6. *Transplantability of MCT and M-MCT in syngeneic C57BL/6 mice*

MCT and M-MCT cells were inoculated subcutaneously into syngeneic C57BL/6 mice. The transplantability of M-MCT cells was lower than that of MCT (Table 4). Four tumors obtained by inoculation of M-MCT cells were cultured in vitro and examined for the presence of virus. No viral antigen was detected in any of the four cultures of tumor cells by immunofluorescence staining, even after 10 subcultures in vitro. Moreover no viral antigen could be induced by incubating the cells at 34°C or treating them with various concentrations of 5-iododeoxyuridine.

DISCUSSION

In this work persistent infection was established in mouse tumor cells and some general properties of the cells were examined. Some of the properties of these cells resemble those of the persistently infected cells reported previously by others (Henle et al., 1958; Walker and Hinze 1962; Truant and Hallum, 1977a; 1977b). These properties are as follows: No particular inhibitors were added for the establishment and maintenance of persistent infection. Persistently infected cells are morphologically indistinguished from the uninfected cells. Many or all the cells contain viral antigen. The infected cells are resistant to superinfection with homologous virus and partially resistant to heterologous virus. The cells release infectious virus into the culture medium.

Almost all M-MCT cells showed intracellular mumps virus antigen by immunofluorescence staining, though the extent of staining varied from cell to cell. Truant and Hallum (1977a) reported nuclear immunofluorescence in their BHK-mumps system. The viral antigen in M-MCT is present almost entirely in the cytoplasm; in some cells the viral antigen was seen throughout cells, but in none was immunofluorescence seen only in the nucleus. No nuclear antigen was seen even soon after plating in M-MCT cells or M-MCT clones, which were isolated by agar suspension culture. This discrepancy between our findings and those of others may be due to differences in the virus strains or cells used, or to differences in the antibodies in the anti-sera. The involvement of the nucleus in persistent infection with mumps virus requires further investigation, because a DNA intermediate has been suggested to be related to viral persistency (Joklik, 1977), and the replication of mumps virus has been reported to be Actimomycin D insensitive (Northrop, 1969), or sensitive (East and Kingsbury, 1971). In contrast with intracellular viral antigen, membrane immunofluorescence was found on only 10–30% of the M-MCT cells (data not shown). Hemadsorption, another expression of the viral genome on the cell membrane, was also shown by only 10–20% of the cells.

M-MCT always released infectious virus into the culture medium, but the titer of the virus fluctuated. The amount of virus released increased with cell multiplication (Fig. 1). A temperature sentitive mutant (ts mutant) is related to many viral persistence systems and has been suggested to have an important role in persistency (Kimura et al., 1975; Youngner et al., 1976; Joklik, 1977). Truant and Hallum (1977a) reported the involvement of a ts mutant in their BHK-mumps system. In our system, MV_{pi} showed some tendency to be temperature sensitive at 40°C, when compared with the parental virus

(MV_0), but this tendency was not sufficient to warrant the conclusion that MV_{pi} is a ts mutant. M-MCT was shifted from 37°C to 40°C at passage 23 and subcultured at 40°C 7 times, to see the effect of higher temperature on viral genome expression. Even at 40°C, almost all the cells contained intracellular viral antigens, infectious virus was released into the medium, and some cells showed hemadsorption and intracytoplasmic inclusion bodies. Thus the persistency of M-MCT could not be cured by culture at high temperature. These findings also suggest that a ts mutant does not play any important role in our M-MCT persistent system.

M-MCT cells were resistant to challenge by homologous virus (Fig. 4), and the replications of HSV-1 and VSV in M-MCT were somewhat restricted. Henle et al. (1958) observed strong inhibition of VSV replication in their system, though they did not test whether this inhibition was due to interferon. Interferon could not be detected in BHK (Truant and Hallum, 1977a) or a human conjunctiva cell system (Walker and Hinze, 1962). In our system, the culture fluid had some interfering activity when examined in a mouse L 929 cell-VSV system by the method of Younger et al. (1966). The presence of interferon in the culture fluid of M-MCT, however, can't explain why the replication of mumps virus in M-MCT was inhibited more than that of VSV, because VSV is one of the most sensitive of viruses to the action of interferon. These findings suggest that interferon is one of factors involved in persistent infection in M-MCT cells, but that other factors that interfere with replication of homologous virus more than with that of heterologous virus, may also be involved.

Thirty-one clones of M-MCT were isolated by culture in soft agar. In some clones intracellular viral antigens were present in all cells, whereas in others antigens were present only some cells. Some clones showed extensive hemadsorption, whereas others showed little. Some clones contained many inclusion bodies, whereas others contained few. Similar differences in expression of the viral genome in different clones were observed in a BHK-mumps persistent system by Truant and Hallum(1977a). But our results show several distinct differences from theirs: First, the cloning efficiency of persistent cells in our system was lower than that of parental uninfected cells. As the growths of MCT and M-MCT in monolayer culture were the same (Fig. 1), M-MCT may have lost anchorage dependency to some extent. This possibility is supported by the fact that M-MCT was less transplantable in syngeneic C57BL/6 mice than MCT (Table 4). In contrast, BHK persistent cells showed higher cloning efficiency than uninfected BHK cells. Second, all antigen positive clones of M-MCT released infectious viruses into the medium, while antigen positive clones of BHK persistent cells did not release infectious virus. These differences suggest that these two infected lines maintain infection by different mechanisms.

The reason for the lower transplantability of M-MCT was not investigated in this experiment. A similar phenomenon has been observed with persistently infected hamster cells infected with hemagglutinating virus of Japan (Yamada and Hatano, 1972) and measles virus (Evermann and Burnstein, 1975). The low transplantability might result from immunological events in vivo, or M-MCT may lose tumorigenicity by some other mechanism as suggested by the fact that the efficiency of colony formation of M-MCT in soft agar medium was lower than that of MCT. The reason for the lower transplantability of M-MCT is an interesting issue to investigate, because modification of tumor cells by virus is of current interest from the viewpoint of cancer therapy (Kobayashi et al., 1977; Okuno et al., 1978).

Interferon seems to be involved in the M-MCT system, but the persistency of mumps virus in M-MCT can't be explained only by the presence of interferon, as discussed above.

A temperature sensitive mutant, which is involved in many persistent systems (Youngner et al., 1976; Joklik, 1977), does not seem to play any important role in M-MCT persistency. We have not examined the participation of defective interfering particles, which have been considered to play an important role in VSV persistence and other persistencies (Holland and Villarreal, 1974; Joklik, 1977). Further studies are required on the mechanism of mumps virus persistency in cultured cells.

ACKNOWLEDGMENTS

We wish to thank Mr. T. Matsuo for excellent technical assistance.

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