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Persistent Infection with Mouse Hepatitis Virus 3 in Mouse Lymphoid Cell Lines

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The sensitivity of mice to mouse hepatitis virus 3 (MHV3) varies according to strain, age, and immune status of the animals. In semisusceptible strains, mice surviving the acute phase of infection develop a chronic disease characterized by the occurrence of paralysis, virus persistence, and immunodeficiency. Persistent MHV3 infections established in vitro in YAC and RDM-4 mouse lymphoid cell lines were characterized by virus production, presence of cytoplasmic viral antigens, and cell lysis. The occurrence of cell "crisis" in YAC cells was manifested by a sharp increase in cell lysis and in the number of fluorescent cells and, concomitantly, by a marked decrease in virus titers. A relationship was observed among the percentage of fluorescent cells, cell lysis, and virus yield and was modulated by renewal of culture media, change in temperature, or inhibition of cellular RNA synthesis. Cell cloning and antibody treatment experiments indicated that viral transmission was performed by viral infection of newly permissive cells produced by the division of uninfected cells in the culture and not by transmission of viral information by infected dividing cells. The biological and biochemical properties of MHV3 variants derived from persistently infected YAC lymphoid cells were characterized. Thermosensitivity and thermolability of cloned viruses originating from persistently infected YAC cells, as well as parent virus suspensions, were studied. A similar heterogeneity was observed when YAC-derived cloned substrains (YAC-MHV3) were compared with parent-derived cloned viruses, indicating that no selection of temperature-sensitive mutants was induced in persistently infected YAC cells. However, the capacity of MHV3 to induce a lethal acute disease when injected into susceptible mice was lost very rapidly. The absence of pathogenicity was related to the induction of a subclinical infection which elicited defense mechanisms. These data suggest, therefore, that MHV3 replication in lymphoid cell lines leads to induction or selection of variants which maintain pathogenicity in vitro but display reduced pathogenic effects in vivo.

The sensitivity of mice to mouse hepatitis virus 3 (MHV3) infection varies according to strain, age, and immune status of the animals (5, 15). Three types of sensitivity are observed: resistance, full susceptibility, and semisusceptibility. After MHV3 infection, semisusceptible mice develop a chronic disease characterized by the occurrence of paralysis and viral persistence, since MHV3 can be recovered from brain, liver, spleen, and lymph nodes in most animals during the first 3 months postinfection (16). During the chronic phase of the disease, a progressive immunodeficiency occurs which is related to lympholysis and inhibition of antigen-driven lymphocyte proliferation (17). The marked involvement of the lymphoid system and the presence of MHV3 in lymphoid cells in infected animals (16), as well as the capacity of the virus to replicate in vitro in lymphocytes (12), led us to develop an in vitro model of MHV3 infection in lymphoblastoid cell lines.

We observed that MHV3 persistent infections can be carried out in vitro in permanent YAC and RDM-4 lymphoid cell lines and that MHV3 variants, derived from persistently infected YAC cells, lose their capacity to induce a lethal disease when injected into susceptible mice.

MATERIALS AND METHODS

Mice. A/J and C57BL/6 mouse strains were purchased from Jackson Laboratories, Bar Harbor, Maine. C57BL/6 newborns were bred in our mouse colony. Animal care and housing have already been reported (15).

Cells. YAC and RDM-4 mouse lymphoid cell lines were obtained from S. Lemieux and D. Oth (Armand-Frappier Institute, Laval, Quebec, Canada). YAC cells (YAC-1 substrain) were derived from a Moloney virus-induced T-cell lymphoma of A/Sn origin and have been propagated as a suspension line in culture for several years (28). RDM-4 cells originated from a B-cell lymphoma of AKR origin and were maintained by passages in vivo as well as in vitro (14). Cells were grown in suspension culture in RPMI 1640 medium (Flow Laboratories, McLean, Va.) containing fetal calf serum at a concentration of 10% for YAC cells and 5% for RDM-4 cells, penicillin (100 U/ml), and streptomycin (100 mg/ml). Subcultures were performed by dilution with fresh culture medium when the cell density reached 5×10^5 cells per ml (YAC cells) and 10^6 cells per ml (RDM-4 cells). The doubling times of YAC and RDM-4 cells were 18 and 24 h, respectively. L2 cells, a continuous mouse fibroblast cell line (provided by R. Anderson, University of Western Ontario, London, Ontario, Canada) were grown in Eagle minimal essential medium with glutamine (2 mM), 5% fetal calf serum, and antibiotics. L2 cells were used for propagation, cloning, and titration of MHV3.

Peritoneal exudate cells were obtained after washings of the peritoneal cavity with 8 ml of RPMI 1640 medium containing 1% heparin (Allen & Hanbury, Toronto, Ontario, Canada). Peritoneal exudate cells were seeded in microtitration plates (5×10^5 cells per well) and cultured at 37°C under 5% CO_2 for 3 days.

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In all cultures, cell counts and viability were regularly determined. Cell viability was assessed by the trypan blue exclusion test (21).

Cell cloning. Cloning of infected and uninfected cells was performed by the limit dilution assay (1 cell per 0.2 ml of medium) in 96-well microtitration plates. Plates were centrifuged at 500 × *g* for 10 min. All plates were checked daily, and the number of cells in each clone and the number of clones were recorded. When the number of cells per clone reached 300, cell clones were divided among three wells, and the plates were further incubated for 2 to 3 days until use.

Indirect immunofluorescence test. Sedimented cells of infected and uninfected control cultures were used for indirect immunofluorescence after the addition of 0.1 ml of Haygman medium (20 volumes of 20% bovine albumin, 60 volumes of phosphate buffered saline [PBS] [pH 7.2], 8 volumes of 5% EDTA), pH 6.8. Cells deposited in wells of Teflon glass slides (12 wells; Flow Laboratories) in a volume of 0.025 ml per well were air dried and kept at 4°C until fixation. Cell fixation was performed in a solution of methanol-acetic acid-PBS (89:1:10), pH 7.2, at 20°C for 30 min. Glass slides were washed in PBS, pH 7.2, for 15 min under agitation and air dried. Four units of specific antiserum (0.025 ml per well) was added to fixed cells in each well. Slides were incubated in a humidified atmosphere at 37°C for 30 min, washed twice in PBS under agitation for 15 min, and then air dried. Four units of either fluorescent anti-mouse immunoglobulin G or anti-mouse Fab_2 antiserum (Cappel Laboratories, Cochranville, Pa.) was added to each well. After further incubation and washings, slides were treated with glycerol-PBS (9:1) and examined under fluorescent microscopy. The intensity of the fluorescence was assessed on a scale ranging from 0 to 4. The percentage of cells with granular cytoplasmic fluorescence of 2 or more was determined for a minimum of 200 cells per sample. Each experiment included four controls: (i) similarly treated uninfected control cells, (ii) infected cells incubated with fluorescent anti-mouse immunoglobulin G antiserum alone, (iii) positive control cells, and (iv) negative control serum.

Anti-MHV3 antibody. Anti-MHV3 antibody was raised in resistant A/J strain mice after two intraperitoneal injections of 1,000 50% lethal doses of virus given 2 weeks apart. Sera were collected 3 days after the last injection, pooled, titrated using the complement fixation test (1:1,024), and kept at −70°C. Antisera were decomplemented at 56°C for 30 min before use. For antibody treatment, anti-MHV3 antibody at a final concentration of 1:25 was added to persistently infected or uninfected cell cultures. At each cell passage, antibody was added at the same concentration. After anti-MHV3 antibody treatment, cell cultures were washed three times in an excess of RPMI 1640 medium and resuspended in fresh medium.

ACTD treatment. The toxicity of actinomycin D (ACTD) (Sigma Chemical Co., St. Louis, Mo.) was tested at different drug concentrations in normal lymphoid cell cultures. According to experiments, various doses of ACTD were added in persistently infected and uninfected cell cultures.

Virus production and titration. MHV3 was a cloned substrain and was produced as already described either in vivo in susceptible mice or in vitro on L2 cell cultures (6). For titration, viral suspensions, diluted serially in 10-fold steps, were tested on L2 cells cultured in 96-well microtiter plates. Virus titer was expressed as 50% tissue culture infective dose ($TCID_{50}$). In lymphoid cell cultures, extracellular virus titers were determined in supernatants after centrifugation at 1,000 × *g* for 15 min. Sedimented cells, after washings and resuspension at the initial volume, were used for the determination of cell-associated virus titers.

Virus cloning. Supernatants of L2 cell cultures infected with parental MHV3 and MHV3 derived from persistently infected YAC cells (YAC-MHV3) were used as starting materials for the preparation of virus clones. Virus cloning was performed on L2 cells using the limit dilution assay.

Thermosensitivity of cloned virus. Determination of thermosensitivity was carried out in L2 cells in microtitration plates infected with cloned viruses (multiplicity of infection [MOI] 0.01) and maintained in culture at 33, 37, or 39.5°C for various lengths of time. Each titration was done in triplicate. A virus yield ratio was calculated according to the difference in virus titers obtained when cells were cultured at two different temperatures for 18 or 24 h.

Thermolability of cloned virus. Thermolability was determined by the rate of reduction of $TCID_{50}$ per milliliter after incubation of virus samples at 37, 45, and 50°C. Undiluted virus stocks were incubated in a water bath at the three different temperatures, and samples were taken at various times and frozen at −70°C. Residual infectivity was determined by virus titration in L2 cells.

Defective interfering particle assay. The search for defective interfering particles was carried out by using an in vivo test. A volume of 0.1 ml of 10-fold dilutions of virus suspension was injected intraperitoneally into each of three adult C57BL/6 mice (susceptible to MHV3) per dilution. The number of dead animals per group was recorded.

Interferon assay. Supernatants of persistently infected cell cultures were treated by UV irradiation (30 min) or heat (50°C for 15 min) to destroy virus infectivity before testing. The interferon assay was carried out by published methods (4).

In vivo pathogenicity assay. In vivo pathogenicity was determined by intraperitoneal injection of 0.1 ml of virus suspensions into susceptible C57BL/6 newborn and/or adult mice. Each group consisted of 5 to 10 mice, and the number of animals which survived during the first 2 weeks after injection was recorded.

Experimental design. In all experiments, studies were carried out in triplicate samples of MHV3-infected and uninfected cell cultures or cell clones. The three identical samples were used for (i) cell count and viability, (ii) indirect immunofluorescence, and (iii) determination of extracellular and cell-associated virus titers.

RESULTS

Establishment of MHV3 persistent infection. Persistent infections were established in YAC and RDM-4 cells and maintained for up to 100 days in some experiments. They were stopped at that time for convenience. In all cultures, the number of viable cells was regularly counted, and virus persistence was determined by virus production and detection of cellular viral antigens by immunofluorescence. Induction and maintenance of persistent MHV3 infections in YAC and RDM-4 cells were tested at various MOI (0.001 to 100). In YAC cells, a persistent infection occurred only at an MOI of ≥ 1, whereas all MOI induced viral persistence in RDM-4 cells. Figure 1 shows the results observed with 1 MOI. No difference in cell number was observed when infected and uninfected RDM-4 cells were compared. A marked difference, however, was observed in YAC cells (Fig. 1C). In addition, a cell "crisis," characterized by a drastic decrease in cell number, occurred in infected cell populations at various times (Fig. 1C). Indirect immunofluorescence examination of infected and control cells revealed that the

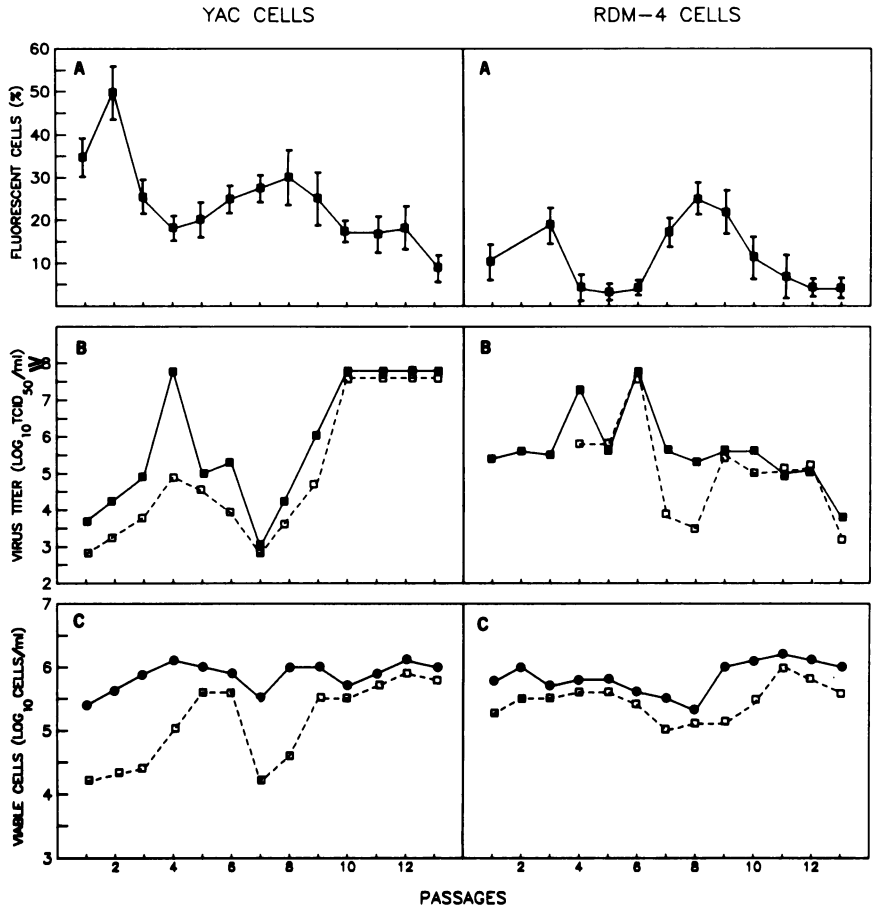

FIG. 1. MHV3 persistent infection induced in YAC and RDM-4 cells. A, Percentage of fluorescent cells; B, free (■) and cell-associated (□) virus production; C, cell count of MHV3-infected (□) and uninfected (●) cell cultures.

patterns of fluorescence varied according to time of infection (Fig. 1A). Large fluorescent syncytia were frequently seen during the first two passages postinfection, whereas small unfused fluorescent cells were observed thereafter. Infectious virus was produced by both persistently infected cell lines but reached a higher titer in RDM-4 cells. Titers varied with time, and no significant difference was seen between free virus and cell-associated virus titers (Fig. 1B).

To study the effect of fresh medium on the development of persistent infection, culture medium was changed every other day starting at the time of infection of YAC and RDM-4 cells with 1 MOI. In infected YAC cells, this procedure resulted in a marked increase in cell lysis, in the number of fluorescent cells, and in virus titers. No significant variations were observed in RDM-4 cells.

Cell cloning. Cell cloning of persistently infected and noninfected YAC and RDM-4 cells was performed by the limit dilution method. Cloning efficiency was estimated as the ratio of the number of growing clones to the number of expected clones. The cloning efficiency of infected (I) versus uninfected control (C) cells was expressed as an I/C ratio. In the first two experiments, the I/C ratios for YAC cells were 0.77 and 0.72. In experiment 3, a normal ratio of 1.01 was related to an abortive infection, since YAC cells had been inoculated with a low MOI (0.1). The percentages of fluorescent cells in experiments 2 and 3 were 20 and 0%, respectively. Virus did not persist in any of the clones tested, as evidenced by the absence of virus production (0 of 272), of immunofluorescent cells (0 of 160), and of resistance to superinfection (0 of 155). In two experiments performed with RDM-4 cells, the I/C ratios were 0.94 and 1.08. Similarly, neither production of virus (0 of 168) nor resistance to superinfection (0 of 101) was observed.

Effect of anti-MHV3 antibody. To evaluate the role of extracellular transmission of infection in virus persistence in YAC cells, anti-MHV3 antibody at a final concentration of 1:25 was added during four successive passages. The number of cells in infected cell populations progressively reached a level similar to that of uninfected populations, whereas the number of fluorescent cells progressively decreased (Fig. 2). Free or cell-associated virus was not recovered after the first passage. Cell cultures were maintained during nine subsequent passages after extensive washings and culture in antibody-free medium. Infectious virus, cell-associated virus antigens, and resistance to superinfection were not observed, indicating virus disappearance.

Role of cellular metabolism. Since the growth of MHV3 is equal at 33 and 37°C, to evaluate the role of cellular metabolism in the maintenance of MHV3 persistence, YAC cell cultures were incubated at 33 and 37°C for two successive passages. When cultured at 33°C, the number of viable cells in infected cultures became similar to that of control cultures. A switch of temperature from 33°C during the first passage to 37°C during the second passage resulted in a

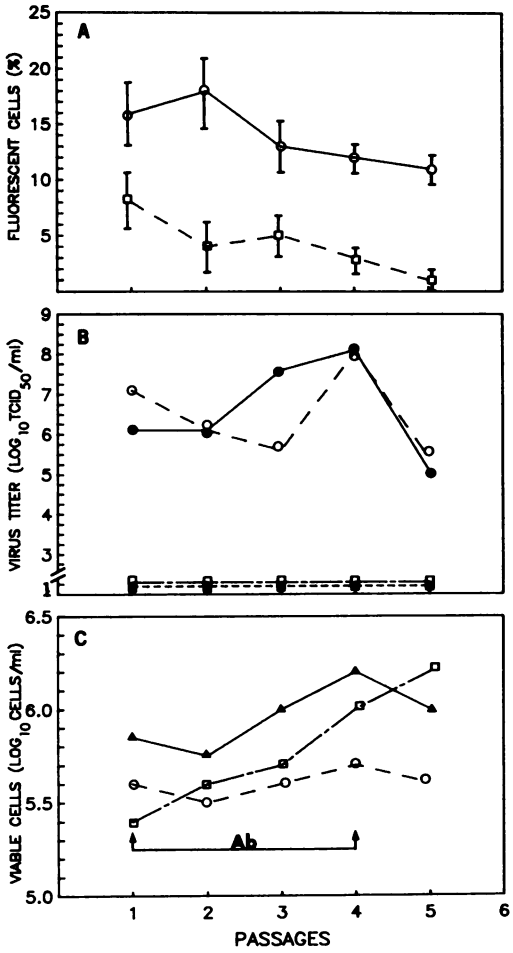

FIG. 2. Anti-MHV3 antibody treatment. MHV3-infected YAC cells were cultured with (□,■) or without (○,●) antibody. A, Percentage of fluorescent cells; B, free (□,○) and cell-associated (■,●) virus production; C, cell count of MHV3-infected (□,○) and uninfected (▲) cell cultures.

marked increase in cell lysis. Although such temperature changes did not result in variations in virus titers, they caused marked differences in the number of fluorescent cells. During two successive passages at 33°C, the number of fluorescent cells regularly increased from 15 to 75%. A switch in temperature from 33°C during the first passage to 37°C during the second passage produced a rapid decrease (from 45 to 25%) in the number of fluorescent cells, which remained at 25% during two subsequent passages. Similarly, infected cells cultured only at 37°C during the same period of time displayed a constant number of fluorescent cells (ca. 20 to 25%).

Low doses (0.025 and 0.050 μg/ml) of ACTD were added to YAC cells at two different stages of culture. No modification of fluorescent cell number and an increase of virus titers were observed when ACTD was added at a stage associated with a high number of fluorescent cells and a low virus titer (Fig. 3). However, ACTD added at a stage of low fluorescent cell number and high virus titer resulted in a rapid increase in the number of fluorescent cells and a marked decrease in virus titers (Fig. 3).

Interferon was not detected in any of six undiluted culture supernatants of MHV3 persistently infected YAC cells at various times of culture.

Thermosensitivity. The search for thermosensitive mutants was carried out in cloned virus isolates originating from either infected L2 cells or from YAC cells persistently infected with MHV3 for 45 days. Virus populations derived from L2 or YAC cell cultures at 33, 37, and 39.5°C exhibited heterogeneous patterns of thermosensitivity (Table 1).

The kinetics of heat inactivation of 10 isolates obtained from persistently infected YAC cells was compared with that of the parent virus. Parental MHV3 was completely inactivated at 45 and 50°C in 20 and 10 min, respectively. In addition, a 1.5 $\log_{10}$ reduction of virus titer was obtained after 3 h of incubation at 37°C. Eight cloned viruses originating from persistently infected YAC cells exhibited a similar thermolability. When compared with the parent virus, however, one virus isolate appeared to be more thermolabile at 37 and 45°C, and another appeared to be more thermostable at 37°C.

In vivo pathogenicity. The capacity of YAC cell-derived MHV3 isolates to induce an acute disease upon injection into susceptible mice was tested using culture supernatants of YAC cells inoculated with parental MHV3 at an MOI of 1. At various times postinfection, in vitro virus titers and in vivo pathogenicity were determined. Although virus replication was regularly expressed in YAC cells during 45 days of culture with a yield between 10^4 and 10^7 $TCID_{50}$/ml, the property of in vivo pathogenicity rapidly disappeared (Table 2). Eight days after injection, YAC cell culture supernatants had lost their ability to induce an acute disease when injected into susceptible C57BL/6 mice. Since the disappearance of in vivo pathogenicity may be related to an accumulation of defective interfering particles (8), nonpathogenic (NP) MHV3 was diluted (10^{-1} to 10^{-6}/ml), and in vivo pathogenicity was tested. No recovery of pathogenicity was obtained after NP virus dilutions.

In an attempt to determine whether in vivo injection of NP MHV3 could lead to virus elimination or to induction of a subclinical infection, liver and peritoneal macrophages of C57BL/6 mice injected with NP MHV3 were examined for the presence of virus. A persistent virus infection developed in C57BL/6 mice infected with NP MHV3, as evidenced by focus formation in peritoneal exudate cells and by the detection of low titers of virus in the liver (Fig. 4). Subsequent experiments revealed that virus was recovered from peritoneal macrophages, liver, and brain in 3 of 45 animals from 4 to 6 months postinfection.

The in vivo pathogenicity of cloned MHV3 progeny derived from YAC or L2 cells was determined. Uncloned and cloned YAC-MHV3 were partially pathogenic for newborns but were not pathogenic for adult C57BL/6 mice, whereas full pathogenicity was observed with uncloned and cloned L2-MHV3 (Table 3). In addition, as observed with uncloned MHV3, the injection of NP MHV3 clones into susceptible mice led to virus persistence. To determine whether NP MHV3 could be used as an immunogenic agent, groups of 10 to 15 C57BL/6 mice were injected with NP MHV3 on day zero and challenged with wild-type MHV3 at various intervals. When challenged with wild-type MHV3 between days 1 and 4 postinfection, 40 of 40 animals died, whereas all mice challenged from days 5 to 45 survived (65 of 65).

DISCUSSION

The chronic disease induced by MHV3 in semisusceptible mouse strains is characterized by the occurrence of paralysis

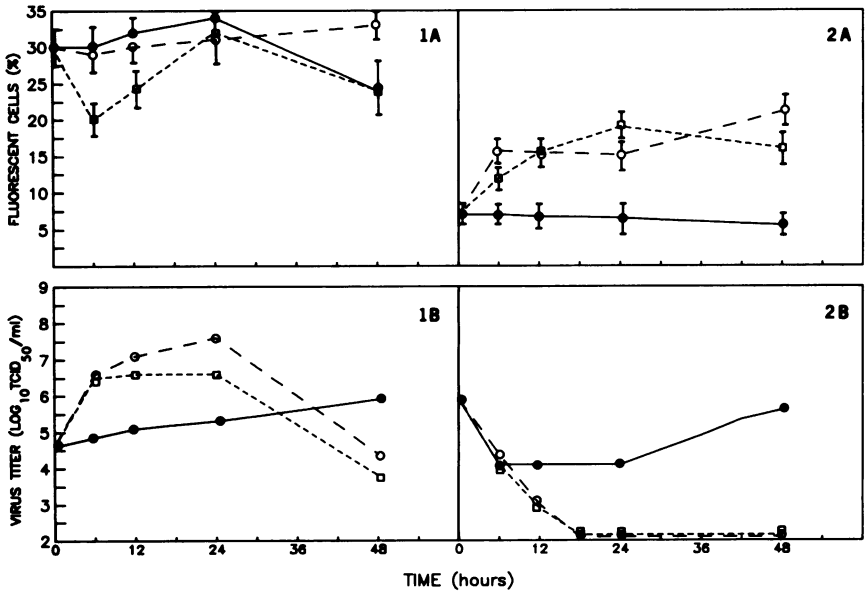

FIG. 3. Effect of ACTD on MHV3 persistent infections in YAC cells at two different stages of culture (stage 1, high number of fluorescent cells; stage 2, low number of fluorescent cells). Cells were incubated without (●) or with 0.025 μg (□) or 0.050 μg (○) of ACTD per ml. Results are expressed as the percentage of fluorescent cells (A) and total virus titers (B).

and virus persistence (17). Extremely low titers of virus were recovered from liver, brain, spleen, and lymph nodes. Immunological study of chronically infected animals revealed a marked impairment of B- and T-cell number and function (5). Several lines of evidence point to the direct role of MHV3 persistence in the immune depression: the marked decrease in lymphocyte numbers during chronic disease (17), the failure of MHV3-infected lymphocytes to undergo blast transformation in vitro upon stimulation with mitogens or allogeneic cells (unpublished data), and the replication of MHV3 into lymphocytes (12). Thus, it was important to see whether MHV3 persistence could be established in vitro in lymphocyte cultures and whether such a model could be utilized for the study of MHV3-cell interactions and for a better understanding of the mechanism of MHV3 persistence.

MHV3 persistence could be readily established in vitro in the two lymphoid cell lines tested: YAC cells, which is a line of T-cell origin, and RDM-4 cells, which derive from a B-cell lymphoma. Although some differences were seen between YAC and RDM-4 cells, a general relationship was observed among virus production, occurrence of cytoplasmic virus antigens, and cell lysis. High virus titers were associated with a decrease in the number of fluorescent cells and in cell lysis. Conversely, low virus titers were associated with a high number of fluorescent cells and increased lysis. Such a relationship suggests sequential events in which virus production would be followed by accumulation of virus antigens and cell death. Massalski et al. (19) have demonstrated an accumulation of core material after an abrupt cessation of virus assembly in L2 cells infected by the JHM strain of murine coronavirus. Preliminary electron microscopy studies of infected YAC cell cultures revealed a low viral production in morphologically intact cells and the presence of clustered cores in the cytoplasm of vacuolized cells or in cells undergoing lysis. Infected RDM-4 cells showed an apparent exclusion of clustered cores by polarization of viral antigens in the cytoplasm. These data indicate that only a

TABLE 1. Replication at different temperatures of virus clones isolated from MHV3-infected L2 and YAC cells

Yield ratio[a] (log_{10})	No. of clones (%)					
	33–37°C		37–39.5°C		33–39.5°C	
	YAC-MHV3	L2-MHV3	YAC-MHV3	L2-MHV3	YAC-MHV3	L2-MHV3
−1	3 (7.5)	0 (0)	1 (3)	0 (0)	0 (0)	0 (0)
0	1 (2.5)	2 (13.3)	2 (6)	2 (18)	2 (6)	1 (9)
1	10 (25)	1 (6.7)	9 (28)	5 (45)	1 (3)	0 (0)
2	7 (17.5)	3 (20)	9 (28)	3 (27)	3 (10)	0 (0)
3	6 (15)	3 (20)	8 (25)	1 (9)	3 (10)	1 (9)
4	7 (17.5)	5 (33.3)	3 (9)	0 (0)	7 (23)	1 (9)
5	5 (12.5)	1 (6.7)	0 (0)	0 (0)	4 (13)	5 (45)
6	1 (2.5)	0 (0)	0 (0)	0 (0)	8 (28)	2 (18)
7	0 (0)	0 (0)	0 (0)	0 (0)	4 (13)	1 (9)

[a] Yield ratio was calculated as difference in virus titers obtained when cells were cultured at various temperatures for 18 h (37 to 39.5°C) or 24 h (33 to 37°C).

TABLE 2. In vivo and in vitro pathogenicity of YAC cell-derived MHV3[a]

Days postinfection	In vivo		In vitro ($TCID_{50}$/ml)
	No. surviving/no. tested	%	
0	0/12	0	$10^{5.1}$
1	2/6	33	$10^{3.1}$
2	0/6	0	$10^{6.1}$
4	0/6	0	$10^{7.8}$
7	2/6	33	$10^{7.8}$
8	6/6	100	$10^{6.2}$
11	6/6	100	$10^{5.1}$
15	6/6	100	$10^{6.1}$
20	12/12	100	$10^{6.6}$
25	12/12	100	$10^{4.1}$
45	12/12	100	$10^{6.1}$

[a] YAC cell cultures were infected with MHV3 at an MOI of 1. At various times postinfection, culture supernatants were tested for virus titer and in vivo pathogenicity. Culture supernatants (0.1 ml) were injected intraperitoneally into C57BL/6 adult mice.

small number of infectious virus-producing cells can be found during persistent infection at any given time. Similar observations were noted by Roumillat et al. (24), Robey et al. (23), and Graze and Rayston (7) with lymphoblastoid cell lines persistently infected by various herpesviruses. The relationship between virus production and cell lysis was particularly remarkable in YAC cells, where crisis occurred. Such crises were manifested by a sharp increase in cell lysis and fluorescent cell number and, concomitantly, by a marked decrease in virus titers. Similar cell crises have already been demonstrated in mouse L cells persistently infected with vesicular stomatitis virus (22, 29) and in human lymphoblastoid cells infected with parvoviruses (1) or with herpes simplex virus (3). A cell crisis may be triggered by several factors, e.g., decreased interferon production (25), enhanced virulence of the virus (10, 27), or a sudden decrease in the number of defective interfering virus particles (8). Such factors, however, were not found in MHV3-infected YAC cell cultures. Regular changes of culture medium resulted in increased cell lysis, whereas low incubation temperature or ACTD treatment led to an accumulation of fluorescent cells. These factors caused important changes in the cellular metabolism. It was found that expansion and modulation of virus receptors may determine the outcome of certain virus infections in lymphoid cells or cell lines (20).

The results obtained with anti-MHV3 antibody treatment and cell cloning in two sets of experiments strongly suggested that, in persistent MHV3 infection of YAC and RDM-4 cells, the type of viral transmission was horizontal and not vertical and was related to cell-cell infection by infectious virus released in culture medium. Persistent infection induced with MHV3 in lymphoid cell lines is therefore characterized by a viral "carrier state" in which infectious virus particles produced by infected cells can only infect permissive cells. Persistent virus infections induced in vitro with coronaviruses (MHV3 and JHM strains) have mainly been established in continuous cell lines of neural origin and in mouse myeloblast or rat hepatoma cells (18, 26). The mechanisms involved in such persistent infections are still unknown.

During persistent infections, different patterns of virus evolution have been described in which cytolytic virus infections are converted to more temperate host-virus interactions. Such variations could be related to production of defective interfering particles, antigenic drift, selection of mutants, or interaction with the interferon system (30). Persistent infections induced in lymphoid human cell lines by measles or herpesvirus (3, 9) have been shown to promote the selection of thermosensitive mutants. We found that parental MHV3 produced virus populations that were heterogeneous with respect to thermosensitivity. Viral heterogeneity was maintained in persistently infected YAC cell cultures, and no selection of thermosensitive mutants appeared to be induced. Similar negative results were obtained with neuroblastoma cells persistently infected with JHM virus (26). In addition, Lucas et al. (18) demonstrated that the rapid inhibition of MHV3 and JHM synthesis resulting from a shift to high temperature was not associated with the appearance of thermosensitive mutants but was related to a host factor.

Initiation and maintenance of persistent infections in cells capable of interferon production may be related to the development of defective interfering particles or virus mutants which stimulate an increased capacity of cells to produce interferon (25). Neither interferon nor defective interfering particles were detected in MHV3 persistently infected YAC cell cultures. Similar negative results were obtained in persistent infections induced in vitro with other coronaviruses in animal (26) and human (2) cell lines.

Virus evolved from persistently infected lymphoid cell cultures can differ from the parent virus with respect to virulence markers such as in vivo pathogenicity. In persistent infections, the selective advantage of virus mutants of lower pathogenicity is apparent since it allows host survival (30). MHV3 variants produced in persistently infected YAC cell cultures differed from the parent virus in their lack of pathogenicity when injected into susceptible mice. Similar results were observed in MHV3 persistent infection carried out in RDM-4 mouse lymphoid cells (data not shown). The

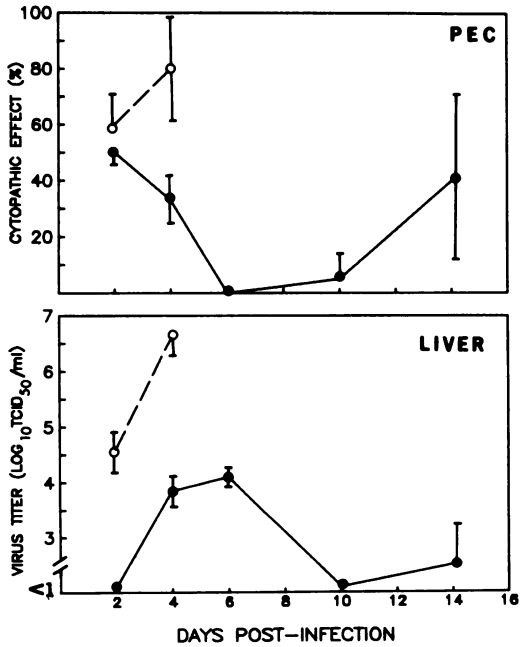

FIG. 4. Virus infection induced by pathogenic L2-MHV3 (○) and NP YAC-MHV3 (●) injected into C57BL/6 adult mice as expressed by virus titers in liver and viral cytopathic effects in peritoneal exudate cells (PEC) after 48 h of culture.

TABLE 3. In vivo and in vitro pathogenicity of YAC and L2 cell-derived MHV3

Origin of cell-derived MHV3 (no. of clones)	No. of passages (days) in vitro	In vivo pathogenicity[a] (no. surviving/no. tested) (%)		In vitro virus titer ($TCID_{50}$/ml)
		Newborns	Adults	
YAC-MHV3 (uncloned)		5/20 (25)	19/19 (100)	$10^{5.5}$
YAC-MHV3 (21)	2 (2)	11/135 (8)	21/21 (100)	$10^{6.5}$
L2-MHV3 (uncloned)		ND	0/15 (0)	$10^{5.0}$
L2-MHV3 (14)	2 (2)	ND	0/42 (0)	$10^{6.0}$

[a] Culture supernatant (0.1 ml per animal) was injected intraperitoneally into C57BL/6 mice. ND, Not done.

disappearance of the in vivo pathogenicity of YAC-MHV3 occurred rapidly and was detected in all virus clones tested. The loss of pathogenicity was related not to the state of virus persistence per se, but rather to the origin of the replicating cells, as this was observed in lymphoid cells but not in fibroblasts (results not shown). The sequencing by oligonucleotide mapping of mouse coronaviruses exhibiting various pathogenicities suggested that genetic sequences associated with viral pathogenicity were present in mRNA species 1 and 3, which corresponded to genes coding for the major envelope glycoprotein and for RNA polymerase (13). Mechanisms involved in the viral pathogenicity associated with such genes have not been determined. Envelope glycoproteins are responsible for adsorption and penetration into cells of myxovirus and paramyxovirus. Proteolytic cleavage of glycoproteins is required for the expression of biological activity. Glycoproteins of pathogenic virus strains are cleared in a wide spectrum of different host cells, whereas those of NP strains are activated in a few host systems only (11). Preliminary results suggested that treatment of NP MHV3 with proteases did not restore the in vivo pathogenicity.

These data indicate, therefore, that MHV3 replication in lymphoid cells leads to induction or selection of variants which maintain in vitro pathogenicity but display reduced in vivo pathogenic effects. Such variants seem to be responsible for the development of subclinical infection in susceptible adult mice. They display antigenic properties, as they can elicit defense mechanisms which enable infected animals to survive a challenge with virulent MHV3.

The ability of MHV3 to induce in vitro persistent infections in lymphoid cell lines suggests that a similar mechanism may be involved in vivo. Carrier-type transmission of infection may produce atrophy of lymphoid organs during the chronic phase of the disease and progressive immunodepression (17). Conversely, high anti-MHV3 antibody titers should block transmission of infection and cure the animals. Persistent MHV3 infection in lymphoid cell lines represents an interesting model for studying virus-lymphocyte interactions as well as cellular mechanisms involved in the loss of pathogenicity. The in vivo significance, however, of the latter phenomenon has to be established, and its existence in normal lymphocytes would be an observation of major importance.

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