JOURNAL OF VIROLOGY, Apr. 1986, p. 26–30
0022-538X/86/040026-05$02.00/0

Vol. 58, No. 1

Spontaneous Curing of a Minute Virus of Mice Carrier State by Selection of Cells with an Intracellular Block of Viral Replication

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Received 24 October 1985/Accepted 2 January 1986

We previously described a persistent infection established by the lymphotropic minute virus of mice in mouse L cells at the level of the cell population (D. Ron, P. Tattersall, and J. Tal, J. Virol. 52:63–69, 1984). This carrier state is maintained by a series of consecutive phenotypic changes which take place in both the cells and the virus and is cured spontaneously after 150 to 200 cell generations (D. Ron and J. Tal, J. Virol. 55:424–430, 1985). We show here that the cure was caused by the selection of virus-resistant cells in the culture. The resistance of these survivor cells to virus replication was due to an intracellular block. Infection of a spontaneously cured culture with the fibrotropic parental minute virus of mice resulted in a restrictive infection in which the viral replicative-form DNA was formed and amplified, but the synthesis of single-stranded progeny DNA was markedly reduced. The lymphotropic strain was blocked in these cells at an earlier stage, with little or no amplification of viral replicative-form DNA observed. These data indicate that the replication of minute virus of mice requires host-coded helper functions in at least two stages of its growth cycle.

Parvoviruses are small viruses containing a single-stranded DNA genome about 5 kilobases in size. The mammalian parvoviruses are divided into two subgroups on the basis of their requirement for helper viruses. Members of the adeno-associated virus subgroup are entirely dependent on adenovirus or herpesvirus for their replication, whereas the nondefective, or autonomous, parvoviruses are capable of productive replication without the aid of a helper virus (2, 31). Minute virus of mice (MVM), a member of the autonomous parvoviruses, is endemic to mice, as shown by serological surveys of wild and laboratory mouse colonies (16). Studies of its biology suggest that it is capable of establishing persistent infections in mouse populations (22).

Two strains of MVM have been isolated from mice: a fibrotropic strain, designated MVM(p), which grows in mouse fibroblasts but is restricted in T-lymphocyte cell lines, and a lymphotropic strain, MVM(i), with reciprocal tissue specificity, i.e., it grows lytically in T lymphocytes but is restricted in fibroblasts (24, 27). MVM(i), however, can readily establish persistent infections in A9 cells, a subline of mouse L cells. The persistence of the virus is at the population level, i.e., its passage from one cell to another involves an extracellular stage. These cultures can therefore be cured of the infecting virus by including anti-MVM serum in the growth medium (18). In the absence of anti-MVM serum, these carrier states last between 20 and 30 passages (150 to 200 cell generations) and are then cured by a spontaneous disappearance of the virus.

We previously showed that in the $A9_{MVM(i)}$ carrier state, the virus and the cells underwent extensive changes. Viral host range mutants which had adapted to growth in fibroblasts and eventually lost the ability to grow in lymphocytes were formed. One such mutant, designated *hr*301, isolated from a late stage in the persistent infection, was studied in detail and shown to be identical to MVM(i) by restriction enzyme analysis, although its host range was identical to that of MVM(p) (19). Concomitantly, cells within the infected culture which had increased resistance to the replication of the resident virus in the culture were selected (18). Our data suggest that virus-cell coevolution provided the mechanism for this persistent infection. The $A9_{MVM(i)}$ carrier state thus provided a useful tool for studying the host cell response to a viral infection in the absence of external factors, such as the host immune response. Several properties of this system make it attractive. The virus involved is small and structurally simple, and the nucleotide sequences of both MVM(p) and MVM(i) are now known (1, 20). In addition, any stage in the persistent infection can be reconstructed from stage-specific cells and their corresponding virus pools (18).

In this work, we studied the properties of the cells which emerged from the spontaneously cured culture, designated A9(s) (survivors). These cells were devoid of intracellular virus or subviral components and resisted superinfection by either MVM strain. Comparison of the interaction of MVM(p) and MVM(i) with A9 and A9(s) cells showed that the selection of cells resistant to the resident mutant virus triggered an increased resistance toward both the lymphotropic and the fibrotropic virus strains. On the basis of these results, we suggest that both fibrotropic and lymphotropic strains interact with the same cellular components; whether this interaction provides the virus with a full or partial helper activity or no helper activity depends on the type and strength of this interaction. Finally, we identified an early stage in virus replication which requires a host cell helper function.

MATERIALS AND METHODS

Cells and viruses. A9 cells, a variant of mouse L cells (13), and EL4 cells, a mouse lymphoma cell line (21), were grown in monolayer or suspension cultures. Both lines were maintained in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% fetal calf serum (Seralab). Hyb 1/11 cells, a hybrid between ouabain-resistant A9 cells and EL4 cells (27) were grown in the presence of 10% fetal calf serum and hypoxanthine-aminopterin-thymidine solution (12). MVM(p) (25) and MVM(i) (3) were grown in A9 and EL4 cells, respectively, and purified as previously described (19, 28).

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Assays. Quantitation of total virus particles was done by a hemagglutination assay with 1% human erythrocytes. To quantitate infectious virus, we plaque assayed both strains in 324K indicator cells (27) as described elsewhere (25). Virus-producing cells were quantitated by an infectious-center assay (26). The immunofluorescence assay was performed essentially as described by Tattersall (26). Anti-MVM serum was prepared in rabbits by using highly purified empty virus capsids. Fluorescein-conjugated goat anti-rabbit immunoglobulin G was purchased from Miles Yeda, Rehovot, Israel.

Propagation of persistently infected cell cultures. $A9_{MVM(i)}$ cells were maintained at 37°C in monolayer cultures. The cells were subcultured whenever the monolayer reached confluency by trypsinization and transfer of 25 to 50% of the cells to fresh dishes.

Binding assay. [^{3}H]thymidine-labeled viruses were incubated with cells in 1 ml of Dulbecco modified Eagle medium containing 20 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES) (pH 7.2) but not $NaHCO_3$, as described by Linser et al. (11). After incubation at 4°C for 1 h, a 0.4-ml sample was withdrawn, and total radioactive counts were determined by trichloroacetic acid precipitation (bound plus unbound virus, *A*). The cells in the rest of the culture were pelleted, and 0.4 ml of the supernatant was withdrawn for radioactive counting (unbound virus, *B*). Nonspecific binding was determined by treating the cells after virus binding with neuraminidase. The percentage of bound virus in each sample was calculated as $[(A - B)/A] \times 100$ minus the percentage of nonspecifically bound virus.

Dispersed-cell assay. The dispersed-cell assay used was that of Lavi and Etkin (10). In short, samples of 8×10^5 cells were trapped on nitrocellulose filters, denatured, neutralized, baked at 80°C for 4 to 8 h, preincubated, and hybridized in Denhardt solution (4) containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 100 μg of denatured herring sperm DNA per ml at 67°C for 15 h. The probe for hybridization was ^{32}P-labeled, nick-translated DNA (2×10^8 cpm/μg) from plasmid pPT206, which contains 95% of the MVM genome (15). After hybridization, the filters were washed, air dried, and autoradiographed at −70°C with a Du Pont Cronex intensifying screen for 10 to 24 h or, alternatively, counted in a liquid scintillation counter.

Analysis of viral replicative intermediates. Prior to infection, cells were parasynchronized by propagating the cultures in the presence of 2 mM thymidine for 15 h (30). At 14 h later, samples of 5×10^6 cells were seeded in 9-cm dishes and allowed to adhere for about 30 min. The cells were washed three times with phosphate-buffered saline and infected with virus. Low-molecular-weight DNA was obtained from the infected cells by the Hirt procedure (5), adapted to the extraction of MVM DNA (14), with the omission of the DNA denaturation step. The DNA preparations were electrophoresed on a 1% agarose gel and blotted onto nitrocellulose membrane filters (23). The probe for hybridization was nick-translated plasmid pPT206 DNA.

Preparation of virus-resistant cells. A fresh culture of A9 cells was infected per cell with 1 PFU of MVM(p) or $MVM(i)_{A9}$, a virus stock from the late stage of the persistent infection (designated pool L virus [18]), under conditions identical to those in which an MVM(i) persistent infection is established. Extensive cytopathic effects appeared within 2 to 3 days, and the cells were maintained in the original medium for 2 to 3 weeks until colonies of survivor cells appeared. Once colonies appeared, the medium was changed every 3 days. The culture was then trypsinized and subcultured again in the presence of 1% anti-MVM serum (hemagglutination inhibition of 1:1,600) for two passages and in the absence of anti-MVM serum for two additional passages. The resistant cells obtained did not contain any detectable infectious virus, as determined by plaque assaying lysates of 2×10^6 cells.

TABLE 1. Spontaneous curing of the $A9_{MVM(i)}$ culture

No. of days postinfection	Infectious centers (%)	Infectious virus[a] (PFU/ml)	Fluorescent nuclei[b](%)	Viral DNA[c]
125	0.35	2×10^4	0.4	+
150	$<10^{-5}$	$<2 \times 10^{-7}$	$<10^{-3}$	−

[a] A lysate of 5×10^6 cells in 5 ml of 10 mM Tris hydrochloride–mM EDTA (pH 7.4).
[b] Between 1,000 and 1,500 cells were screened in each infection.
[c] The presence (+) or absence (−) of viral DNA was determined by DNA dot hybridization (9) under conditions in which one viral copy per cell was detectable.

RESULTS

Virus and subviral components in A9(s) cells. In a previous work, we reported that cells derived from a late stage of the persistent infection produced high levels of infectious progeny virus without apparent cytopathic effects (18). Passaging of these cells resulted in a gradual decrease in infectious-virus levels and eventually led to a complete cure of the culture. This spontaneous cure process is shown in Table 1. Cells derived from cultures 125 and 150 days postinfection were examined for the production of infectious virus and subviral components. The results showed that infectious virus, viral antigens, and viral DNA, which were readily detectable at 125 days postinfection, were under the limits of detection by 150 days. The spontaneous nature of the curing process suggested that it resulted from the selection of virus-resistant mutant cells. To examine this possibility, and in an attempt to detect their origin, we studied the properties of A9(s) cells.

Superinfection of A9(s) cells with MVM. A9(s) and parental A9 cells were infected with MVM(p), MVM(i), and $MVM(i)_{A9}$ virus derived from a 125-day-old culture. The infections were done at high input multiplicities (100 PFU per cell), and the infected cells were incubated for 3 days, a time span sufficient for several cycles of virus replication. Infectious-virus production was then assayed in 324K indicator cells, and cell viability was determined by examining dye exclusion with trypan blue. The results (Table 2) showed that A9(s) cells were at least 100-fold less permissive than

TABLE 2. Virus production in A9 and A9(s) cells[a]

Infecting virus	Cells	Cell viability (% of control)	Virus production (PFU/ml)
MVM(p)	A9	<0.1	2×10^6
	A9(s)	98	10^4
MVM(i)	A9	83	2×10^2
	A9(s)	100	ND
$MVM(i)_{A9}$	A9	<0.1	2×10^7
	A9(s)	99	4×10^3

[a] Monolayer cultures (5×10^5 cells each) were infected at 100 PFU per cell. Three days later, the cells were trypsinized and counted in the presence of trypan blue. Virus production was determined by plaque assaying the medium and the cell lysates in 324K indicator cells. ND, Not detected (less than 1 PFU per 10^6 cells).

TABLE 3. Virus binding and viral DNA synthesis in A9 and A9(s) cells

Infecting virus	Cells	Specific binding to cells (% of input cpm[a])	Intracellular viral DNA (cpm) at indicated h postinfection[b]	
			4	24
MVM(p)	A9	66	5,410	78,080
	A9(s)	22	4,118	37,882
MVM(i)	A9	69	1,063	10,481
	A9(s)	21	1,039	1,456

[a] For each binding assay, 2×10^5 cells were infected with 2×10^6 PFU of ^{3}H-labeled virus (about 1,000 cpm). The virus used was a purified fraction of infectious ("light full") particles obtained from a glycerol gradient as described earlier (19). The input multiplicity of infection (25 PFU per cell) was only sufficient to saturate about 1% of the total binding sites for MVM in the culture (11). Nonspecific binding (4%) was subtracted as the background level.

[b] Determined by the dispersed-cell assay.

parental A9 cells to MVM(p) and MVM(i)$_{A9}$ replication, exhibiting limited cytopathic effects, and were completely restrictive to MVM(i) replication.

To determine whether the reduction in virus production was due to membranal barriers or to intracellular blocks, we compared the capacity of A9 and A9(s) cells to bind [^{3}H]thymidine-labeled viruses. MVM(p) and MVM(i) were bound to A9 cells at similar efficiencies (as was expected, since both viruses are known to recognize the same cellular receptors [24]) (Table 3). The binding of both viruses to A9(s) cells was ca. threefold lower than that to parental A9 cells, but this reduced binding was not sufficient to account for the differences observed in virus production between these cell lines. Furthermore, measurements of intracellular viral DNA at 4 h postinfection (before the onset of viral DNA synthesis) revealed only slight differences in the penetration of MVM(p) and MVM(i) into both A9 and A9(s) cells, suggesting that the restriction of virus replication in A9(s) cells is an intracellular event.

Measurements of intracellular viral DNA at 24 h postinfection (i.e., after viral DNA amplification) showed that A9(s) cells supported the amplification of MVM(p) replicative-form (RF) DNA but not of MVM(i) DNA (Table 3). These results suggested that the restriction of the growth of MVM(i) in A9(s) cells was an early one, prior to viral DNA synthesis, whereas the block of the replication of MVM(p) in these cells was subsequent to viral DNA synthesis. To examine this, we analyzed the intracellular forms of the viral DNAs in A9(s) cells. Parasynchronized cells were infected with viruses at high input multiplicities, and the low-molecular-weight DNAs were extracted and analyzed by Southern blotting (Fig. 1). For quantitation purposes, DNA replication in each infection was determined by performing the dispersed-cell assay at 4 and 24 h postinfection (lower boxes in Fig. 1). As shown, large amounts of monomer and dimer RF DNAs were synthesized in both A9 and A9(s) cells infected with MVM(p). Single-stranded progeny DNA synthesis was readily detected in A9 cells but only barely detected in A9(s) cells. The faint band at the single-strand position in MVM(i)-infected A9(s) cells was most probably a remnant of the infecting parental viral DNA. It is noteworthy that under these infection conditions, A9(s) cells produced between 10^5 and 10^6 less infectious virus than did A9 cells. Both forms of viral RF DNA were also made in MVM(i)-infected A9 cells, but only the monomer form could be detected in MVM(i)-infected A9(s) cells. The low intensity of the MVM(i) monomer RF band is in agreement with the low

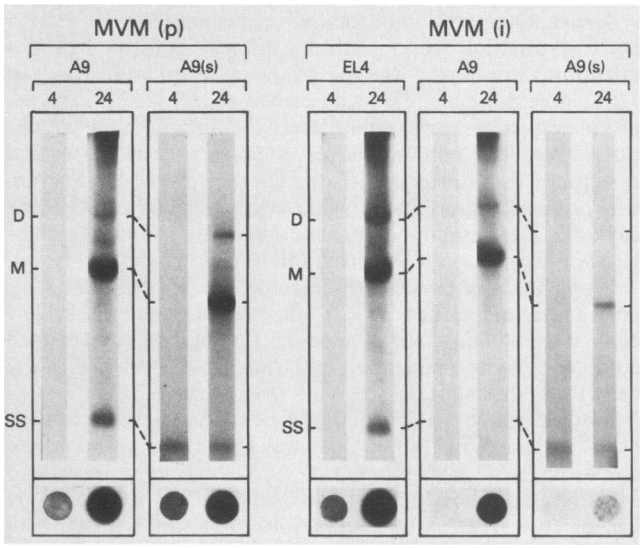

FIG. 1. Replicative intermediates of MVM(p) and MVM(i) DNAs in A9 and A9(s) cells. Parasynchronized A9 and A9(s) cells were infected at 20 PFU per cell with MVM(p) or MVM(i). EL4 cells were also infected with MVM(i) as a control for MVM(i) permissive infection. Hirt supernatants (14) were prepared at 4 and 24 h postinfection and subjected to Southern blotting analysis. The blots were hybridized to nick-translated pPT206 DNA (10^8 cpm/μg) for 15 h at 67°C. For each infection, intracellular viral DNA was determined by the dispersed-cell assay, and the filters containing the viral DNAs were hybridized to the same DNA probe. These data are shown in the lower boxes. SS, M, and D, single-stranded, double-stranded monomer, and double-stranded dimer DNA forms of MVM, respectively.

levels of intracellular DNA in this infection. Thus, A9(s) cells contain two separate blocks of the replication of fibrotropic and lymphotropic MVM strains.

Origin of A9(s) cells. There are two possible explanations for the origin of A9(s) cells. Either they were present in the original A9 culture in very small proportions or they arose by mutation(s) during the course of the persistent infection. Since every culture of A9 cells contains a small fraction of cells which are resistant to MVM(p) (unpublished data), a comparison of these naturally occurring resistant cells with A9(s) cells could provide an answer to whether the latter preexisted in the parental culture. To make this comparison, we isolated A9 cells resistant to either MVM(p) or pool L virus [a pool of resident MVM(i)$_{A9}$ virus obtained from the late stage (72 days of culturing) of the persistent infection (18)] and designated them A9(PR) and A9 (LR), respectively. The isolation procedure is described in Materials and Methods. A9(s), A9(PR), and A9(LR) cells, as well as parental A9

TABLE 4. Production of infectious virus and viral DNA synthesis in survivor cell lines following MVM(p) infection

Cells	Cytopathic effects	Infectious virus (PFU/ml)[a]	Intracellular viral DNA (cpm) at indicated h postinfection[b]	
			4	24
A9	++	2×10^6	NT	105,723
A9(s)	−	2×10^4	NT	28,960
A9(PR)	−	ND	53	92
A9(LR)	−	ND	65	115

[a] All infections were done at 100 PFU per cell. ND, No infectious virus detectable in a lysate containing 10^6 cells.

[b] Determined by the dispersed-cell assay. NT, Not tested.

cells, were infected with MVM(p) at 100 PFU per cell and examined for infectious-virus production and for viral DNA synthesis. Table 4 clearly shows that A9(s) cells were distinct from the naturally occurring A9(PR) and A9(LR) cells. The absence of viral DNA synthesis and the low input viral DNA found in A9(LR) cells at 4 h postinfection suggested that, in contrast to A9(s) cells, these cells were deficient in membrane receptors for the virus.

DISCUSSION

From a mechanistic point of view, persistent infections can be divided into two types: those which are maintained at the level of the individual cell and those which are maintained at the population level. Carrier cultures of the first category are characteristic of viral defective-interfering-particle involvement. In these persistent infections, 100% of the cells always produce viral antigens, and the infections are long and indefinite in duration and cannot be cured easily by antibodies against the infecting virus. Viral carrier states which are maintained at the population level are short-term ones, a low and variable percentage of the cells produce viral antigens, and the carrier states can be cured by antiviral serum and also tend to cure spontaneously (for a review, see reference 6). In vivo the cure can result from the involvement of factors such as the host response and cell differentiation. The mechanisms underlying the spontaneous curing of carrier cultures in vitro are obscure (6).

Previously, we showed that a viral carrier state established by MVM(i) in A9 cells and maintained at the level of the cell culture is cured spontaneously after 150 to 200 generations (18, 19). In the present work, we show that the cure is effected by the selection of mutant cells designated A9(s). In contrast to parental A9 cells, A9(s) cells are highly restrictive to infection by MVM(p) and MVM(i). They are also highly resistant to $MVM(i)_{A9}$, a pool of resident virus from a 125-day-old carrier culture which resembles *hr*301 virus in its growth properties.

The emergence of the A9(s) cells is a result of a multistep process of selection, the selective pressure being the resident virus $MVM(i)_{A9}$. It is not a simple selection of preexisting resistant cells by eradication of the sensitive cells in the culture. This conclusion is based on several observations. First, every culture of A9 cells studied to date has contained a fraction of cells which are resistant to infection by MVM(p) or by fibrotropic host range mutants of MVM(i) (D. Ron and J. Tal, unpublished results). These cells were readily obtainable from a high-multiplicity infection, whereas A9(s) cells evolved during the course of 20 to 30 passages of persistently infected cells. Second, we showed here that the naturally occurring resistant cells do not absorb MVM(p), possibly owing to the lack of membrane receptors. In contrast, A9(s) cells do absorb MVM(p) and allow partial replication of its DNA in the cell nuclei (Table 4). Finally, we previously reported that MVM(i) can readily establish persistent infections in freshly isolated clones of A9 cells (18). These carrier states, too, terminate spontaneously, yielding A9(s) cells. Taken together, these data show that A9(s) cells are distinct from the naturally occurring resistant cells in A9 cultures.

Spontaneous curing of persistently infected cells has been shown to occur in various systems, such as vesicular stomatitis virus-infected HeLa and L cells (7, 8), poliovirus-infected HeLa cells (29), and simian virus 40-infected AGMK cells (17). The block of simian virus 40 replication in AGMK cells was shown to be an early event in the virus replication cycle, such as penetration or uncoating, since it was possible to overcome it by transfecting the cells with viral DNA. In the other systems, the curing processes were not studied, nor are the roles of the host cells understood.

A9(s) cells emerged from parental A9 cells in response to selective pressures exerted on the culture by fibrotropic host range mutants of MVM(i). Unlike A9 cells, A9(s) cells were restrictive to MVM(p) replication and were by far more restrictive to MVM(i) replication. The block of MVM(p) replication was located after viral RF DNA amplification. The block of MVM(i) replication, which was located in A9 cells at the initiation of transcription (B. Spalholtz and P. Tattersall, personal communications; D. Ron and J. Tal, unpublished results), was located in A9(s) cells at an earlier stage, at or before viral RF DNA synthesis (Fig. 1). Thus, the selection process was toward greater resistance to both viral strains. On the basis of these observations, we suggest that in A9 cells, MVM(p) and MVM(i) both interact with the same intracellular helper component(s). This interaction provides a full helper activity for the replication of MVM(p). The interaction with MVM(i), which may be weaker, is capable of facilitating partial DNA replication but not RNA and progeny DNA syntheses. We further hypothesize that during the persistent infection, either the cell-coded helper functions were modified or their expression was reduced. As a result, MVM(p) replication in the surviving A9(s) cells was blocked after viral RF DNA amplification, and MVM(i) replication was blocked before or at viral RF DNA synthesis.

Although we showed here that A9(s) cells restrict virus replication intracellularly (Table 4), our data do not exclude the possibility that the A9(s) culture is enriched with naturally occurring resistant cells which are deficient in their ability to absorb the virus. Compared to A9 cells, A9(s) cells are about 60% less efficient in virus binding (Table 3), suggesting that such a fraction may comprise as much as two-thirds of the A9(s) culture. This is unlikely, however, because the absorption of input viral DNA by the two cell cultures was almost identical. Furthermore, the threefold difference in binding cannot account for the large differences in virus production between the two cell lines.

The infection of A9(s) cells with MVM(i) enabled us to identify yet another stage in virus replication which requires a cellular helper function(s). This function operates early in the infection, before or at viral RF DNA synthesis. A9(s) cells restrict the replication of MVM(i) at this early stage. This block is not an artifact created by the selection in vitro of virus-resistant A9(s) cells. We recently studied the replication of MVM(p) and MVM(i) in two mouse testicular cell lines and found that they modulated the replication of both viruses very differently (E. Guetta, D. Ron, and J. Tal, submitted for publication). One of these cell lines restricted MVM(p) and MVM(i) replication at stages similar to those found in A9(s) cells, indicating that these deficiencies in cellular helper functions occur naturally. The data also showed that, like the transcriptional helper function, this early function is also developmentally regulated. The nature of these helper functions and their mode of interaction with the replicating virus are intriguing problems, since these cellular components may be involved in the differentiation process.

ACKNOWLEDGMENTS

We thank Nitza Peleg for technical assistance and Jean Rommelaere and Jim Trempe for reviewing this manuscript.

This work was supported by a grant from the U.S.-Israel Binational Science Foundation. J.T. is a Fellow in Cancer Research

supported by the David Laser Memorial Fund of the Israel Cancer Research Fund.

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