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Coevolution of Cells and Virus as a Mechanism for the Persistence of Lymphotropic Minute Virus of Mice in L-Cells

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Infection of L-cells with minute virus of mice (i), a lymphotropic strain of minute virus of mice, resulted in the emergence of host range mutant viruses capable of a lytic infection that destroys the initially restrictive parental cells. Despite that, the culture was not lysed completely; instead, a persistent infection resulted which lasted at least 150 days. Throughout the persistent infection, extensive changes occurred in both the tissue tropism of the progeny virus and in the phenotypic properties of the cells. Mutant cells were selected which were increasingly restrictive to the replication of the resident virus, but concomitant changes in the virus enabled it to replicate in a subpopulation of the restrictive cells. The persistent infection could be reconstructed by infection of mutant cells with mutant virus; in contrast, neither infection of parental cells with mutant virus nor infection of mutant cells with parental virus led to persistence. On the basis of these results, we suggest that virus-cell coevolution provides the primary mechanism for the initiation and the maintenance of the persistent infection.

A persistent infection by a normally virulent virus is generally established when a cytolytic viral infection is converted to a more temperate host-virus interaction which allows the virus to reside within the culture without destroying it. Although the mechanisms underlying virus attenuation are not clear, interferon induction in the infected cells (11, 17) and the formation of interfering viral mutants (5, 30) were shown to be involved in the establishment and maintenance of various viral carrier states. Some DNA viruses and reverse-transcribing RNA viruses may persist latently in cells by integration into the host genome (29).

Persistence has been implicated in the biology of the small, single-stranded-DNA-containing parvoviruses (27). 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, but in the absence of helper virus, they can integrate their DNA into the host genome without causing cell transformation (2, 29). The nondefective, or autonomous, parvoviruses are capable of productive replication without the aid of a helper virus. Most members of this subgroup are innocuous to adult host animals, yet they are believed to establish long-term, asymptomatic infections in vivo (27). One autonomous parvovirus, the Aleutian disease virus, produces disease by establishing a persistent infection in minks. The temperature-sensitive nature of the replication of Aleutian disease virus is believed to contribute to the maintenance of this persistent infection in vivo (16).

We have been studying the interaction of two strains of the autonomous parvovirus, minute virus of mice (MVM) with mouse L-cells in culture. Although these two MVM strains are serologically indistinguishable (25) and share almost complete DNA sequence homology (12), they possess allotropic host range properties. MVM(p), the prototype virus, establishes a lytic infection in L-cells in vitro, but its replication in T-lymphocyte cell lines is restricted. In contrast, MVM(i), the lymphotropic strain, grows well in T-lymphocyte-derived cell lines, but its replication in L-cells is restricted (25). In both heterologous infections, the restriction is intracellular (18, 21). In short-term infections (48 to 72 h) of A9 cells (an L-cell-derivative line) with MVM(i), virus growth is restricted after viral DNA replication (18, 21) but before viral mRNA and progeny DNA synthesis (D. Ron and J. Tal, unpublished results; P. Tattersall, personal communication) and produces only minute quantities of mature progeny virus. However, 7 to 10 generations later, persistent infections are readily established. These persistent infections are short-term ones, and the cells are spontaneously cured after about 120 to 160 generations. The virus shed by the carrier culture was shown to contain host range mutants of MVM(i). One such mutant, designated *hr*301, had adapted to growth in A9 cells and lost the ability to grow in EL4 cells, a mouse lymphoma cell line. Restriction enzyme analysis of *hr*301 DNA did not distinguish it from that of MVM(i), indicating that the mutation responsible for the host range alteration did not involve extensive changes in the viral genome (18).

The present study was aimed at gaining a better understanding of the mechanism underlying this persistent infection. Our results show that during the persistent infection, virus mutants with host range properties similar to those of MVM(p) and of *hr*301 had gradually evolved. Concomitantly, the persistently infected culture became increasingly restrictive to the replication of the resident virus. These observations suggest that changes in the virus and in the cells provide the mechanism for the maintenance of this persistent infection.

MATERIALS AND METHODS

Cells and viruses. A9 cells, a variant of the mouse L-cell line (10), and EL4, a mouse lymphoma cell line (20), were grown in monolayer or in suspension cultures. Both lines were maintained in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 5% fetal calf serum (Seralab). hyb 1/11 cells, a hybrid cell line between A9 ouab^r cells and EL4 cells (25), was grown in the presence of

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10% fetal calf serum and hypoxanthine-aminopterin-thymidine solution (9). The MVM(p) (23) and MVM(i) (3) strains of MVM were grown in A9 and in EL4 cells, respectively, and purified as previously described (18, 26). Quantitation of total viral particles was done by hemagglutination assay with 1% human erythrocytes. To determine infectious virus, both strains were plaque assayed in hyb 1/11 indicator cells as described elsewhere (25). Virus-producing cells were determined by infectious center assay (24).

Cloning of cells. Cell clones were obtained by growing cured cells in 5-cm dishes. The cells were diluted to give an average of 1 cell per dish, and clones were isolated from dishes containing single clones.

Indirect immunofluorescence. Immunofluorescence assays were performed essentially as described by Tattersall (24). Anti-MVM serum was prepared in rabbits by using highly purified empty virus capsids. Fluorescein-conjugated goat anti-rabbit immunoglobulin G (IgG) was purchased from Miles Yeda, Rehovot, Israel.

Dispersed cell assay. The method used in this work was that of Lavi and Etkin (8). 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) of the plasmid pPT206 (received from P. Tattersall) which contains 95% of the MVM genome (13). 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.

Curing of cultures from infectious virus. The persistently infected cells [designated $A9_{MVM(i)}$] were grown for two to three passages in the presence of 1% anti-MVM serum. To determine whether the cells were indeed cured, we subjected them to infectious center assay, and cell lysates were assayed for plaque formation on A9 cells. Both tests revealed less than 1 PFU per 10^6 cells. The cells were also shown to be free of any traces of viral capsid proteins by immunofluorescent staining (less than 0.1% fluorescent nuclei) and of viral DNA by hybridization of ^{32}P-labeled viral DNA probe (7×10^7 cpm/μg) to cellular DNA dot blots on nitrocellulose membranes.

RESULTS

Possible role of attenuating factors in the persistent infection. Defective interfering (DI) particles (5, 7), viral temperature-sensitive (*ts*) mutants (30), and induction of interferon in the infected cells (11, 14, 17) have been shown to be involved in the establishment and maintenance of persistent viral infections in vitro. In a search for the mechanism underlying the persistence of MVM(i) in A9 cells, we investigated the possible involvement of each of these factors. Defective interfering particles are amplifiable subviral genome-containing particles that exert specific inhibitory effects on the viral genomes which generated them. Cells containing DI particles enable the viral genome to replicate slowly and undergo extensive genetic changes within cells that otherwise would have been killed quickly (6). The characteristics of persistent infections involving DI particles are of long-term or indefinite duration, antigens are present in most of the cells in these carrier cultures, the persistence of the virus is at the individual cell level, and an attenuated lytic cycle of the normal viral genomes exists in these cells, making it difficult to cure them from infectious virus by antiviral serum (5). None of these properties was observed in the persistent infection of MVM(i) in A9 cells. The percentage of cell nuclei which were positive for viral antigens in immunofluorescent staining fluctuated between 10 and 17% at the peak of the virus-producing stage (40 to 70 days postinfection) and between 0.1 and 4% at the late stage (70 to 150 days postinfection [19]). Also, the culture could be readily cured of infectious virus by using anti-MVM serum, indicating that the persistent state in this case was maintained at the population level (namely, the transmission of virus at an extracellular stage) rather than by a vertical transmission to daughter cells.

Further experimental evidence argues against the involvement of DI particles in the MVM(i) persistent infection. First, we determined the ratio between infectious virus (PFU) and total progeny virus (hemagglutinating units [HAU]) at various times after infection. Since DI particles are not infectious, their accumulation in the culture is expected to result in a gradual decrease in the PFU/HAU ratio. However, this ratio remained relatively stable throughout the infection (data not shown). Secondly, persistent infections in A9 cells were readily established by infecting them with MVM(i) which was isolated from individual plaques on hyb 1/11 cells, whereas EL4 cells, infected by these same clonal isolates, were lysed within 48 h.

To determine whether a *ts* mutant of MVM(i) was involved in this persistent infection, $A9_{MVM(i)}$ cells from a late stage in the infection were incubated simultaneously at 33 and 37°C and assayed for virus production and for cell death over a period of 20 days. The results showed that similar amounts of virus were produced, that only about 5% of the cells at both temperatures were dead by trypan blue staining criterion, and that a temperature shift-down from 37 to 33°C did not result in lysis of the culture. Furthermore, if a viral *ts* mutant was present, it would be expected to interfere with the lytic infections at 37 and 39°C and to enhance the lysis of the culture at 33°C. To test this, six individual virus clones from a late stage in the infection, including *hr*301, were used to infect fresh A9 cells, and the infected cultures were incubated simultaneously at 33, 37, and 39°C. As a result, all three cultures were lysed completely within 48 to 72 h, producing similar yields of infectious progeny virus as determined by plaque assays of the cell lysates. These experiments indicate that an involvement of MVM *ts* mutants is highly unlikely in this persistent infection.

The possible role of interferon in this system was examined through the ability of the interferon-sensitive mengovirus to replicate in persistently infected cells. $A9_{MVM(i)}$ cells from 6, 36, and 72 days postinfection were superinfected with mengovirus at 0.1, 1, and 10 PFU per cell. Cured $A9_{MVM(i)}$ cells obtained at 6, 36, and 72 days of the persistent infection, as well as uninfected A9 cells, were also infected with mengovirus. All of the mengovirus-infected cultures developed cytopathic effects (CPE) and degenerated within 48 h. Interferon involvement in the MVM(i) persistent infection was therefore also ruled out.

Experimental strategy for the study of virus-cell coevolution. As mentioned earlier, infection of A9 cells with MVM(i) at 1 PFU per cell does not bring about an immediate destruction of the culture [as does MVM(p) infection of these cells], which continues to grow at a normal rate. The abrupt burst of virus production and our ability to isolate a host range mutant (*hr*301) from the progeny virus (18) suggested that similar host range variants of MVM(i), now

capable of lytic replication in A9 cells, had arisen in the culture. The eventual establishment of a persistent infection rather than a complete lysis of the culture also suggested that not all of the cells in the culture were sensitive to the new viral variant(s). These observations suggested that in the persistently infected culture, changes in the virus occurred concomitantly with the selection of A9 cells with new phenotypes. To examine this hypothesis, three stages in the persistent infection were chosen for scrutiny: early stage (10 days after infection, passage 1), middle stage (50 days after infection, passage 3), and late stage (72 days after infection, passage 9). From each of these stages, cured cultures were prepared by growing them in the presence of anti-MVM serum as described above. These stage-specific cultures were designated $A9_{Epi}$, $A9_{Mpi}$, and $A9_{Lpi}$, respectively. Progeny viruses from each of these three stages were also collected and designated pool E, pool M, and pool L, respectively.

The criteria chosen for detecting changes in the virus and in the cell phenotypes were (i) the ability of these virus stocks to establish productive infections in the stage-specific cell populations and (ii) the synthesis of virus-specific DNA and proteins. All the infections were done at high input multiplicities (i.e., 20 PFU per cell) to ensure synchronized events in virus replication.

Evidence for changes in the virus host range. A9 and EL4 cells were infected with MVM(i) and with each of the three virus pools. The infected cultures were examined daily for the appearance of CPE, and 5 days after the infection, virus production was measured by the hemagglutination assay. MVM(i) grew in EL4 but not in A9 cells, pool E virus was produced at similar efficiencies in both cell lines, and pools M and L grew well in A9 cells but lost their ability to replicate in EL4 cells (Fig. 1). These results show that gradual changes in the tissue tropism of the progeny virus took place. This conclusion is further supported by measurements of viral DNA synthesis in each of these infections. MVM(i) DNA was replicated in both A9 and EL4 cells (in agreement with previous results [18]), but pool M and L DNAs were amplified in A9 cells only (Fig. 2). Pool E DNA synthesis in both cell lines was poor, a result which is in apparent contradiction with the efficient virus production observed in these infections (Fig. 1). It is noteworthy, however, that a full correlation between DNA synthesis and virus production in these experiments cannot be drawn, because DNA synthesis was assayed at 24 h postinfection and virus production was determined at 5 days postinfection. This apparent discrepancy, therefore, may reflect kinetic differences in viral DNA synthesis.

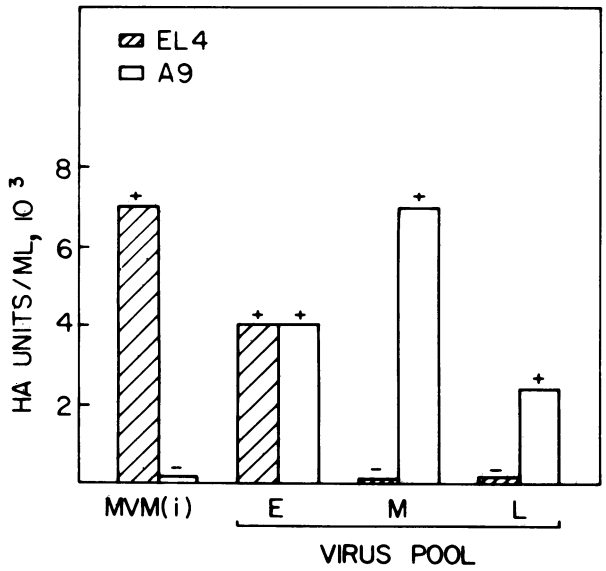

FIG. 1. Virus production in A9 and EL4 cells. A9 and EL4 cells (5×10^5 cells of each) were infected with MVM(i) and with virus pools E, M, and L at input multiplicities of 20 PFU per cell. The cells were incubated at 37°C and examined daily for the appearance of CPE. The cells were harvested 5 days postinfection, and viral particles were released and assayed by hemagglutination. Symbols indicate appearance (+) or absence (−) of CPE.

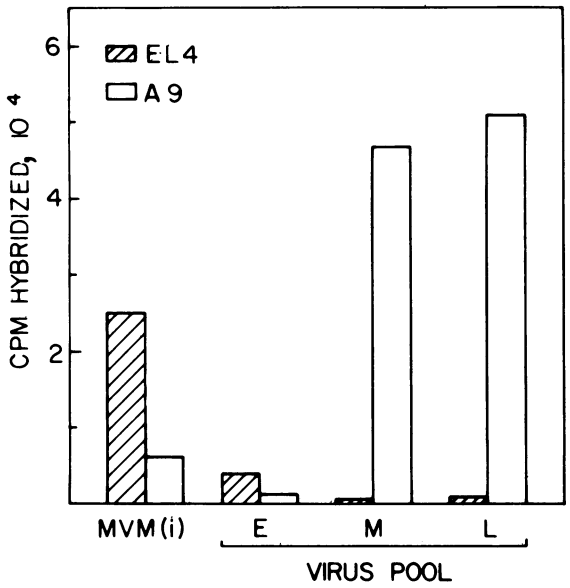

FIG. 2. Viral DNA synthesis in A9 and in EL4 cells. Cells were infected in suspension at input multiplicities of 20 PFU per cell. At 4 and 24 h postinfection, samples (8×10^5 cells each) were withdrawn and assayed for viral DNA content by the dispersed cell assay, and the filters were counted after hybridization. The values in the figure represent the hybridized counts per minute obtained at 24 h, from which the 4-h counts (input DNA) were subtracted.

The properties of the virus progeny produced during the course of the persistent infection were also examined by infecting the stage-specific cell cultures with each of the virus pools and measuring intracellular viral DNA synthesis. Identical numbers of cells were subjected to dispersed-cell assay at 4 and 24 h postinfection. Since viral DNA synthesis does not start before 6 h, the 4-h assay provided the relative amount of intracellular parental viral DNA, and the 24-h assay provided the relative amplification of viral DNA in these cells. Viral DNAs of pools M and L were amplified well in $A9_{Epi}$ and $A9_{Mpi}$ cells, but pool L DNA and not pool M DNA was replicated in $A9_{Lpi}$ cells (Fig. 3). The filters were also counted after autoradiography to determine the input viral DNA (i.e., counts per minute at 4 h postinfection) and the amplification ratio (i.e., counts per minute at 24 h/counts per minute at 4 h) in each individual infection. The counts of the 4-h filters revealed only small variations among the various infections (data not shown), indicating that in restrictive cells, the block to the replication of the viral DNA was not a consequence of poor penetration of parental input DNA into the cells. Figure 3 thus shows that subsequent to the alteration of the virus tissue tropism during the early

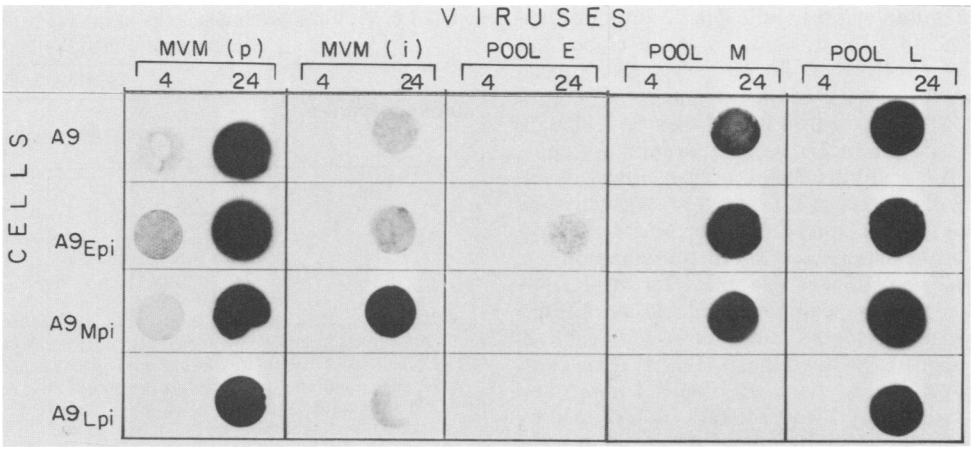

FIG. 3. Viral DNA synthesis in stage-specific $A9_{MVM(i)}$ cells. A9 cells and stage-specific cells were infected in suspension at an input multiplicity of 20 PFU per cell with each of the virus stocks indicated. Dispersed cell assay was performed with 8×10^5 cells at 4 and 24 h postinfection.

stages in the persistent infection, further changes in the virus took place. The virus population that emerged as a result of these changes (pool L) resembled MVM(p) in its ability to replicate its DNA in all three stage-specific cultures.

To corroborate these observations, infectious virus production in each of the infections was determined by plaque assay on A9 indicator cells. The results of these assays (Table 1) show that in $A9_{Lpi}$ cells, production of pool L virus was 100-fold better than that of pool M virus, and production of pool M virus was 10-fold better than that of pool E virus, a further indication that the virus progeny had evolved during the course of the persistent infection.

Evidence for phenotypic changes in the persistently infected culture. Examination of DNA synthesis and virus production after infection of stage-specific cell cultures with the three virus pools provided evidence that the cells, too, had evolved during the persistent infection; pool E viral DNA was amplified best in $A9_{Epi}$ cells and pool M DNA was amplified in $A9_{Epi}$ and $A9_{Mpi}$ cells but not in $A9_{Lpi}$ cells (Fig. 3). It seems, therefore, that each stage-specific virus pool was capable of replicating its DNA in cells derived from its own stage or from previous stages. The limited DNA replication of pool E and M viruses in $A9_{Lpi}$ cells further suggests that the persistently infected culture evolved by the selection of cells restrictive to productive infection by virus progeny from previous stages (i.e., resident virus). This selection, however, did not affect the replication of MVM(p) or of MVM(i) DNAs, which in all three stage-specific cultures were comparable to that measured in parental A9 cells. Exceptions to this were $A9_{Mpi}$ cells, which supported efficient MVM(i) DNA amplification. The nature of this transient elevated synthesis is not clear. Hence, although no changes in the cell phenotype could be seen by infecting cells with the parental viruses, such changes become apparent when stage-specific cultures are infected with stage-specific virus pools.

Measurements of infectious virus production in infected stage-specific cultures provided further support to the cell evolution hypothesis. Table 1 shows that the production of pool E virus was about 300-fold lower in $A9_{Lpi}$ cells than in $A9_{Mpi}$ cells and, similarly, production of pool M virus was 100-fold lower. Furthermore, although $A9_{Lpi}$ cells were efficient producers of pool L virus, no CPE or cell lysis was detected in these cells as late as 5 days postinfection. Instead, virus progeny was accumulated intracellularly and could be released by mechanical disruption of the cells. This phenomenon, a further manifestation of the changes that took place in the cells, is discussed further below.

Expression of viral functions in $A9_{Lpi}$ cells. The results presented so far are consistent with a process in which the persistently infected culture gradually became restrictive to productive infection by the resident virus. This can happen in one of two ways: by progressive reduction in the capacity of all the cells to produce virus or by the gradual selection of cells which are restrictive to virus replication. To study these possibilities, we isolated from $A9_{Lpi}$ cells individual clones of cells. The clones were passaged once and then examined for permissivity to infection by MVM(p), MVM(i),

TABLE 1. Virus production in stage-specific cells

Cell	Production of the following virus[a]					
	Pool E		Pool M		Pool L	
	CPE	PFU/ml	CPE	PFU/ml	CPE	PFU/ml
A9	+	1×10^6	+	2×10^6	+	NT
$A9_{Epi}$	+	1×10^6	+	1×10^6	+	1.5×10^6
$A9_{Mpi}$	+[b]	2×10^6	+	5×10^6	+	6×10^6
$A9_{Lpi}$	−	6×10^3	−	5×10^4	−	6×10^6

[a] +, Positive for CPE; −, negative for CPE; NT, not tested.
[b] Light CPE detected.

TABLE 2. Production of MVM(p) and *hr*301 in A9 and $A9_{Lpi}$ clones

Cells	Production of the following virus[a]					
	MVM(p)			*hr*301		
	CPE	HAU/ml	Total PFU	CPE	HAU/ml	Total PFU
A9	+	1,040	1×10^8	+	800	4×10^6
$A9_{Lpi}$ clone						
4	−	60	NT	−	2	1.8×10^4
5	−	32	NT	−	3	2.9×10^4
6	+	560	NT	−	1,600	5×10^6
7	−	720	NT	−	1,200	5×10^6
10	−	560	NT	−	1,200	5.1×10^6

[a] +, Positive for CPE; −, negative for CPE. NT, Not tested.

and the host range mutant, *hr*301. In Table 2, virus production and appearance of CPE in some of these clones are compared with those observed in A9 cells. The results revealed two types of clones: clones 4 and 5 were poor producers of both MVM(p) and *hr*301; clones 6, 7, and 10 were as efficient virus producers as the parental A9 cells. Like the parental $A9_{Lpi}$ culture, none of the clones developed CPE within 5 days after *hr*301 infection, and only one of the clones (clone 6) developed CPE after MVM(p) infection. None of the clones supported MVM(i) replication.

The $A9_{Lpi}$ clones were further examined for viral DNA synthesis, measured by the dispersed cell assay. Clones which were poor virus producers (clones 4 and 5) (Table 2) were also poorer in *hr*301 DNA synthesis (Fig. 4). However, differences in DNA synthesis between clones 4 and 5 and clones 6, 7, and 10 were only 5- to 10-fold (as determined by filter counting) compared with 200-fold differences in virus production between the two groups. Furthermore, MVM(p), too, is poorly produced in cells of clones 4 and 5, despite the fact that its DNA is synthesized in these cells as efficiently as in the fully permissive A9 cells. It follows, therefore, that in the persistently infected culture, cells were selected in which virus production is inhibited both at and after viral DNA synthesis.

Reconstruction of the persistent infection. As an approach to study the late stage in the persistent infection, $A9_{Lpi}$ and parental A9 cell cultures were infected with 1 PFU per cell of pool L virus, and the infected cultures were examined for virus-producing cells by infectious center assay and by immunofluorescence. The results (Table 3) show that although persistent infection was reestablished in the cured $A9_{Lpi}$ cells, the similarly infected parental A9 cells were lysed by the virus within 72 h. Although some clones of surviving A9 cells were usually visualized at 10 to 15 days postinfection, they did not produce virus, nor could we detect subviral components in these survivor cells. In the reconstructed persistent infection, about 20% of the cells in the culture were engaged in virus production after 7 days. Nevertheless, neither cell lysis nor CPE was detected, in agreement with results presented earlier (Table 2). Extensive cell lysis did occur, however, when the culture was passaged after trypsinization. Furthermore, infectious center assay and plaque assay of cell lysates both showed that, once released from the cells, this virus is infectious, indicating that the attenuated lysis of these cells did not result from interference with virus assembly. At 18 days postinfection, virus-producing cells constituted only 0.4% of the culture, but practically all the cells contained virus-specific antigens. The cytoplasmic localization of the fluorescence suggests that infectious viral particles were not assembled, although viral proteins were synthesized. The nature of this transient but extensive phenomenon is currently under investigation. As was the original persistent infection, the reconstructed persistent infection was gradually cured thereafter (Table 3): by 52 days postinfection (equivalent to 130 days postinfection in the original culture, little, if any, infectious virus was detected, and several passages later, the cells were free from detectable immunofluorescence as well.

It should be noted that the reconstruction of the persistent infection required mutant (pool L or *hr*301) virus. MVM(i), which can readily establish a persistent infection in A9 cells, was incapable of doing so in $A9_{Lpi}$ cells.

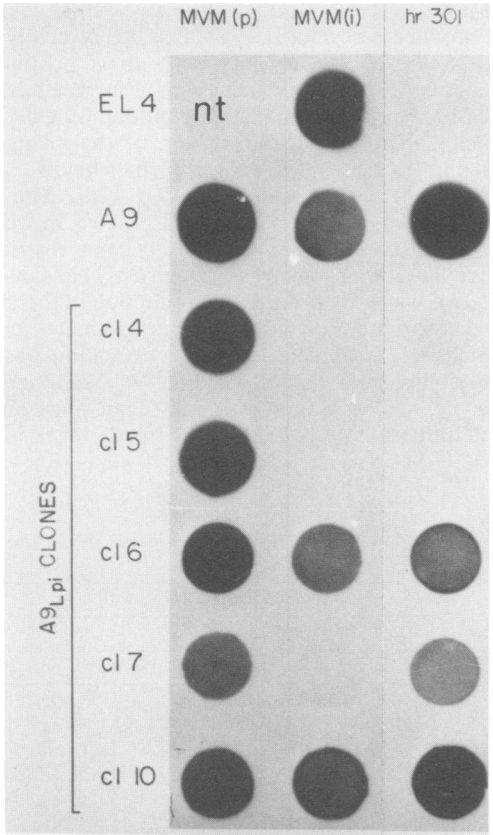

FIG. 4. Viral DNA replication in $A9_{Lpi}$ clones. Infections and assay were done as described in the legend to Fig. 3. The dispersed cell assay was performed at 24 h postinfection.

TABLE 3. Virus-producing cells in the reconstructed late stage of the persistent infection[a]

Days postinfection	% of $A9_{Lpi}$ cells with:		
	Infectious centers	Fluorescence in:	
		Cytoplasm	Nuclei
4	NT[b]	14	9
7	20	21	19
18	0.4	100	0.5
24	NT	50	0.3
52	0.01	21	0.65
59	NT	3	0.15

[a] A9 and $A9_{Lpi}$ cells were infected with pool L virus at input multiplicity of 1 PFU/cell. Less than 0.001% of the surviving A9 cells had infectious centers on all days tested (not tested 4 days postinfection).
[b] NT, Not tested.

DISCUSSION

The complexity of the virus-host relationship is only partially reflected in cell culture systems, since factors which play important roles in the maintenance of persistent infections in vivo, such as host response and cell differentiation, are missing from in vitro systems. Yet, we show in this work that selective pressures exerted on the virus and on the host cell in vitro are sufficient to bring about extensive changes in both virus and host. Our results suggest that these changes are the major contributors to the maintenance and possibly also to the establishment of the persistent infection of MVM(i) in A9 cells.

In the virus, two categories of changes were observed. Initially, a host range transition took place, as shown from

the ability of virus pools M and L to grow in A9 but not in EL4 cells. Pool E grew in both cell lines, probably indicating that the acquisition of the fibrotropic phenotype during the course of the persistent infection preceded the loss of the lymphotropic properties. Alternatively, pool E virus may contain a mixture of unaltered MVM(i), capable of growing in EL4 cells, and of fully mutated virus which replicates in A9 cells. We favor the first possibility for two reasons. First, we have recently isolated from *hr*301-infected EL4 cells a viral mutant, designated *hr*302, which can grow lytically in both A9 and EL4 cells (D. Ron, Y. Segev and J. Tal, unpublished data). Hence, besides viruses with altered host range, changes in the MVM(i) genome can give rise to viruses with extended host range. We speculate, therefore, that pool E comprised *hr*302-like mutants and that these extended host range viruses constitute an interim stage in the host range transition of MVM(i) from a lymphotropic to a fibrotropic phenotype. Secondly, the growth rate of pool E is peculiar: although it could grow well in some cell lines such as EL4, A9, and $A9_{Mpi}$ (Fig. 1; Table 1), the levels of viral DNA synthesis measured in these infections were very low (Fig. 2 and 3). Since DNA synthesis was measured at 24 h postinfection, whereas virus production was assayed at 5 days postinfection, this apparent discrepancy probably reflects a low rate of pool E DNA synthesis. This consistent finding seems to be an intrinsic property of pool E virus, suggesting that it constitutes a genetically uniform population of viruses as opposed to a mixture of parental and mutated ones.

After the host range transition of MVM(i), subtle changes took place which were detected only by growing these virus pools in stage-specific cells and not in the A9 and EL4 parents. For example, although all three virus pools grew equally well in parental A9 cells, pool L virus could replicate in $A9_{Lpi}$ cells and pool E and M viruses could not (Fig. 3; Table 1). Furthermore, of the three virus pools, only pool L viral DNA replication and virus production in stage-specific cells were quantitatively similar to those of MVM(p) in these cells. Thus, pool L virus is very similar in its host range properties to MVM(p) and to the clonal isolate *hr*301 (18).

The viral mutants which had arisen to a detectable level as early as 10 days after the infection were highly cytocidal to parental A9 cells (Fig. 1; Table 1). This raises the question, why was the culture not lysed completely at this stage? Our interpretation of this finding is that by the time this mutant virus population appeared in the culture medium, the cell culture had already undergone phenotypic changes. These changes resulted in the selection of a subpopulation of cells which were restrictive to the newly formed mutant virus, thus leading to the establishment of a persistent infection. Although such changes are presumably taking place in the culture all the time, they never lead to the establishment of a persistent infection when these cells are infected with MVM(p), *hr*301, or pool L virus (D. Ron and J. Tal, unpublished data). As the data in this paper show, further changes in the cells and in the virus then took place during the subsequent course of the persistent infection. Resident virus at any given stage in the infection could thus establish a productive infection in subpopulations of the culture that survived infection at a previous stage. The changes observed in the cells were manifested by the selection of cells which were restrictive to the resident virus as well as to virus from previous stages. This is shown by the restricted replication of virus pools E and M but not pool L in $A9_{Lpi}$ cells. We have also shown that the reconstruction of the persistent infection requires both mutant cells ($A9_{Lpi}$) and mutant virus (pool L) and that persistent infections are not obtained by infecting mutant cells with MVM(i) or by infecting parental A9 cells with mutant virus. Taken together, these results provide evidence that changes in both the virus and in the cells are required for the maintenance of this persistent infection. Our findings that viral *ts* mutants and DI particles, as well as interferon, do not play a major role in this carrier state are in agreement with this proposed mechanism.

The nature of the phenotypic changes, which took place in the cells, is not clear. One possibility is that the mutations in the resistant cells affected the cell-coded helper function(s) necessary for MVM replication (25). Another possibility is that MVM, like the defective parvovirus adeno-associated virus, can integrate its DNA into the genome of the host cells and thereby affect their susceptibility to viral infection. This possibility was examined by hybridizing nick-translated viral DNA probe to dot blots of high-molecular-weight genomic DNA extracted from $A9_{Epi}$, $A9_{Mpi}$, $A9_{Lpi}$, and A9(s) cell clones. The results did not reveal viral sequences in these cell DNAs to a level of less than 0.1 copy per genome (D. Ron and J. Tal, unpublished data). Furthermore, since their establishment, the cured cells were maintained in culture for over 15 passages with no apparent recurrence of virus or subviral components, thus arguing against integration.

Virus evolution during persistent infections is a widely reported phenomenon, but the infecting viruses are usually selected for avirulence, thus allowing them to coexist with the culture without causing its total destruction. In contrast, $MVM(i)_{A9}$ (represented in this work by the virus pools E, M, and L), consists of an altogether different type of mutant virus whose virulence to the parental A9 cells increases rather than decreases during the course of the persistent infection.

In most studies of persistent infections, attention was primarily focused on changes in the virus. The evolution of conditional or deletion mutants was generally considered to be the major contributor to the establishment and maintenance of these carrier states (6, 30), and although cell mutants were isolated from various persistently infected cultures (15, 22, 28), they were not shown to be relevant to the maintenance of these persistent infections. In recent years, it has become increasingly clear that the host cells, too, play important roles in persistent infections by various viruses. In one recent work, Ahmed et al. (1) showed that in L-cells persistently infected with reovirus, both mutant cells and mutant virus arose. The mutant virus, which readily lysed parental L-cells, was only capable of establishing a persistent infection in mutant cells. Thus, the role of the host cells in the maintenance of persistent infections may prove to be greater than is presently known.

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