Measles virus from a long-term persistently infected human T lymphoblastoid cell line, in contrast to the cytocidal parental virus, establishes an immediate persistence in the original cell line

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To investigate the mechanisms of measles virus (MV) establishment and maintenance of persistence in lymphoid cells, we have established a long-term persistent infection with MV, Edmonston strain, in the human T lymphoblastoid cell line MOLT4, which has been in continuous culture for over 8 years. In this culture, designated MOMP1, more than 98 % of cells display viral antigens. The MOMP1 culture is immune to superinfection with MV and is not cured by anti-MV antibodies. No evidence of defective interfering particles was obtained. The persistently infected culture releases an infectious virus showing a miniplaque and thermoresistant modified phenotype that, unlike the parental virus Edmonston strain which produces a lytic infection with extensive cell fusion, establishes an immediate persistence in MOLT4 cells with neither significant loss of cell viability nor cell fusion. This suggests that the modification in the virus suffices to maintain the state of persistence without requiring a coevolution of the host cell during the infection, as has been reported in other persistent virus infections.

Introduction

Measles virus (MV) is capable occasionally of producing persistent infection (p.i.) in humans, most notably subacute sclerosing panencephalitis (SSPE), a fatal disease. Although the causal effect of MV in this progressive disease is well established, the mechanisms of virus persistence in the host remain largely unknown. In particular, it is not known where the virus is harboured nor how it reaches the central nervous system (Norrby & Oxman, 1990; ter Meulen et al., 1983).

In vitro culture infection has demonstrated that MV can infect human lymphocytes and lymphoblastoid cell lines, producing p.i.s. in some of them (Joseph et al., 1975; Barry et al., 1976). Virus isolation has indicated that MV infects human lymphocytes in vitro and in some cases can be isolated from SSPE patient lymph nodes (Gresser & Chany, 1963; Horta-Barbosa et al., 1971). More recently there have been reports of the presence of MV RNA in peripheral blood mononuclear cells of SSPE patients (Fournier et al., 1985; R. Fernandez-Muñoz & M. L. Celma, unpublished results). Cell culture experiments with MV and other viruses suggest that virus variants, such as defective interfering (DI) particles (Roux & Holland, 1979; Holland et al., 1980) and temperature-sensitive mutants (Youngner & Preble, 1980), could produce the establishment and maintenance of p.i. On the other hand, host cell factors may also determine the establishment of persistence and there are indications in certain systems that the appearance and selection of cell variants during infection (cell coevolution) might play an essential role in the maintenance of p.i.s. (Ahmed et al., 1981; Ron & Tal, 1985; de la Torre et al., 1988). To study possible virus and host cell factors involved in the establishment and maintenance of MV persistence in lymphocytes, we have established a long-term steady-state MV p.i. in the human T cell lymphoblastoid cell line MOLT4. The virus released by this culture, in the absence of detectable DI particles, is itself able to establish an immediate p.i. in MOLT4 cells, suggesting that the viral genome determines the persistence with no requirement for host cell coevolution.

Methods

Cells. The human T lymphoblastoid cell line MOLT4, originally obtained from a patient with acute lymphocytic leukaemia (Minowada et al., 1972) was kindly supplied by Dr George Klein of the Karolinska Institute, Stockholm, Sweden. Cells were grown in RPMI 1640 supplemented with 2 mM-glutamine, 8% heat-inactivated (56 °C for 30 min) foetal calf serum (FCS), gentamicin, penicillin and 007% NaHCO3. Monolayer cultures of Vero cells from green monkey kidney, obtained from the ATCC, were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% FCS, glutamine and
antibiotics. All cell cultures were incubated in a humidified atmosphere of 5% CO₂ in air at 37 °C, unless otherwise stated. Twice a week, lymphocyte cell cultures were diluted with fresh medium to 2 × 10⁴ to 3 × 10⁵ cells/ml.

Viruses. The Edmonston strain of MV, kindly supplied by Dr P. Choppin, was plaque-purified twice on Vero cells. Stock virus was produced by growth in these cells at an m.o.i. of 0-01 to 0-05 p.f.u./cell. Infected cultures were collected when most of the monolayer showed c.p.e.; cells were disrupted by repeated cycles of freezing and thawing or ultrasoundization, and cell extracts were clarified and frozen at −80 °C. Vesicular stomatitis virus (VSV), strain Indiana, from the ATCC was grown in BHK cells.

Antisera. Anti-MV hyperimmune serum was obtained by intramuscular and subcutaneous immunization of rabbits with MV grown on Vero cells and purified in sucrose gradients. The neutralization titre of these sera was assayed by plaque reduction on Vero cells.

Virus titration. Infectious MV was determined by plaque assay on confluent Vero cells. Briefly, 10-fold dilutions were adsorbed onto the cells for 90 min at 21 °C, 5 ml of 1% (w/v) Noble agar in DMEM containing 2% FCS was added to 60 mm Petri dishes and the dishes were incubated at 37 °C for 4 days; 3 ml 0-01% neutral red in DMEM containing 1% Noble agar was added and the plaques were counted after 24 h.

Infection of lymphoid cells. Cells were sedimented by centrifugation at 200 g for 7 min and the supernatant was carefully aspirated. Virus dilutions, giving an m.o.i. of 1 p.f.u./cell in 2 ml of medium, unless otherwise stated, were added to the pellet, and were allowed to adsorb at 37 °C for 90 min. Cells were washed twice in medium, adjusted to a concentration of 1 × 10⁴ cells/ml and held at 37 °C in a humidified 5% CO₂ atmosphere. Samples of cells and supernatants were removed at the times indicated for viable cell counting (as determined by trypan blue exclusion), virus titration and visualization of viral antigens. Percentage survival was calculated by dividing the viable cell count of the infected culture by the viable cell count of a mock-infected culture; cell counts were performed at equivalent times post-infection.

Immunofluorescent staining. Cells were deposited on glass slides, dried at room temperature, fixed in acetone at −20 °C for 10 min and stained by an indirect immunofluorescence assay using anti-MV hyperimmune rabbit or guinea-pig sera and, in some experiments, human convalescent sera of post-measles encephalitis patients. Specific fluorescein isothiocyanate-conjugated rabbit immunoglobulins from Meloy or Tago were used. Uninfected cells were included as controls.

Electron microscopy of cell sections. Cells were pelleted and fixed with 5% glutaraldehyde and 1% cacodylate buffer pH 7-3 for 2 h at 4 °C. The pellet was post-fixed in 1% osmium tetroxide in cacodylate and embedded in epoxy resin. Sections were cut with an ultramicrotome, stained with 2% uranyl acetate and lead citrate, and examined with a Philips TE400 electron microscope.

Haemagglutination and haemadsorption assays. In both cases, fresh red blood cells from Macacus rhesus were used. For haemagglutination assays, 25 µl of twofold dilutions of virus samples in PBS were incubated with 25 µl of 1% (w/v) suspension of red blood cells at 37 °C for 60 min in polystyrene microtitre plates; calibrated controls were included. In haemadsorption assays, one volume of a suspension of 10⁶ cells/ml plus one volume of 1% (w/v) suspension of red blood cells were incubated at 37 °C for 30 min and haemadsorbing cells were counted in a haemocytometer.

Interferon (IFN) assay. α-IFN and γ-IFN levels were determined by radioimmunoassays (Danabott and Boots Celltech) of the culture medium of 5 × 10⁶ cells 1 day after medium change. Antiviral activity was determined by a VSV plaque reduction test using Madin-Darby bovine kidney cells and National Institute of Health standard Ga 23-901-532.

Purification of viral nucleocapsid RNA. We followed the procedure described by Leppert et al. (1979). Cells were radiolabelled when indicated with [3H]uridine in the presence of 4 µg/ml actinomycin D (Calbiochem) and disrupted with a buffer containing 0-15 M-NaCl, 50 mM-Tris-HC1 pH 7-8 and 0-6% Triton X-100 (Sigma). The cytoplasm was adjusted to 6 mM-EDTA, 0-1% sodium deoxycholate, layered over a composite gradient of 20 to 40% (w/v) CsCl in 20 mM- Tris-HC1 pH 7-8, 4 mM-EDTA and 5% (w/v) sucrose in 20 mM-Tris-HC1 pH 7-8, 4 mM-EDTA, 100 mM-NaCl and then subjected to centrifugation in an SW41 rotor (Beckman) for 17 h at 35000 r.p.m. at 10 °C. The nucleocapsid band (density, 1-3 g/ml) was collected, dialysed against 25 mM-Tris-HC1 pH 7-8, 50 mM-NaCl, 1 mM-EDTA and adjusted to 0-2% SDS. The proteins were digested with 200 µg/ml proteinase K (Merck) and RNA was isolated after phenol-chloroform extraction and ethanol precipitation. The 52S viral nucleocapsid RNA was isolated by sucrose gradient centrifugation in a linear 15 to 30% sucrose–NETS (100 mM-NaCl, 2 mM-EDTA, 10 mM-Tris-HC1 pH 7-8, 0-2% SDS) gradient in an SW28 rotor (Beckman) at 17000 r.p.m. at 25 °C for 17 h. RNase T1 fingerprinting was performed according to Celma et al. (1977).

Formaldehyde–agarose gel electrophoresis. Tritiated RNA (up to 20 µg/cm slot) was dissolved in distilled H₂O and denatured for 15 min at 55 °C in the presence of 50% deionized formamide (Eastman), and 6.5% formaldehyde (Mallincrodt) in electrophoresis buffer (20 mM-MOPS pH 7-0, 5 mM-sodium acetate, 0-1 mM-EDTA pH 8-0). After addition of 10% loading buffer (50% glycerol, 1 mM-EDTA, 0-4% xylene cyanol), the RNA was fractionated by horizontal 1-2% gel electrophoresis. After 3H enhancement with New England Nuclear ENHANCE, the gel was dried at 60 °C and autoradiographed with an intensifying screen at −70 °C.

Results

Establishment and characteristics of the long-term p.i.

MOLT4 cells are susceptible to MV, producing a lytic infection with extensive cell fusion. Oldstone and co-workers (Joseph et al., 1975) rescued infected cell survivors after several weeks of infection by repeated frequent feeding; in contrast, Albrecht and colleagues (Barry et al., 1976) were unable to rescue any cell survivors by this technique. We have succeeded in establishing a long-term p.i., designated MOMP1, with the MV Edmonston strain in MOLT4 cells. MOLT4 cells were infected at an m.o.i. of 0-5 to 1 p.f.u./ml as described in Methods and the infected cells were left undisturbed for 6 days. On day 6, there were abundant polykaryons and more than 80% of cells were dead; the culture was centrifuged to separate dead cells, and the live cells were washed and cultured in fresh medium. The infected culture was split into several subcultures (MOMP1, MOMP2, etc.). During the following 6 weeks,
cells were centrifuged every 4 to 5 days. After 2 months, the cell culture stabilized, although it suffered several crises in which it was necessary to eliminate dead cells. Finally, after 6 months of continuous culture, viability was stabilized.

The MOMP1 cell line has been growing in continuous culture for over 8 years, with a growth rate approximately 80% of that of the parental MOLT4; MOMP1 duplication time in exponential growth is 36 h at 37 °C. MOMP1 cells show the same morphology as MOLT4 and less than 1% of the cells form syncytia. Most, if not all, MOMP1 cells contain both MV antigens, as determined by indirect immunofluorescence assays with polyclonal anti-MV sera (Fig. 1), and MV RNA, as determined by in situ hybridization assays with 3S-labelled riboprobes for the MV N gene (results not shown). About 70% of the cells express haemagglutinin on their surface, as measured by M. rhesus red blood cell haemadsorption assays. By electron microscopy of cell sections, MOMP1 cells contained aggregated viral nucleocapsids in their cytoplasm (Fig. 2). In contrast to MOLT4, when MOMP1 cells are superinfected with the
Table 1. Characteristics of MOMP1 persistently infected culture

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tr>
<td>Time in continuous culture</td>
<td>8 years</td>
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<tr>
<td>Growth rate</td>
<td>36 h</td>
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<tr>
<td>Cells with viral antigens</td>
<td>&gt;98%</td>
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<tr>
<td>Effect of anti-MV serum treatment</td>
<td>Not cured</td>
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<tr>
<td>Superinfection with MV</td>
<td>Immune</td>
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<tr>
<td>Superinfection with VSV</td>
<td>Cell lysis</td>
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<tr>
<td>IFN production</td>
<td>&lt; 20 IU of IFN/ml</td>
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<tr>
<td>Virus yield per day in supernatants titrated at 33 °C</td>
<td>0.15 p.f.u./cell</td>
</tr>
<tr>
<td>Virus yield per day in supernatants titrated at 37 °C</td>
<td>0.15 p.f.u./cell</td>
</tr>
<tr>
<td>Virus yield per day in supernatants titrated at 39 °C*</td>
<td>0.03 p.f.u./cell</td>
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<tr>
<td>Properties of released virus</td>
<td></td>
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<tr>
<td>Plaque morphology</td>
<td>Small</td>
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<tr>
<td>Thermalability†</td>
<td>125-fold more stable</td>
</tr>
<tr>
<td>Infectivity‡</td>
<td>20-fold less infectious</td>
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* For MOMP1V the PE ratio was 0.2 and for Edmonston strain was 0.5 (PE ratio: titre at 39 °C/titre at 33 °C).
† Titre MOMP1V/titre Edmonston strain on Vero cells after 120 min incubation at 45 °C.
‡ For MOMP1V the ratio p.f.u./haemagglutinin units was 8 × 10³ and for Edmonston strain this ratio was 1.6 × 10⁵.

original MV Edmonston, no c.p.e. (cell fusion or cell killing) is observed (Fig. 3); this also indicates that in the persistent culture all cells are infected. However, MOMP1 cells and MOLT4 cells are equally susceptible to the lethal effect of VSV (Fig. 3).

To find whether IFN is involved in the maintenance of MV persistence in MOMP1 cells, we measured the IFN activity released into the medium by MOMP1 and MOLT4 cells by the plaque reduction assay, and we found similar low levels, less than 7 international units (IU)/ml in each case. By specific IFN radioimmunoassays both types of cells produced 10 to 15 IU/ml of α-IFN and less than 2 IU/ml of γ-IFN. Moreover, the fact that MOMP1 cells remain susceptible to VSV indicates that there is no significant increase in IFN production, since VSV is highly sensitive to its effect (Table 1). Treatment of MOLT4 cells with anti-human α-IFN, anti-human β-IFN and anti-human γ-IFN during Edmonston and MOMP1 virus infection or treatment of MOMP1 cells produced no apparent changes in viral cytopathology (results not shown). MOMP1 cells produced infectious extracellular MV, approximately 0.6 p.f.u./cell/day, a yield about 100-fold less than that produced in the lytic infection of MOLT4 cells at the point of maximal virus production.

Effect of anti-MV serum on the persistently infected culture MOMP1

MOMP1 cells were treated with heat-inactivated rabbit anti-MV hyperimmune sera. Polyclonal rabbit anti-MV serum was added to a culture of MOMP1 cells at a concentration (10%, v/v) sufficient to neutralize 10-fold more infectious virus than that released by MOMP1 cells; in parallel, another culture was adjusted to 10% (v/v) with preimmune serum. After 4 weeks of treatment, when anti-MV antiserum was removed, most, if not all, MOMP1 cells showed MV antigens by immunofluorescence and most expressed surface viral haemagglutinin protein. Virus production was increased and 50-fold higher titres were observed. Thus, anti-MV antibody treatment did not cure the MOMP1 culture, confirming that this is a steady-state persistent culture and not a carrier culture.

Absence of detectable DI particles in the MOMP1 culture

An MV strain prepared under conditions that minimized the possibility of generation or amplification of DI particles was used. Thus, MOLT4 cells were infected with Edmonston virus stock prepared by low multiplicity infection of Vero cells using a three times plaque-purified virus. However, it is still possible that DI particles were generated during the continuous culture of MOMP1. To
Establishment of immediate persistence by MV

To test this possibility, MV RNA was labelled in vivo with [3H]uridine; nucleocapsids were purified on CsCl equilibrium gradients (Leppert et al., 1979) and analysed by sucrose gradient centrifugation. Fig. 4 shows that most, if not all, nucleocapsid RNA larger than 4S sedimented as a 52S peak. When this RNA was subjected to denaturing gel electrophoresis only one major band, migrating as expected for genomic RNA, was detected; occasionally in some of the experiments (for example Fig. 4c) a faint band migrating between the 28S and 18S markers was observed after overexposure of the film. Although we can not exclude the presence of subgenomic RNA species, since a low proportion of short viral RNAs of less than 1000 nucleotides might have escaped detection, no evidence of regular production of subgenomic RNA species as described in other cultures (Rima et al., 1977; Calain & Roux, 1988; Enami et al., 1989) was obtained in MOMP1 cells. An RNase T1 fingerprint of the in vivo [32P]-labelled major sucrose band RNA showed little sequence variation between 52S RNA from lytic and persistent infections (results not shown), thus excluding the possibility of large deletions and repetition of sequences in MOMP1 viral RNA.

Simultaneous infections with both Edmonston and MOMP1 MV (MOMP1V) at equal m.o.i. did not indicate interference by the latter in the yield of Edmonston large plaque extracellular virus: 2 x 10⁷ p.f.u./ml compared to 3 x 10⁷ p.f.u./ml in mixed infection. Cell killing by Edmonston MV after 3 days of mixed infection was 90% of the Edmonston control infection (Fig. 5). The residual surviving cells are most likely those which were infected only by MOMP1V, and thereupon became resistant to Edmonston virus superinfection. These results suggest that MOMP1V establishment of persistence is not due to the presence of DI particles.

**Characteristics of the virus released by the persistently infected MOMP1 culture**

The virus produced by MOMP1 cells (MOMP1V) has some phenotypic properties different from those of the original virus, Edmonston strain. When we plaque-assayed MOMP1V in parallel with the Edmonston strain of MV at 37 °C and 39 °C, we observed slightly increased sensitivity of MOMP1V to higher temperatures (Table 1). Plaque-assaying MOMP1V at 33 °C and 37 °C did not indicate cold sensitivity (Table 1). Even when plaque-isolated clones of virus from MOMP1 cells were plaque-assayed at these three temperatures, no significant differences were found with respect to the whole population (results not shown).

The number of virus particles produced by MOMP1 was approximately fivefold less than that produced by lytically infected MOLT4 cells, as determined by haemagglutinin activity in cell culture supernatants and [35S]methionine-labelled virion proteins immunoprecipitated by anti-MV antibodies. The average amount of infectious virus produced 5 h after medium replacement by lytically infected MOLT4 cells 48 h post-infection was approximately 2 p.f.u./cell, whereas MOMP1 cells yielded 0.02 p.f.u./cell, as determined by plaque-assaying cell culture supernatants 5 h after medium replacement. Thus, the infectivity of MOMP1V particles (p.f.u./viral proteins) was about 20-fold less than that of virus released during lytic infection. The number of virus particles and infective particles per cell did not change significantly during these years of continuous culture,
once the culture had stabilized after 6 months of infection.

Some structural component of the MOMP1 virion
must be altered with respect to lytic infection, since it
showed a significantly higher thermostability (Fig. 6 and
Table 1). MOMP1V when infecting Vero cells showed a
slower and less efficient cell fusion effect than did the
Edmonston MV strain. Plaque morphology of the
MOMP1V variant on Vero cells was also altered,
showing a significantly smaller size than the lytic virus
produced in MOLT4 cells (Fig. 7).

Immediate establishment of a p.i. in MOLT4 cells with
MV released by MOMP1 culture

Infection of MOLT4 cells with the Edmonston strain of
MV grown in either simian Vero cells or human MOLT4
cells caused lytic infection with extensive cell fusion and
cell killing (Fig. 8b and 9b), with more than 90% of cells
dying 1 week after infection. In contrast, when MOLT4
cells were infected at the same m.o.i. (1 p.f.u./cell) with
MOMP1V, neither cell fusion nor cell destruction was
observed (Fig. 8b and 9c). MOMP1V-infected MOLT4 cells did not reach MOLT4 cell counts, owing to slower cell growth but not to cell killing. Virtually all MOLT4 cells did not show any decrease in Edmonston virus production similar to that of the persistently infected culture MOMP1 (Fig. 8a). The latter culture remained stable for over 1 year with no growth crises. The same immediate p.i.s. were obtained after plaque purification of MOMP1V and infection at an m.o.i. of 0.01 p.f.u./cell.

**Discussion**

Although it is known that MV infects and can persist in human lymphoid cells, the mechanisms responsible for the establishment and maintenance of the persistence remain largely unknown. A number of possible mechanisms have been proposed for such viral infections; some refer to virus mutants (variants) produced during the infection, such as temperature-sensitive mutants (Ju et al., 1978; Youngher & Preble, 1980) or cold-sensitive mutants, as described for an SSPE-isolated MV (McKimm-Brechkin et al., 1982) and a persistently MV-infected mouse neuroblastoma (Rager-Zisman et al., 1984). Interference by defective virus has been described as one mechanism in the establishment and maintenance of persistent paramyxovirus infections (Roux & Holland, 1979; Rima et al., 1977).

The properties of the host cell may also be relevant to the outcome of the infection. Ahmed et al. (1981) have described an in vitro p.i. with reovirus 3, in which parallel evolution of virus and infected cells, so-called cell coevolution, seems to be required for the maintenance of viral persistence. Other authors such as Ron & Tal (1985), using murine L cells infected with the parvovirus lymphotropic minute virus of mice, and more recently, de la Torre et al. (1988), with the picornavirus foot-and-mouth disease virus in BHK cell cultures, have reported experiments suggesting that simultaneous virus and cell evolution during infection may be a more general requirement for maintenance of persistence.

The MOMP1 culture described here is a true steady-state p.i. in which all cells are infected, rather than a carrier culture in which released virus continuously infects virus-free cells, since (i) in the MOMP1 culture, most, if not all, cells carry MV antigens and RNA, (ii) the MOMP1 culture shows complete immunity to superinfection with MV and (iii) there is no curing by anti-MV neutralizing rabbit antiserum.

This steady-state equilibrium between cell and virus has proven stable over the past 8 years of continuous culture, with no growth crises or virus cytopathic reactivations. This remarkable stability makes this culture useful for the assay of possible factors inducing reactivation of MV from p.i. of lymphoid cells. As the MOMP1 culture is not cured by anti-MV serum, it is possible to study MV persistence in this lymphoblastoid cell line in the presence of hyperimmune sera. Modulation of viral gene expression by anti-MV antibodies, first described by Fujinami & Oldstone (1980) in acutely infected cells, is currently under study in our laboratory in the persistently infected MOMP1 culture.

Edmonston and MOMP1V mixed viral infections of MOLT4 cells did not show any decrease in Edmonston virus production, indicating that no interfering particles were present in MOMP1V preparations. On the other hand, we have found no evidence of MV subgenomic RNAs as described in other MV infections (Rima et al., 1977; Calain & Roux, 1988; Enami et al., 1989), although we cannot completely exclude a low proportion of non-interfering small subgenomic RNAs.

The MV mutant MOMP1 shows some phenotypic changes with respect to the parental Edmonston strain. Thus, it has a miniplaque morphology on Vero cell monolayers and shows increased thermostability, as has been described for other MV infections (Ju et al., 1978). It shows no apparent cold sensitivity and only moderate thermostability. IFN has been implicated in the establishment and maintenance of MV persistence in lymphocytes (Jacobson & McFarland, 1982), but measurements of released IFN and IFN neutralization revealed no apparent role for these molecules in the maintenance of MV persistence in MOMP1 cells.

The most relevant property of MOMP1V is, however, that in contrast to the parental Edmonston strain, it does not fuse or kill MOLT4 cells but establishes immediate persistence. Thus the virus itself carries the genetic information sufficient to establish and maintain the persistent state of infection. This situation is in contrast with other p.i.s., in which the virus produced does establish a p.i. in cured persistently infected cells, but not in the original cell line (Ahmed et al., 1981; Ron & Tal, 1985; de la Torre et al., 1988). Our results suggest that cell coevolution is not a general requirement for maintenance of long-term persistent virus infections.

MOMP1V is less pathogenic and establishes rapid persistence in a number of human lymphoblastoid cell...
lines (R. Fernandez-Muñoz & M. L. Celma, unpublished results). This property of MOMP1 may be helpful in obtaining p.i.s of these cells to study such virus–lymph cell interactions as lymphokine or immunoglobulin production. Comparing the structure of Edmonston virus and the non-fusogenic MOMP1V would help to characterize the viral components responsible for cell killing and cell fusion.

We believe that the possibility of reproducing the establishment of immediate persistence with MOMP1V, in parallel with a lytic infection with the parental MV Edmonston in MOLT4 cells, will be helpful in the study of early infection events, when the establishment of persistence is probably determined.

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