Key words: measles virus/nucleoprotein/nucleus/mutant

Nuclear Accumulation of Measles Virus Nucleoprotein Associated with a Temperature-sensitive Mutant

By LINDA W-L. CHUI, RAIJA VAINIONPÄÄ, R. MARUSYK,* A. SALMI AND E. NORRBY

Viral Pathogenesis Research Unit, Department of Medical Microbiology and Infectious Diseases, Faculty of Medicine, University of Alberta, Edmonton, Alberta T6G 2H7, Canada and

Department of Virology, Faculty of Medicine, Karolinska Institute, Stockholm, Sweden

(Accepted 6 June 1986)

SUMMARY

A measles virus (MV) Lec strain conditional-lethal (temperature-sensitive) mutant, designated MV ts38, has been isolated from 5-fluorouracil-mutagenized stock. The mutant has been characterized with regard to growth characteristics at 32 °C (permissive temperature) and 39 °C (non-permissive temperature). Virus-specific RNA transcription and/or translation appeared to be blocked at the non-permissive temperature as no virus-specific products could be detected by biochemical or immunological procedures. Following initiation of viral replication at 32 °C, with subsequent shift-up to 39 °C, presynthesized nucleoprotein (NP) was transported to, and accumulated in the cell nucleus whereas other viral proteins could not be detected there. A corresponding accumulation of NP in the nucleus is most often seen in association with MV neurotropic subacute sclerosing panencephalitis isolates in vivo and in vitro.

INTRODUCTION

Measles virus (MV), a member of the genus Morbillivirus of the family Paramyxoviridae (Kingsbury et al., 1978), is a negative-sense, single-stranded RNA virus (M, 4.5 × 10⁶ to 5.2 × 10⁶; Baczko et al., 1983; Tucker et al., 1984) with seven structural proteins, including cellular actin (Tyrrell & Norrby, 1978). In addition to the structural proteins, a non-structural protein C (M, 18000 to 20000) has been detected in infected cells (Bellini et al., 1985).

In cell culture, MV generally causes a lytic infection with giant cell (syncytium) formation leading to cell death. However, MV is also known to cause persistent infections both in vivo and in vitro. MV infection usually results in an acute illness but with non-immunized children who contract the disease in early childhood, there is a 1:100000 incidence of subacute sclerosing panencephalitis (SSPE), a rare, degenerative disease of the central nervous system. MV antigen has been detected in SSPE brain by immunological techniques (Oyanagi et al., 1970; Haase et al., 1985; Norrby et al., 1985). In ultrastructural studies of SSPE brain sections (Oyanagi et al., 1970; Dubois-Dalcq et al., 1974; Iwasaki & Koprowski, 1974) or co-cultivated SSPE brain cells (Oyanagi et al., 1971), nuclear accumulations of smooth filaments have been identified. The smooth filaments have been identified as MV nucleocapsid. The mechanism, and significance, of nuclear MV nucleocapsid accumulation is not understood, but the phenomenon is associated with MV persistent infection both in vivo and in vitro (Minagawa et al., 1976; Fournier et al., 1981; Robbins, 1983; Uchiyama et al., 1985).

We report here the isolation of an MV conditional-lethal (temperature-sensitive) mutant and an analysis of the mutant's replication at permissive (32 °C) and non-permissive (39 °C) temperatures. At the restrictive temperature, the mutant displayed properties which resemble those of SSPE isolates previously described in the literature.
METHODS

Cells. Vero cells, obtained from the American Type Culture Collection, were grown in glass Blake bottles or plastic culture flasks in Eagle's MEM (Auto-Pow MEM, Flow Laboratories) supplemented with 2% calf serum, glutamine and antibiotics.

Virus. The Lec SSPE strain (originally obtained from Dr H. Koprowski, Wistar Institute, Philadelphia, Pa., U.S.A.) was twice plaque-purified and stock virus was grown to a titre of 107 p.f.u./ml in Vero cells. Virus stocks were stored at -70 °C.

Virus assay. Virus was assayed by a standard plaque assay method on Vero cell monolayers grown in 60 mm diam. plastic Petri dishes. As required for experimental purposes, virus-infected cells were incubated at 32 or 39 °C in a 5% CO2 atmosphere. After 10 days of infection plaques were visualized by staining of 20% acetic acid-fixed monolayers with a crystal violet solution.

Virus mutagenesis. Vero cells were infected with plaque-purified Lec virus at a multiplicity of 0-1 p.f.u./cell. After 1 h adsorption, MEM containing 200 μg/ml 5-fluorouracil was added. The cells were incubated at 32 °C for 24 h and the medium replaced with fresh MEM lacking 5-fluorouracil. Following incubation for a further 48 to 72 h, virus was harvested and assayed for infectivity as described above.

Isolation of mutants. Following mutagenesis and plaque assay at 32 °C, visible plaques were selected and resuspended in 0-5 ml serum-free culture medium. Cells were infected with 0-1 ml of each plaque suspension and incubated at 32 °C and 39 °C. Those plaque isolates which produced c.p.e. at 32 °C and to a lesser extent at 39 °C were then plaque-assayed at the two temperatures and the efficiency of plaquing determined. The isolates with a plaquing efficiency (plaque titre at 32 °C/plaque titre at 39 °C) of greater than 102 were retained for further study.

Immunoprecipitation. Monoclonal antibodies against the MV structural components haemagglutinin (H), phosphoprotein (P), nucleoprotein (NP), fusion protein (F) and matrix/membrane protein (M) were prepared as previously described (Norrby et al., 1982). Antiserum against MV was prepared by intramuscular injection of purified MV in Freund's complete adjuvant (1:1). A 1 ml intravenous booster was administered 5 weeks later and the animal exsanguinated 10 days following the final injection.

Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse or anti-rabbit antibodies (Nordic Laboratories, Tilburg, The Netherlands) were used at a dilution of 1:100.

For immunofluorescence, Vero cells were grown and infected on multi-well slides (Flow Laboratories). At appropriate intervals, infected cells were fixed in cold acetone and incubated with antisera for 2 h at room temperature and stained with FITC conjugates for 30 min at 37 °C in a humidity chamber. All slides were examined with a Leitz Dialux 22 incident light fluorescence microscope.

Immunoprecipitation. The synthesis of MV-specific proteins in Vero cells was analysed by radiolabelling the cells at 48 h post-infection with 40 μCi/ml [35S]methionine (sp. act. 1120 Ci/mmol, New England Nuclear) in methionine-deficient MEM containing 10% complete medium. After radiolabelling for 24 h, cells were washed with phosphate-buffered saline (PBS) and disrupted with RIPA buffer (10 mm-Tris–HCl pH 8-2, 150 mm-NaCl, 600 mm-KCl, 0.5 mm-MgCl2, 2% Triton X-100, 1% aprotinin, 1 mm-phenylmethylsulphonyl fluoride, 0-3 mm-iodoacetamide). The lysate was then centrifuged for 1 h at 100000 g in a Beckman SW60 rotor to remove insoluble proteins. In the immunoprecipitation assay, 100 μl of radiolabelled viral antigens were incubated overnight at 4 °C with 100 μl 1:50 diluted rabbit anti-MV serum with gentle shaking. Protein A–Sepharose CL-4B beads (Pharmacia) were added and the suspension was incubated on ice for 2-5 h. Unadsorbed material was removed by centrifugation at 10000 g for 15 to 20 s. The antigen–antibody complexes were then washed three times with RIPA buffer followed by a further wash with 10 mm-Tris–HCl pH 8-2, containing 0-1% SDS. After the final wash, dissociating buffer (0-1 m-Tris–HCl pH 6-8, 5% SDS, 5% 2-mercaptoethanol) was added and the mixture was immersed in boiling water for 5 min. The immunoprecipitates were analysed by SDS–polyacrylamide slab gel electrophoresis for 220 min at a constant current of 30 mA (Marusyk & Cummings, 1978). After electrophoresis, the gel was fixed, dried and an autoradiogram prepared using Kodak Royal X-Omat film exposed at -70 °C.

Haemadsorption. Tube cultures of Vero cells were incubated for various times after infection at 32 °C and 39 °C. The infected cells were washed with warm PBS and covered with a 0-5% suspension of Cercopithecus aethiops erythrocytes and incubated for 1 h at 37 °C. The preparations were examined immediately.

Electron microscopy (EM). Samples for EM were prepared as previously described (Mofford & Marusyk, 1984). Preparations were examined in a Philips EM300 or a Siemens Elmiskop 102 electron microscope.

RESULTS

Mutagenesis and isolation of temperature-sensitive mutants

Replication of parental virus stock in the presence of 200 μg/ml 5-fluorouracil resulted in an approximately 100-fold decrease in infectious titre. Of the plaque isolates selected as possible mutants, 265 were tested for growth at the permissive and non-permissive temperatures, and three were selected as suitable for further study. One mutant, designated MV ts38, was
characterized in this study. The plaquing efficiency of MV ts38 was calculated to be $4.8 \times 10^5$ (plaque titre at 32 °C/plaque titre at 39 °C).

**Thermolability**

No significant difference in heat sensitivity was observed when MV ts38 was compared with the parental virus stock. Both titres decreased by 1 log$_{10}$ after incubation at 42 °C for 30 min. Consequently, apparent temperature-dependent differences in capacity to replicate were not related to thermolability of the virus.

**Detection and localization of viral proteins**

When MV ts38 was grown at the permissive temperature of 32 °C, typical MV c.p.e. and positive haemadsorption were detected at 48 h post-infection. However, when similar cultures were incubated at the non-permissive temperature of 39 °C, neither c.p.e. nor haemadsorption was observed. We concluded, therefore, that biologically active haemagglutinin was not membrane-associated (not synthesized?) at the non-permissive temperature.

When MV ts38-infected Vero cells were incubated for 32 h at the permissive temperature and then stained with rabbit anti-MV serum, fleck- and globular-type cytoplasmic fluorescence was detected (Fig. 1a; Table 1). By 52 h post-infection, c.p.e. had increased dramatically and fluorescence was seen throughout the cytoplasm, but only very weak and diffuse-type nuclear fluorescence was observed (Fig. 1b).

Infected cells that were incubated initially at the non-permissive temperature (39 °C) for 24 h and then shifted-down to the permissive temperature (32 °C) for 8 h showed no fluorescence (Fig. 2a). At 28 h after shift-down, globular- and fleck-type fluorescence was found in the cytoplasm with rabbit anti-MV serum (Fig. 2b).

Eight h after shift-up from the permissive temperature, the fluorescence was confined to the cytoplasm, but by 28 h after shift-up, there was an accumulation of intranuclear inclusions, which with time became more predominant as the cytoplasmic staining decreased (data not
Fig. 2. Immunofluorescence of MV ts38-infected Vero cells shifted-down from non-permissive (39 °C) to permissive (32 °C) temperature and stained with rabbit anti-MV serum and FITC-conjugated anti-rabbit serum 8 h (a) and 28 h (b) after shift-down.

Table 1. Summary of immunofluorescence staining results of MV ts38-infected Vero cells 56 h post-infection

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Fluorescence</th>
<th></th>
<th></th>
<th>Cytoplasmic</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nuclear</td>
<td>32 °C</td>
<td>39 °C</td>
<td>Shift-up</td>
<td>Shift-down</td>
<td>32 °C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diffuse</td>
<td>Neg.*</td>
<td>Dots</td>
<td>Globular</td>
<td>Diffuse</td>
</tr>
<tr>
<td>Polyclonal anti-MV serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoclonal anti-NP serum (16BD8)</td>
<td></td>
<td>Diffuse</td>
<td>Neg.</td>
<td>Dots</td>
<td>Globular</td>
<td>ND*</td>
</tr>
<tr>
<td>Monoclonal anti-P serum (16BD3)</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Neg.</td>
<td>ND</td>
<td>Globular</td>
<td>Flecks</td>
</tr>
</tbody>
</table>

* Neg., Negative; ND, not done.

shown). To identify the nature of intranuclear staining observed, monoclonal antibodies against MV NP and P components were used for subsequent staining. Staining with NP monoclonal antibodies confirmed that the syncytial intranuclear inclusions contained nucleocapsid proteins (Fig. 3a). On the other hand, staining with P monoclonal antibody was confined to the cytoplasm (Fig. 3b). EM examination of MV ts38-infected cells at the permissive temperature revealed the cytoplasmic accumulation of ‘rough’ nucleocapsids and round or globular-like nuclear accumulations of ‘smooth’ nucleocapsids (Fig. 4). The ‘rough’ form was
Fig. 3. Immunofluorescence of MV ts38-infected Vero cell syncytia shifted-up from permissive (32 °C) to non-permissive (39 °C) temperature and stained 28 h after shift-up with monoclonal antibodies against NP (a) and P (b).

located randomly only in the cytoplasm while the ‘smooth’ form was found in parallel arrays in the nuclei. Although the EM studies were also done on infected cells grown at the non-permissive temperature, no observable changes were obtained. This might have been due to blockage at the early stage of virus synthesis. In the shift-up and shift-down experiments, only a few cells were infected, as confirmed by immunofluorescence, therefore explaining the negative results obtained.

Viral protein synthesis

Results from immunoprecipitation tests indicated that viral proteins were not synthesized at the non-permissive temperature (Fig. 5, lanes 3 to 6), whereas all the major MV structural components were immunoprecipitated from infected cells at 32 °C. Immunoblot analysis of infected cell lysates confirmed the absence of viral polypeptides in ts38-infected cells at the non-permissive temperature (data not shown). In vitro translation also showed that mRNA transcription occurred normally at 32 °C but not at 39 °C (data not shown). Also, MV-specific proteins were synthesized following shift-down to 32 °C albeit at a much lower rate than in control cultures maintained at 32 °C.

DISCUSSION

The MV Lec strain-derived temperature-sensitive mutant, MV ts38, replicated to a lesser extent at 39 °C than at 32 °C, and this restriction in growth appeared to be related to a shut-down of mRNA transcription of the major virus structural components. The absence of these viral proteins at 39 °C was confirmed by immunoprecipitation and Western blotting techniques. Furthermore, the growth restriction of the MV ts38 mutant is not related to heat lability, as no difference in growth pattern between the mutant and the parental virus was detected following heat inactivation assays (data not shown).

Following a shift from the permissive temperature (32 °C) to the non-permissive temperature (39 °C), increasing amounts of nucleocapsid protein accumulated in the nucleus with time after
Fig. 4. Electron micrograph of MV ts38-infected Vero cells at the permissive temperature (32 °C), 72 h post-infection. S, ‘Smooth’-type nucleocapsids; R, ‘rough’-type nucleocapsids. Bar marker represents 1 μm.
infection. A similar accumulation of the nucleocapsid protein has been reported in several in vivo and in vitro cell systems. The nuclear inclusions are, however, found more frequently in persistently infected cells than in lytically infected cells (Bouteille et al., 1965; Rustigian, 1966; Dubois-Dalcq et al., 1974; Martinez et al., 1974). In SSPE biopsy or post-mortem material, nuclear viral inclusions have been consistently found in brain tissues and have been proposed as a diagnostic marker of the disease (Martinez et al., 1974). The role of nuclear invasion by the virus is, however, poorly understood. Robbins (1983) reported that there appears to be a clear correlation between the extent of viral nuclear invasion and the progression of morbidity.

EM examination reveals that there is a morphological difference between the cytoplasmic and nuclear nucleocapsids (Fig. 4). The ‘rough’ form of the nucleocapsid is found only in the cytoplasm and the ‘smooth’ form only in the nucleus. Intranuclear inclusions of the ‘smooth’ type of nucleocapsid have always been found in association with MV SSPE strain-infected cells (Oyanagi et al., 1971), SSPE virus persistently infected cell lines (Dubois-Dalcq et al., 1973) and SSPE brain cell cultures (Oyanagi et al., 1970). The different appearance of the nucleocapsid inclusions in the cytoplasm and in the nucleus is thought to be the result of the association of different protein components with the nucleocapsid structure. The cytoplasmic nucleocapsids contain NP, P, the large protein L and possibly also M protein, while it has been speculated that the nuclear nucleocapsids contain only NP. It would appear then that during the first 24 h of replication of the MV ts38 mutant at the permissive temperature, initial synthesis of both NP and P begins. When infected cells are then shifted to, and further incubated at, the non-permissive temperature, only NP is transported into the nucleus, finally accumulating as inclusion bodies which are seen as the dot type fluorescence. In the light of similar observations of others, the transport to and accumulation of NP in infected cell nuclei might have a role in persistence. That putative role, and the mechanism(s) involved are not, however, understood. Alternatively, the data indicate that the transport to, and accumulation of, NP in the cell nucleus...
is a late event in the virus–cell interaction and may have no role in virus replication or persistence.

In this study, we report the characterization of an MV temperature-sensitive mutant (MV ts38), the nucleocapsids of which accumulate in infected cell nuclei at non-permissive temperatures and which mimic phenomena typically associated with persistently infected cells. The MV ts38 mutant serves as a good model for studying the steps involved in nuclear accumulation and possibly in the mechanism of establishment and maintenance of the persistent state.

We thank Drs G. Lund and B. Ziola for valuable discussions during the course of this work. We very much appreciate the gift of monoclonal antibodies from Dr T. F. Wild, and we gratefully acknowledge the valuable assistance of Halyna Marusyk with the EM observations. This study was supported in part by the Medical Research Council and the Alberta Heritage Foundation for Medical Research.

REFERENCES


*(Received 24 March 1986)*