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Intraperitoneal inoculation into sensitive BALB/c mice of D85, a thermosensitive (ts) mutant, provokes acute hepatitis followed by recovery of the mice. The ts mutant was able to replicate in the liver. However, the maximal viral titre was obtained 2 days later than was the case with the wild-type (wt) MHV 3 infection; the viral antigens remained localized within small foci and no invasion of the entire liver was observed. The hepatocytes infected with D85 showed strong steatosis similar to that induced by wt virus, but the other lesions induced by MHV 3 (closing of endothelial cell fenestrae and hepatocytolysis) were not seen. An important feature noticed with the D85 mutant concerned the establishment, in the surviving animals, of persistent infection: this phenomenon was demonstrated by the decrease of viral titre in the liver, viral RNA detection, and the fact that viral antigens gradually decreased until the 3rd month post-infection.

Mouse hepatitis viruses (MHV) are enveloped viruses containing a single-stranded positive RNA genome. The viral RNA encodes three structural polypeptides: the phosphonucleoprotein N (60 kDa), the matrix glycoprotein M (21 kDa) and the glycoprotein of the peplomer S (180–90 kDa) responsible for attachment to receptors on permissive cells and for cell fusion. MHV produces a wide spectrum of diseases dependent on the particular viral strain as well as on host factors such as genetic background, age, route of infection and immune status (1–3). The MHV 3 strain causes acute hepatitis in most sensitive inbred mice including BALB/c mice (4). In this case, the infection of the Kupffer cells and endothelial cells is a prerequisite to the infection of the parenchymal cells in MHV 3 induced hepatitis (5–7). In vitro, all liver cells (Kupffer cells, endothelial cells (8) and hepatocytes (9)), are highly susceptible to MHV 3 which rapidly induces large syncytia in them. In the case of susceptible mice, infection by MHV 3 leads to the appearance of procoagulant activity induced by monocytes/macrophages and controlled by T lymphocytes resulting in abnormalities of microcirculation and the formation of sinusoidal microthrombi (10–13). We have recently isolated a panel of MHV 3 thermosensitive (ts) mutants to study the viral factors involved in the hepatitis induced in sensitive BALB/c mice (14). This paper describes the pathogenicity of one mutant: D85. The replication of this mutant is reduced only at a temperature above 38 °C. At 37 °C, D85 is able to replicate in DBT cells. Biochemical studies showed that it was an RNA positive mutant. No difference was observed in the size of the viral intracellular RNA species or in the molecular weight of viral proteins.

Materials and Methods

Cells and viruses

Mouse L 2 cells and DBT cells were grown separately in a minimum essential medium (MEM) containing 10% fetal calf serum. The wild-type (wt) MHV 3 strain and the ts
mutant used in this study have been previously described (14).

**Animals**

Ten week-old BALB/c mice were inoculated intraperitoneally (i.p.) with 1·10⁷ PFU of wt virus or ts mutant. Mice were killed at various times post infection (p.i.) and blood and livers were collected. The livers were immediately frozen in liquid nitrogen and stored at −80 °C; blood was allowed to clot for 1 h at 37 °C and the serum was collected and stored at −20 °C prior to assay.

**Viruses**

Chilled pieces of liver were homogenized twice for 15 s in PBS buffer (5 mlg of liver) with a Politron homogenizer. The virus titre was determined by plaque assays on L2 cells, as previously described (14).

**RNA extraction and hybridization**

RNA was isolated from 0.3 g of liver from uninfected, MHV 3 infected, and D85 infected mice at different times using the guanidine isothiocyanate method (15). For blot analysis, RNA in NTE (0.01 M Tris-HCl (pH 7.4), 0.1 M NaCl, 1 mM EDTA) was applied to nitrocellulose filters. Prehybridization was performed in 10× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), 10% formaldehyde, 5× Denhardt's solution and 1% bovine serum albumine, 1% Ficoll and 1% polyvinylpyrrolidone) at 60 °C. Hybridization was performed in the same solution with the addition of 1·10⁶ cpm/ml of [³²P]cDNA from the purified denatured genomic RNA of MHV 3. Filters were washed four times at room temperature in 2× SSC and 0.2% SDS, twice in 2× SSC, 0.1% SDS at 55 °C and exposed to Curix film (Agfa Gevaert) at −80 °C.

**Indirect immunofluorescence assay (IFA)**

Frozen sections (1 μm) of liver were cut on a cryostat before being used for indirect immunofluorescent or histological studies. The sections were fixed for 10 min with acetone and washed twice for 5 min in PBS. Anti-MHV 3 antibody (100 μl) was absorbed for 30 min at room temperature. Sections were then washed three times in PBS. Fluorescein-labeled anti-mouse immunoglobulin G (100 μl) (Institut Pasteur Production) was added for 30 min at room temperature. Each slide was washed three times in PBS overlaid with glycerol (PBS/glycerol, 9:1) and examined under a Leitz fluorescent microscope. Anti-MHV 3 antibody was produced in the resistant A/J mice strain by two i.p. injections of 1·10³ PFU/mouse; for most experiments the antibody was used at a final dilution of 1:100.

**Electron microscopy**

**TEM:** Mouse livers were fixed by perfusion with 1.5% glutaraldehyde buffered with 75 mM cacodylate at pH 7.4 through the portal vein according to the method of Wisse (16). Small blocks were post-fixed in phosphate-buffered 1% OsO₄ for 1 h and dehydrated with ethanol. Epon was used as an embedding medium and thin sections stained with uranyl acetate and lead citrate were observed under a Philips EM 410 electron microscope (Eindhoven, The Netherlands).

To determine the extent and localization of the lesions, 1 μm semi-thin sections from the same samples used for TEM were stained with toluidine blue and observed under a light microscope.

**Freeze-fracture**

The perfused livers, cut into very small blocks, were fixed for 30 min more with 2% glutaraldehyde either in a phosphate buffer (pH 7.4) or in a 0.1 M cacodylate buffer containing 1 mM MgCl₂, 1 mM CaCl₂ and 4.5% sucrose (pH 7.3). They were then washed and glycerinated in 30% glycerol in the corresponding buffer for at least 4 h at 4 °C. Each block, placed inside a suitable copper specimen holder (Balzers), was frozen in subcooled nitrogen (close to −210 °C). The frozen specimens were then fractured either immediately or after a period of storage under liquid nitrogen in a Reichert-Jung 190 cryofract freeze-fracture device at −150 °C (Cambridge Instruments, Villepinte, France). The fracture planes were replicated with platinum at 45° and coated with carbon. The replicas were cleaned in sodium hypochloride, washed in three changes of distilled water and picked up on hexagonal 7W mesh gold grids. They were examined in a Philips EM 410 electron microscope. Morphometry was performed on electron micrographs with a Kontro semiautomatic MOP Videoplan analyzer (Eching/Munich, F.R.G.). About 4000 μm² of endothelial cell membranes from untreated liver or from mice liver inoculated with MHV 3 were examined.

**SEM**

After the same fixation and dehydration processes used for TEM, the liver samples were dried in a critical point dryer, coated with gold and observed under a Philips SEM 501 electron microscope.

**Results**

**In vitro multiplication**

At 37 °C, the wt virus and the ts mutant D85 replicate equally well in DBT cells (Fig. 1A). However, at the per-
missive temperature, the maximum titre obtained with the ts mutant was approx. 10-times lower than that of the wt virus. At 39.5 °C, the replication of D85 was completely inhibited (Fig. 1B).

Clinical signs and effects of ts mutant

To compare the pathogenicity of D85 to wt MHV 3, groups of twenty mice were inoculated i.p. with $1 \times 10^4$ PFU/mouse. This route was chosen because it had been demonstrated to result in earlier death of sensitive animals. Whilst wt MHV 3 killed 100% of the mice at 5 days p.i., D85 only killed 15% of the mice during the same period (Fig. 2A). Although the LD$_{50}$ of wt MHV 3 was less than 1 PFU/mouse, inoculation of as much as $5 \times 10^4$ PFU/mouse of D85 only killed 35% of the mice. Daily inspection showed that both groups of mice exhibited general lethargy. This symptom initially appeared 1.5 to 2 days p.i. in the case of the wt virus and 3 days p.i. in that of the mutant, and persisted until death or recovery. The body temperature of the mice remained at the same level throughout the infection (data not shown) indicating that survival following infection with this mutant was not a result of an increase in body temperature.

Viral replication in the liver

Viral titres. Maximal virus titre in liver homogenates was the same with both viruses (Fig. 2B). However, the maximal titre for the D85 mutant occurred 1 to 2 days later than the equivalent point for the wt virus; this coincides with the maximum intensity of the clinical symptoms. During the recovery of D85 infected mice, the viral titre decreased rapidly and was undetectable after day 7. Virus isolated from the livers of D85 infected mice was still thermosensitive.

Viral RNA detection. RNA extracted from livers of two infected mice at different times p.i. was hybridized with a specific probe from genomic viral RNA. The dot blot analysis showed that the presence of MHV 3 RNA in the
Fig. 3. Light microscopy: A–F indirect immunofluorescence performed on frozen liver sections. (A) Viral antigens of wt virus 2 days p.i. (B) 3 days p.i. (C) Uninfected liver. (D–F) Viral antigens of D85 respectively at 5 days (D), 10 days (E) and 3 months p.i. (F). The arrow points to the few hepatocytes stained with anti-MHV 3 (F). (G and H) Semi-thin sections/stained with toluidine blue. (G) Uninfected liver. ×560. (H) Mouse liver infected with wt virus for 52 h. Arrow indicates a necrotic area. ×560.
liver was maximal at 3 days p.i.; with D85 detection of viral RNA increased until 5 days p.i. and then decreased (Fig. 2C). The RNA could be detected 20 days p.i., but from 45 days p.i. no RNA reacted with the probe. The probe did not bind to RNA isolated from uninfected mice livers.

**Viral antigens.** In frozen sections of liver, viral antigens could be detected by indirect immunofluorescence 24 h after intraperitoneal infection of wt virus. Antigens were prominent in the periporal areas and in sinusoidal cells. After 48 h, large amounts of viral antigens were localized in the hepatic parenchymal cells. The amount of antigen increased steadily until the death of the animals (Fig. 3A and B). In mice infected with mutant D85, viral antigens could be detected at 36 h p.i.; however, the foci remained of limited size for 5 days following infection (Fig. 3D) and then decreased until the 10th day (Fig. 3E). For up to 3 months p.i., antigens could still be detected in a very few individual cells (Fig. 3F). This suggests that after acute hepatitis, D85 induced a persistent infection.

**Light microscopy study**

In control mice livers, hepatocytes presented round nuclei and the cytoplasm, rich in glycogen, contained few lipid droplets (Fig. 3G). From 48 h p.i. with wt virus, massive steatosis and numerous necrotic foci; picnotic nuclei and cytoplasmic lesions with accumulation of mononuclear inflammatory cells were generally observed in the vicinity of the portal vein (Fig. 3H). With the D85 strain, necrotic areas were neither as numerous nor as extensive compared to those induced by the wt virus. The majority of the hepatocytes did not appear damaged apart from the areas of extensive steatosis (not shown).

**Electron microscopy study**

In the liver of control mice, observed at TEM level, the cytoplasm of the hepatocytes displayed a normal appearance: glycogen and mitochondria were numerous and few lipid droplets were visible. The sinusoid, devoid of red blood cells was lined by endothelial cells, in its lumen Kupffer cells were present (Fig. 4A). At the SEM level endothelial cells displayed numerous fenestrae arranged in sieve plates (Fig. 4B and insert). These were also visible on the E and P faces of the fracture of endothelial cell membranes (Fig. 4C). Three hundred and twenty-one fenestrae were counted in this figure for a surface of 37.5 \( \mu \text{m}^2 \) of endothelial cell membrane. After wt virus infection, sinusoidal lesions were evident at the TEM level (Fig. 5A). Sinusoids were invaded with red blood cells, plasma, cellular debris and fibrin deposits often associated with numerous viral particles (Fig. 5A, insert). Kupffer cells were no longer visible and the endothelial lining was sometimes interrupted. In addition, SEM examination revealed that when endothelial cells were present in the sinusoids invaded with red blood cells (Fig. 5B), they displayed only a few fenestrae (insert Fig. 5B) compared to uninfected liver endothelial cells (insert Fig. 4B). After freeze-fracture, TEM examination confirmed this surprising decrease in the number of endothelial cell fenestrae (Fig. 5C) which did not involve any other apparent cellular membrane lesions. In this figure only 46 fenestrae were counted for a surface of 32.5 \( \mu \text{m}^2 \). In order to quantify this phenomenon a morphometric study was carried out on 4000 \( \mu \text{m}^2 \) of endothelial cell membranes of both control and infected mice livers and these results are summarized in Table 1. A 2-fold decrease in the number of fenestrae per unit surface was found after 3 days of infection with MHV 3.

In addition to these sinusoidal lesions, an important hepatocyte lysis was observed 72 h p.i. (Fig. 5A). Only a few parenchymal cells were still recognizable. They displayed an accumulation of lipid droplets (Fig. 5A), a dilatation of the RER cisternae and picnotic nuclei (not shown). Viral particles were sometimes encountered near the large RER but were most frequently observed at the hepatocyte-sinusoidal border (not shown), or in sinusoids associated with fibrin deposits (Fig. 5A, insert).

After infection with D85, we were able to observe similar necrotic lesions in the sinusoid and in the hepatocytes. But these lesions were found only in a few areas. The only noticeable change in the hepatocytes, apart from glycogen depletion, consisted of a widespread steatosis observed both under TEM (Fig. 6A) and SEM (Fig. 6B). The majority of sinusoidal cells were intact and the endothelial cells exhibited as many fenestrae (Fig. 6B) as those observed in uninfected cells (Fig. 4B and insert).

**Table 1**

| Samples | Surface observed (\( \mu \text{m}^2 \)) | Number of fenestrae | Number of fenestrae (per \( \mu \text{m}^2 \)) |
|---------|---------------------------------|------------------|----------------------------------|
| Uninfected mice livers | | | |
| 1 | 672 | 263 | 3.9 |
| 2 | 3061.5 | 16038 | 5.2 |
| 3 | 352 | 1679 | 4.8 |
| Mouse livers infected with MHV 3 (3 days) | | | |
| 1 | 1062 | 2777 | 2.6 |
| 2 | 316.5 | 650 | 2.0 |
| 3 | 2787 | 7706 | 2.6 |
Fig. 4. Uninfected liver cells. (A) TEM. Ec, endothelial cell; Kc, Kupffer cell; S, sinusoid; H, hepatocyte; L, lipid inclusion. ×4000. (B) SEM. Ec, endothelial cell; H, hepatocyte; f, fenestrae. ×4300 and insert ×7300. (C) TEM after cryofracture. E, E face of the fracture; P, P face of the fracture; Ec, endothelial cell; H, hepatocyte; f, fenestrae. ×16 000.
Fig. 5. Mice liver cells observed 3 days p.i. with wt virus. (A) TEM. The sinusoids (S) were invaded with erythrocytes (E), plasma and fibrin. Hepatocytes (H) filled with lipid inclusions (L) showed a necrotic area (arrow). ×2200. Insert: in sinusoids, MHV 3 particles (*) are often associated with fibrin deposits (f). ×20 000. (B) SEM. The hepatocytes (H) showed numerous cavities corresponding to lipid inclusions (L). The sinusoids (S) were filled with numerous erythrocytes (E). Endothelial cells displayed very few fenestrae (f) (insert), ×10700 and insert ×7500. (C) TEM after cryosection. P face (P) of endothelial cells (Ee), lining and hepatocyte (H) displayed few fenestrae (f). A large surface of the endothelial cell membrane is devoid of fenestrae (*). ×16 000.
Inoculation of wt MHV 3 into BALB/c mice produces a fulminating hepatitis which leads to the death of the animals in 3 or 4 days. This study reports the biological activities of a ts mutant and an attempt to correlate the severity of the disease with histological and ultrastructural observations. The ts mutant D85 was chosen since it is able to replicate in vitro at mouse body temperature (37.5 °C). D85 was clearly an attenuated virus strain since only about 15% of the mice died after i.p. inoculation of 1000 PFU. Histological observations of liver injury after wt virus infection were nearly identical to that previously reported with MHV 3 by Levy et al. (6). They also noticed the damage of the sinusoidal barrier, the presence of inflammatory foci and necrotic areas, and extensive intra-vascular coagulation. The only differences we observed in hepatocyte lesions were the significant modifications of the peroxysomes (17) and the accumulation of lipids in the liver, which seems specific to our virus strain. The origin of lipid accumulation in the liver is unknown, but biochemical determinations in the whole liver have shown an increase in the level of triglycerides and cholesterol (unpublished data).

Electron microscope observations confirmed that, after wt MHV 3 infection, the first lesions appeared in the hepatic sinusoid where the destruction of Kupffer cells and endothelial cells preceded the appearance of the hepatocyte lesions (7). We also observed a 2-fold decrease in the number of fenestrae in endothelial cells. For a morphometric study we used the freeze-fracture technique (the best method to study membranes). Similar findings have been obtained in vivo and in vitro after infection of isolated endothelial liver cells (18). The ‘closing’ of 50% of the fenestrae was probably the result of the fusion activity of the S glycoprotein incorporated into the cytoplasm membrane of the infected endothelial cells and could be explained by the disturbed fluidity of endothelial cell membranes in infected mice livers. Recent results have shown that the cholesterol content in these membranes was considerably decreased (19).

D85 infection induced a strong steatosis which was quite similar to that induced by wt MHV 3. This accumulation of lipids did not seem to be correlated with sinusoidal damages since it appeared in D85 infected mice which presented only few sinusoidal cell lesions and the majority of endothelial cell membranes showed normal fenestration.

An important feature of D85 infection was the establishment in the surviving animals of persistent infection: after the 7th day p.i., the virus titre was no longer detectable in the liver but the RNA could be detected 20 days p.i. and a very small number of hepatocytes with viral antigens could be observed after 1.5 months. The differences between non-infectious particle production, viral RNA, and protein detection could be explained by various factors: (i) differences in the sensibility of the three detection techniques; (ii) blockage of translation, since viral mRNAs seemed to be intact; (iii) selective degradation of viral mRNAs by host enzymes, while allowing remaining mRNAs to continue coding for proteins; and (iv) inhibition of viral maturation, either due to genomic mutations or to modification of glycosylation of S and M proteins and phosphorylation of N protein. At this stage, D85 infection appeared similar to the persistent infection of semi-sensitive C3H mice infected with the wt MHV 3 (20).

The induction of a persistent infection is common to various viruses as well as to ts mutants (Sinbis Virus (21), Measles Virus (22), Vesicular Stomatitis Virus (23)). In

**Discussion**

Inoculation of wt MHV 3 into BALB/c mice produces a fulminating hepatitis which leads to the death of the animals in 3 or 4 days. This study reports the biological activities of a ts mutant and an attempt to correlate the severity of the disease with histological and ultrastructural observations. The ts mutant D85 was chosen since it is able to replicate in vitro at mouse body temperature (37.5 °C). D85 was clearly an attenuated virus strain since only about 15% of the mice died after i.p. inoculation of 1000 PFU. Histological observations of liver injury after wt virus infection were nearly identical to that previously reported with MHV 3 by Levy et al. (6). They also noticed the damage of the sinusoidal barrier, the presence of inflammatory foci and necrotic areas, and extensive intra-vascular coagulation. The only differences we observed in hepatocyte lesions were the significant modifications of the peroxysomes (17) and the accumulation of lipids in the liver, which seems specific to our virus strain. The origin of lipid accumulation in the liver is unknown, but biochemical determinations in the whole liver have shown an increase in the level of triglycerides and cholesterol (unpublished data).

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The induction of a persistent infection is common to various viruses as well as to ts mutants (Sinbis Virus (21), Measles Virus (22), Vesicular Stomatitis Virus (23)). In
the case of Coronavirus infections, some ts MHV 4 or MHV A59 mutants are able to induce a chronic demyelinating disease, whereas the wild-type provokes acute encephalitis (24–28).

The ability of D85 mutant to induce a persistent viral infection was probably the result of host factors. The delay in D85 multiplication could allow for a specific or non-specific immunological antiviral response which is able to inhibit viral replication. The factors involved could be T cells and macrophages which are the main elements of defense against MHV 3 infection (29). The ultrastructural observations showed that the sinusoidal cells were largely intact after D85 infection. In fact, Kupffer cells were able to play their protective role during D85 infection and endothelial cells were still able to maintain the exchange between hepatocytes and blood. The small inflammatory foci observed in the liver infected with this mutant could be due to the induction or regulation of immunological mediators (interleukin-1, tumor necrosis factor, and prostaglandins).

The persistent viral infection could also be due to the intrinsic properties of the D85 strain. In tissue cultures, D85 induced small plaques. In contrast, wt virus provoked large syncytia. This probably reflected an alteration of the fusion function provided by the S glycoprotein. The ts phenotype of D85 could reflect the preservation of the fenestrated areas in the endothelial cells. In this case, the endothelial cells role as filter was intact and could explain the modification of the disease.

Our study focused on the fact that a mutation in the C gene coding for S protein probably transformed lethal, fulminating hepatitis into a mild persistent infection. A similar mutation of MHV 4 transforms an acute encephalomyelitis to a subacute demyelinating disease (30–33). The ts D85 model may be helpful to further our understanding of host and viral factors implicated in acute and persistent disease. Our MHV 3 viral strains are a unique experimental model for viral induced steatosis in adult mice.

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