Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Res. Virol.
1991, 142, 5-15

Increased susceptibility to mouse hepatitis virus type 3 (MHV3) infection induced by a hypercholesterolaemic diet with increased adsorption of MHV3 to primary hepatocyte cultures

J. Braunwald, H. Nonnenmacher, C.A. Pereira and A. Kirn

Laboratoire de Virologie de la Faculté de Médecine, Université Louis Pasteur et INSERM U74, 3 rue Koeberlé, 67000 Strasbourg (France)

SUMMARY

The administration of a hypercholesterolaemic (HC) diet rendered genetically resistant A/J mice susceptible to mouse hepatitis virus 3 (MHV3) infection. The animals died of acute hepatitis with high viral titres in the liver accompanied by many necrotic foci and high serum transaminase levels. Resistance to virus was re-established by refeeding HC mice with a normal diet for 2 weeks. This modification of pathogenesis was accompanied by an increase in the susceptibility of hepatocyte cultures from HC mice to MHV3 and could be explained by an enhancement in virus adsorption. We hypothesize that the incorporation of cholesterol into the plasma membranes of hepatocytes of HC mice, thereby decreasing the membrane fluidity, may lead to an increase in the availability of virus receptors.

Key-words: MHV3, Cholesterol, Hepatocyte; Membrane, Hypercholesterolaemic diet, Mouse, Cell culture, Pathogenesis.

INTRODUCTION

Mouse hepatitis virus 3 (MHV3), a member of the Coronavirus group, is an interesting model for studying the pathogenesis of virus-induced hepatitis in mice. Susceptibility to MHV3 infection is genetically determined (Le Prévost *et al.*, 1975a, b; Virelizier, 1980). Accordingly, BALB/c mice, a susceptible strain, develop acute hepatitis and die after 3-4 days of infection, whereas A/J mice, a resistant strain, develop mild disease with the appearance of a small number of necrotic foci in the liver which disappear 8 to 12 days later. The degree of virus replication in different tissues varies from one strain of mouse to another (Virelizier and Allison, 1976; Arnheiter *et al.*, 1982; Pereira *et al.*, 1984a).

Submitted April 20, 1990, accepted January 11, 1991.

Data from our laboratory have already shown that administration of a hypercholesterolaemic diet (HC) increases the mortality produced by MHV3 in A/J mice and that, under these conditions, the Kupffer cells lose their ability to be activated *in vitro* by lipopolysaccharide (LPS) (Pereira *et al.*, 1986, 1987). It seemed interesting, firstly, to study whether the return to a normal diet ended the susceptibility and re-established resistance, and secondly, if there was a change in the *in vitro* behaviour of hepatic cells from HC animals towards the virus.

In order to do this, hepatocytes from HC mice were isolated and cultured and their susceptibility to MHV3 studied. This paper shows that resistance to MHV3 infection may be diet-modified and that this modification is also reflected in hepatocyte cultures.

MATERIALS AND METHODS

Animals

Ten-week-old mice of the inbred A/J Orl strain (Centre d'élevage des Animaux de Laboratoire, CNRS, Orléans, France) were used in all experiments. Control mice were fed laboratory chow (NR) and experimental mice were fed an HC diet for 3 weeks. The composition of the HC diet was as follows: 52 % sucrose, 18.5 % casein, 18 % lard, 1 % cholesterol, 0.5 % cholic acid and vitamin supplements (Usine d'Alimentation Rationnelle, Paris, France). After 3 weeks of the HC diet, a group of mice were refed laboratory chow for 2 weeks (HCNR).

Virus

MHV3 was a clonal isolate produced on L2-cell cultures. Viral suspensions were titrated at 33°C on L2 cells cultured in 96-well microtitre plates (Lamontagne and Dupuy, 1984).

Isolation of mouse hepatocytes

Mouse hepatocytes were obtained using methods based on those of Berry and Friend (1969) and Seglen (1976) but modified for mice. Mice were injected intravenously with heparin (500 U/100 µl) and anaesthetized intraperitoneally with sodium pentobarbital (6 mg/100 µl). The livers were perfused *in situ* via the portal vein with 0.5 mM EGTA (ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid) in Ca^{++}- and Mg^{++}-free Hank's balanced salt solution (pH 7.45) at 37°C. After 5 min at a flow rate of 13 ml/min, the perfusion solution was replaced by 0.05 % collagenase (specific activity 0.21 U/mg, Boehringer, Mannheim, Germany) in Williams medium E (pH 7.45) (Seromed, Berlin, Germany) for 7 to 9 min at a flow rate of 10 ml/min. All solutions were oxygenated and kept at 40°C to maintain the perfusion at 37°C. After perfusion, the livers from which the gallbladders had been removed were placed in a plastic Petri dish containing a small amount of collagenase solution. They were then gently combed with forceps to obtain isolated hepatocytes.

This cellular suspension was filtered through a nylon mesh to remove debris and the cells were allowed to deposit at 4°C for 20 min in Williams medium supplemented with 20 % heat-inactivated foetal calf serum (FCS). The supernatant was then discarded and the cell pellet resuspended in Williams medium supplemented with 20 % heat-inactivated FCS and centrifuged at 50 *g* for 3 min at 4°C.

Hepatocytes were counted in a haematocytometer and a Trypan blue exclusion test determined their viability as being about 80 %. Cells were diluted in medium containing 10 % heat-inactivated FCS and insulin (4 µg/ml). Cells (1×10^6) were seeded on 35-mm Petri dishes (Primaria-Becton Dickinson, Lincoln Park, NJ, USA) in a 1.5-ml volume and placed in a 5 % CO_2 incubator at 37°C. The medium was changed 3 h after seeding. When cells were maintained in culture, the medium was renewed every day.

Infection of animals and hepatocyte cultures

MHV3 was inoculated intraperitoneally into A/J NR, A/J HC and A/J HCNR mice at a dose of

FCS = foetal calf serum.
HC = hypercholesterolaemic.
HCNR = HC diet 3 weeks, NR 2 weeks.
LPS = lipopolysaccharide.
MHV3 = mouse hepatitis virus 3.
NP40 = Nonidet-P40.
PAGE = polyacrilamide gel electrophoresis.
PBS = phosphate-buffered saline.
PFU = plaque-forming unit.
p.i. = postinfection.
NR = normal ration.
SDS = sodium dodecyl sulphate.

2×10^3 PFU/mouse. Each group consisted of about 30 mice. The animals were observed for a week and their mortality was recorded. Animals were sacrificed daily and livers frozen at −80°C for later determination of virus infectivity, or placed in liquid nitrogen and stored at −80°C for subsequent analysis by indirect immunofluorescence.

Hepatocyte cultures, 24-h old, were infected at a multiplicity of infection of 0.05 PFU/cell. The virus was allowed to adsorb at 37°C for 1 h and, after washing twice with Williams medium E, cultures were incubated at 37°C in the same medium supplemented with 2 % inactivated FCS. At different times after infection, cultures were frozen for titration or immunofluorescence studies.

Virus titration

Livers were weighed, homogenized (B. Braun-Melsungen, Western Germany) and the number of viral particles/g liver was determined by plaque assays on L2 cells.

After thawing, the hepatocyte cultures were scraped from the Petri dishes, centrifuged at low speed for 15 s and the viral titre in the supernatant measured by plaque assays on L2 cells.

Indirect immunofluorescence testing

Frozen sections of liver were cut in a cryostat and fixed with acetone for 10 min at −20°C. Hepatocyte cultures were fixed in a methanol/acetone mixture (1/1) for 15 min at −20°C. After rehydration with phosphate-buffered saline (PBS), the samples were then covered with anti-MHV3 serum diluted 1/200 for 30 min at room temperature. After three 5-min washes with PBS, samples were covered for 30 min with sheep anti-mouse-IgG fluorescein-isothiocyanate-conjugated serum (Institut Pasteur, Paris, France) diluted 1/100. They were again washed 3 times (5 min each) with PBS and examined under a "Zeiss" microscope (Axiophot).

Controls consisted of uninfected livers or cells, MHV3-infected hepatocytes or livers from infected animals labelled with normal serum.

Morphometry

Measurements of the number and areas of the necrotic foci observed in liver sections were performed according to photographs made with a "Kontron semi-automatic MOP Videoplan" analyser (Carl Zeiss, Asnières, France).

Determination of transaminase activity

The activity of serum glutamic pyruvic transaminase was determined using a kit based on a colorimetric method (Abbott Laboratories, Irving, Texas, USA).

Viral RNA and protein synthesis determination

For the determination of viral RNA synthesis, hepatocyte cultures were incubated with 2 μg/ml actinomycin D for 1 h, then labelled with 25 μCi/ml 5-^{3}H-uridine (specific activity 27 Ci/mmol, CEA, Saclay, France) for 2 h. The radioactive medium was removed and after 2 washings with PBS, cell monolayers were treated with 0.5 % sodium dodecyl sulphate (SDS) and the insoluble radioactivity measured.

For the determination of viral protein synthesis, cultures were deprived of methionine for 30 min and pulse-labelled with 15 μCi/ml ^{35}S-methionine (specific activity > 800 Ci/mmol, Amersham, Buckinghamshire, England) diluted in methionine-free medium for 1 h. After washing twice with PBS, cultures were treated with 1 ml lysis buffer A (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA (ethylenediamine tetraacetic acid), 500 units Aprotinin, 1 % NP40, 0.2 mg/ml phenylmethylsulphonylfluoride) for 20 min at 4°C (Wege *et al.*, 1984).

Cells were then scraped off and the suspension centrifuged to separate the nuclei from the cytoplasm. The cytoplasmic fraction was subjected to a 100,000-*g* centrifugation for 90 min at 4°C. Viral proteins were immunoprecipitated from the supernatant by incubation overnight at 4°C with MHV3-antibody diluted 1/100, followed by incubation at room temperature with gentle agitation in the presence of 50 μl of protein A/Sepharose CL-4B (Pharmacia, Fine Chemicals, Uppsala, Sweden) for 20 min.

The immune complexes were sedimented by centrifugation and washed 5 times with 1 ml of A buffer. The pellet was solubilized in 30 μl of buffer containing 50 mM Tris-HCl pH 6.8, 2 % SDS, 2 % β-mercaptoethanol, 15 % glycerol and 0.004 % bromophenol blue and then heated at 100°C for 3 min.

After centrifugation, the supernatant was analysed on 12.5 % SDS-polyacrylamide gels (Laemmli, 1970). Electrophoresis was carried out for about 15 h at a constant current (10 mA). Gels were stained for 30 min at 37°C with 0.25 % Coomassie blue dissolved in methanol/water/acetic acid (5/5/0.7 v/v/v) and then de-stained in acetic acid/methanol/water (3/2/35 v/v/v). Gels were then prepared for fluorography (Bonner and Laskey, 1974), dried and ex-

posed to "Kodak X AR-5" film at −80°C for the appropriate exposure time. Molecular weights were determined by comparison with the migration of marker proteins (^{14}C-methylated protein, MW 14,300-200,000, Amersham, Buckinghamshire, England).

Adsorption studies

Primary hepatocyte cultures (24-h old) from NR and HC mice were infected at a multiplicity of 1 PFU/cell with MHV3 at 4°C to avoid the penetration stage. At different times after infection, the inoculum was removed, the cell monolayer was washed several times with PBS to eliminate all viral particles which were not adsorbed. Adsorption was analysed in the following 2 ways.

Titration

Cells were scraped into PBS and frozen at −80°C until titration. Once thawed, the cellular suspension was centrifuged and the adsorbed viral particles in the supernatant were titrated on L2 cells.

Radioimmunoassay

Monolayers were covered for 3 h at 4°C with an anti-MHV3 serum produced in A/J strain mice (Martin *et al.*, 1988) diluted 1/200, then washed several times with PBS and covered for 2 h at 4°C with 0.3 μCi/ml of an anti-mouse-Ig ^{35}S-labelled antibody (specific activity > 200 Ci/mmol, Amersham, Buckinghamshire, England). After several washes with PBS, cells were scraped into PBS and the trichloroacetic-acid-insoluble radioactivity was determined. This provided a measure of the amount of viral particulate material adsorbed to the cell.

Statistical analysis

Data given in the figures represent the mean values of 3 derterminations in duplicate ± SD. Comparisons between groups were carried out using the Mann and Whitney U test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of HC diet on the resistance of A/J mice to MHV3

Three different series of experiments involving 40 NR, 40 HC and 27 HCNR animals were performed to study the effect of cholesterol on MHV3-induced mortality in mice. Animals were inoculated intraperitoneally with 2×10^3 PFU of MHV3 and the mortality recorded.

NR animals began to die 3 days p.i. with a percentage of mortality of 2.4 ± 0.16 % which increased to 9.7 ± 0.3 % (cumulative) by the fifth day, and remained stable till the end of the experiments.

The mortality of HC mice occurred earlier and the rate was higher. Some animals died as early as the second day p.i. (2.5 ± 0.16 %), and by the third day the percentage of mortality was 42.5 ± 0.49 %. On the seventh day p.i., the percentage of mortality was 82.5 ± 0.38 %.

When the HC diet was replaced by a control diet for 2 weeks, the mice regained resistance to MHV3: 4 and 5 days p.i., the mortality rates were 7.4 ± 0.26 % and 18.7 ± 0.39 %, respectively, and remained stable.

From those data, it was concluded that the administration of an HC diet increases the susceptibility of the mice to MHV3 and that this phenomenon is reversible.

Virus titration was carried out on liver homogenates from the 3 groups of mice (fig. 1). In the livers of HC mice, MHV3 particles were found as early as the first day p.i. and the viral titre increased constantly until death of the animals. In the livers of NR and HCNR mice, viral particles were detected at only 2 and 3 days p.i., and after 3 days the virus titre decreased dramatically.

Immunofluorescence studies and morphometric measurements performed on liver sections of NR and HC mice corroborate these data. At three days p.i., the sizes of necrotic foci and their distribution were not identical in NR and HC animals. In the livers of HC mice, their mean surface area was ten times higher (8.02 mm^2) than in NR animals (0.81 mm^2). The repartition of fluorescent foci was relatively homogenous in the livers of NR animals, with average sizes ranging from 0.33 to 0.92 mm^2, whereas in HC livers, there was great diversity in the sizes of the necrotic foci, their surface areas varying from 0.79 to 20.5 mm^2.

The kinetics of the serum transaminase levels were determined together with the virus content of the livers in an experiment involving three mice daily. In the serum of NR animals, 2 days after the inoculation of MHV3, the transaminase level was 569 ± 75 U/l and decreased with time along with the viral titre in the livers. In HC animals, the transaminase level was double (1,200 ± 92 U/l) and constant at 2, 3 and 4 days p.i., as was the viral titre.

Replication of MHV3 in hepatocyte cultures.

Primary hepatocyte cultures (24-h old) were established from NR, HC and HCNR mice and infected with MHV3 at a multiplicity of 0.05 PFU/cell. The replication kinetics of the virus in these cultures are shown in figure 2. In hepatocytes obtained from HC mice, infectious progeny virus was detected in supernatant fluids as early as 6 h after infection with peak level production at 24 h p.i. In NR hepatocytes, the viral growth curve was slower and delayed; it was only at 12 h p.i. that viral particles were detected, and at 24 h p.i., the viral titre was about one hundred times less than that produced in HC hepatocyte culture. In hepatocyte cultures of HCNR mice, the multiplication of MHV3 was similar to that observed for NR hepatocyte cultures.

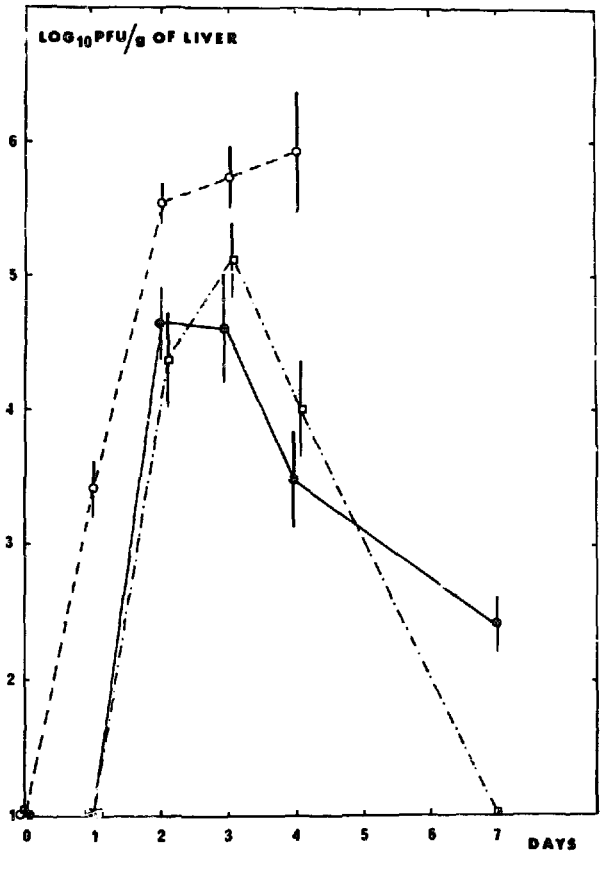

Fig. 1. p.i. virus titres in liver homogenates of NR (●), HC (○) and HCNR (□) MHV3-infected mice.

Each result represents the mean + SD of 3 separate experiments (using a total of 7 animals) for NR and HC groups, and 1 experiment with 2 animals for HCNR mice. Significant difference from NR mice, $P < 0.05$.

These data correlated with observations made for cultures treated for fluorescence studies: in the HC hepatocyte cultures, the fluorescent syncytia were very large and their number was 6-7 times higher than that in the NR hepatocyte cultures.

Viral RNA synthesis was shown to increase more rapidly in HC cells than in NR hepatocyte cultures for up to 4 h p.i., then remain constant for up to 12 h and decrease thereafter. Virus-specific proteins in MHV3-infected hepatocyte cultures from control and HC mice were studied by immunoprecipitation and SDS-PAGE (fig. 3). In HC hepatocyte cultures, viral protein synthesis began earlier: 7-9 h after infection, proteins were already detectable and increased up to 25 h p.i. before decreasing, while in control cultures, they appeared later, at 20 h p.i.

Analysis by SDS-PAGE enabled the viral polypeptides to be specified. Moreover, the presence of two bands corresponding to the spike glycoprotein S, previously named E2 (180 kDa) and the nucleocapsid protein N (60 kDa) was observed in the two types of culture. However, in HC hepatocyte cultures, the intensity of the bands corresponding to the S and N proteins was stronger between 15 and 25 h p.i.

These data show higher rates of transcription and translation in cells from HC mice, which explaines the augmentation of viral polypeptides in these cells. In order to know why transcription was increased and if it correlated with the number of the viral infectious particles capable

of penetrating the cells, we studied the first step of infection, adsorption, more closely.

This study was performed using two methods, titration and radioimmunoassay (see Materials and Methods). Comparison of the adsorption kinetics measured by titration of the 2 groups of hepatocyte cultures shows that the rate of adsorption was already 5 times higher in HC hepatocytes, as early as 15 min after infection (fig. 4A). These data were confirmed by the use of an anti-mouse-Ig ^{35}S-labelled antibody. Indeed, with this method, which shows cell-associated labelling, we noticed a 2-fold greater labelling in HC hepatocyte cultures than in NR hepatocyte cultures (fig. 4B).

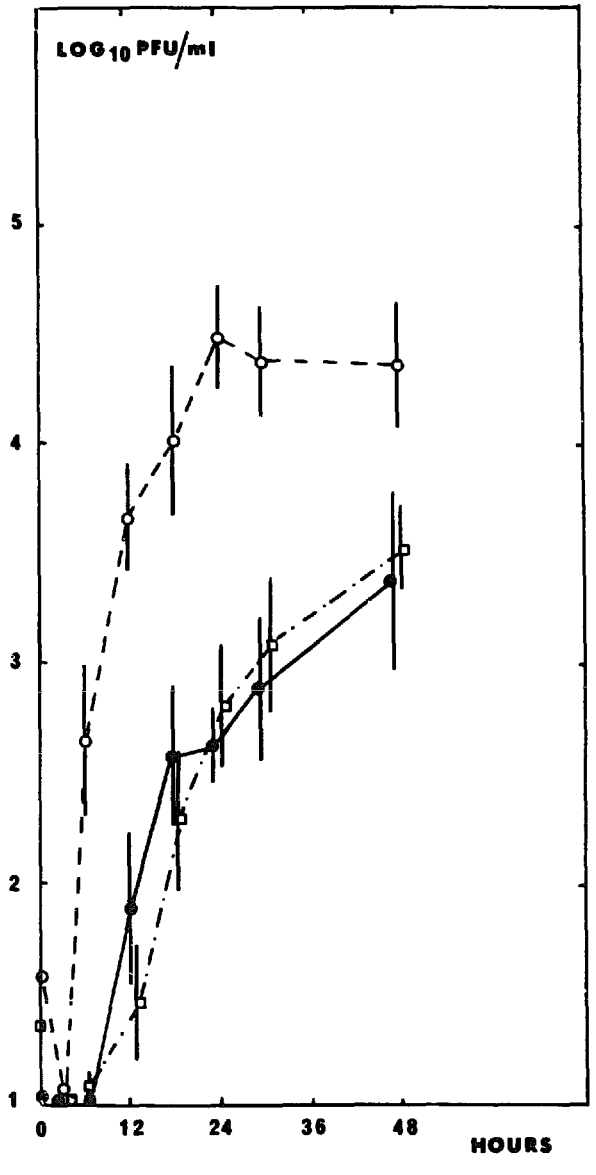

Fig. 2. MHV3 growth in hepatocyte cultures isolated from NR (●), HC (○) and HCNR (□) mice.

At various times p.i., samples were taken for titration. Each assay was carried out in duplicate in 3 separate experiments and each result represents the mean ± SD. Significant difference from NR hepatocyte cultures, $P < 0.05$.

Effect of interferon on MHV3 replication in hepatocytes from NR- and HC-diet-fed mice

NR and HC hepatocyte cultures (24-h old) were treated with 1,000 IU of standard preparation of α-β mouse interferon (Lee Biomolecular, San Diego, California, USA) for 24 h, and then infected with 0.05 PFU/cell of MHV3. The yields of viral particles were titrated 24 h later. It was observed that exogenous interferon induced an antiviral state which was more pronounced in hepatocytes from NR mice than in those from HC animals: the inhibition of viral multiplication was 94.5 ± 4.8 % and 67.7 ± 5.1 %, respectively, indicating that HC hepatocytes showed decreased susceptibility to exogenous interferon.

DISCUSSION

The data presented in this work clearly indicate that A/J mice, which are naturally resistant to MHV3 infection, become susceptible to infection when fed an HC diet for 3 weeks. This pattern of pathogenesis was similar to that found in the genetically susceptible BALB/c strain; indeed, the HC animals developed acute hepatitis with a very high viral titre in the liver associated with many necrotic foci. Immunofluorescence enabled numerous syncytia to be observed whose areas were ten times larger and whose distribution was very much more heterogenous than for normal mice, as shown by analysis morphometry. Hepatocyte necrosis was also demonstrated by determining the transaminase activity which was found to be twice as high in the sera of HC animals. This increased susceptibility to

viral infection induced by an HC diet was also observed in mice fed a diet rich in cholesterol and infected with coxsackievirus B5 (Loria *et al.*, 1976; Campbell *et al.*, 1982).

When the animals were refed a normal diet for a fortnight after the 3-week HC died, they regained resistance to MHV3 infection: 5 days after infection the mortality rate was 18.7 % while in HC mice it was 70 %. These results show that the loss of resistance to MHV3 induced by a cholesterol-enriched diet is a reversible phenomenon and would suggest that the decrease in resistance is consequently not due to genetic modification.

The animals' resistance to viral infection is determined either by their genome (Le Prévost *et al.*, 1975a, b; Virelizier, 1980) or by virus attenuation by mutation in the S gene (Martin *et al.*, 1990), but in both cases, the essential role of liver sinusoidal cells has been clearly demonstrated (Kirn *et al.*, 1982; Steffan *et al.*, 1986).

These cells form a barrier which protects the liver parenchyma from MHV3 infection (Pereira *et al.*, 1984b) and from other viruses, such as vaccinia virus (Steffan and Kirn, 1979). In Kupffer cells from resistant animals cultured *in vitro*, virus multiplication is delayed compared with its multiplication in cells of susceptible animals (Pereira *et al.*, 1984a). On the other hand, the selective destruction of Kupffer and endothelial cells renders resistant mice susceptible to MHV3 (Pereira *et al.*, 1984b). Moreover, Kupffer cells from HC mice lose their ability to be activated by LPS and to inhibit intrinsic MHV3 growth *in vitro* (Pereira *et al.*, 1986, 1987). Under these conditions, it can be suggested that the loss of host resistance induced by an HC diet may be the result of an impairment in the Kupffer cell function.

It was necessary to investigate whether this modification in pathogenesis corresponded to a change in the susceptibility of hepatic cells *in vitro*, and in particular, hepatocytes, towards MHV3 infection. With this in mind, it was essential to isolate hepatocytes from mice. Since MHV3 is a virus specific to the mouse and

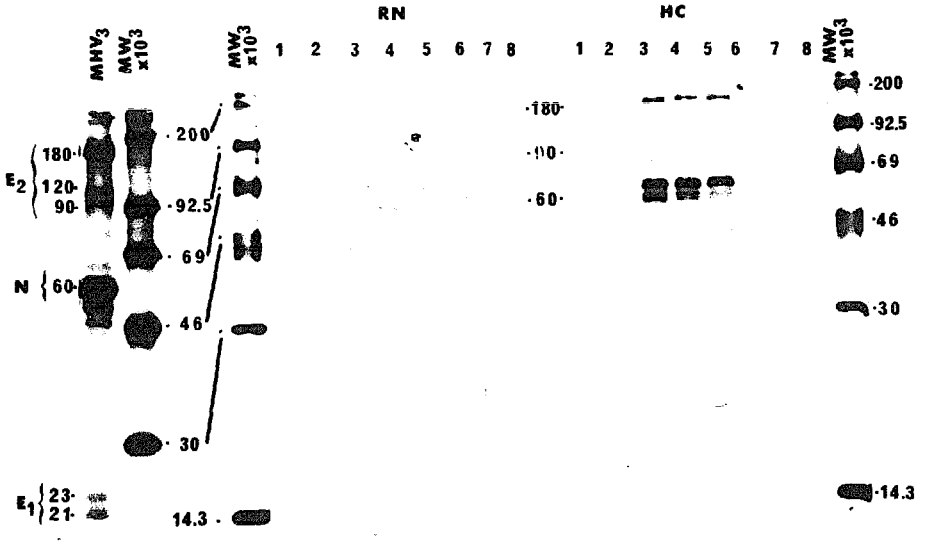

Fig. 3. Comparison of intracellular virus-specific proteins in MHV3-infected hepatocyte cultures isolated from NR and HC mice.

Samples were prepared as described in "Materials and Methods" and the same amounts of NR and HC hepatocytes were loaded on 12.5 % polyacrylamide gel. Analyses of samples were made at 7 (1), 9 (2), 15 (3), 20 (4), 25 (5), 30 (6) and 48 h (7) p.i. Slot 8 represents the analysis of mock-infected hepatocytes.

On the left is the analysis of viral intracellular proteins in MHV3-infected L2 cells by immunoprecipitation in 10 % SDS-PAGE. RN = NR in text.

with very few authors having previously prepared mouse hepatocyte cultures (Renton *et al.*, 1978; Arnheiter, 1980; Klaunig *et al.*, 1981; Maslansky and Williams, 1982), we had to adapt the techniques which are normally described for rat hepatocyte isolation (Deschenes *et al.*, 1980; Guguen-Guillouzo and Guillouzo, 1986).

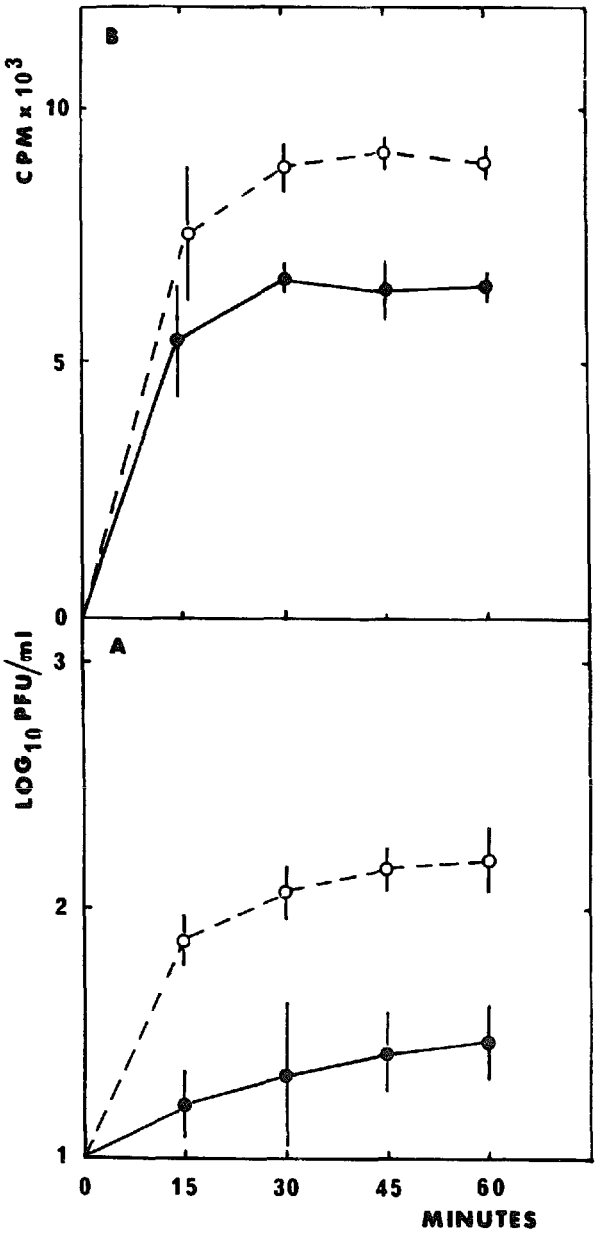

Fig. 4. Adsorption kinetics of MHV3 on NR (●) and HC (○) hepatocyte cultures.

At different times p.i., at 4°C, samples were studied by titration (A) and radioimmunoassay (B) techniques. Each assay was carried out in duplicate in 2 separate experiments and each result represents the mean ± SD. Significant difference from NR hepatocyte cultures, $P < 0.05$.

Here, we followed the methods of Berry and Friend (1969) and Seglen (1976) but modified the use of perfusion buffer and the rate and time of collagenase perfusion. This enabled us to isolate and culture hepatocytes from normal and HC mice and to compare the replication of MHV3 in the two types of culture. On hepatocyte culture from HC mice, there was stimulation of viral multiplication and numerous large-sized syncytia formed, whilst viral RNA and polypeptides appeared earlier and in larger quantities than in control cultures.

These results, showing higher rates of transcription and translation in cells from HC mice, explained the increase in the amount of viral polypeptides in these cells and incited us to study the adsorption stage using two different methods, radioimmunoassay and titration, to determine the amount of physical and infectious particles, respectively. We obtained similar results with both assays: the virus adsorbed more rapidly and in greater amounts to the hepatocyte cultures from HC mice than those of the control cultures.

However, it is difficult to understand why the adsorption difference between the two cultures was smaller using the radioimmunoassay technique, since the latter took into account the physical particles. It is possible that in the viral suspension there are aggregates of particles (as are commonly observed by scanning electron microscopy), and that only the outer virus of the aggregates can capture a molecule of antibody, while in the titration method a plaque is formed by each viral particle.

Nevertheless, in spite of these differences, it is important to note that with both methods the adsorption stage was more efficient in hepatocyte cultures from HC animals than in the control cultures. This may imply that the viral receptors were more available or their number per cell was greater in cultures from HC mice than in the control cultures.

In the case of hepatocyte cultures prepared from HC NR mice, the curve of viral growth

could be superimposed on that observed with hepatocyte cultures from control mice. This observation confirms that the modification brought about by the diet was not genetic since the cells obtained from mice refed a normal diet after a 3-week HC diet responded in the same manner as hepatocytes of normal mice; this also corroborates *in vivo* observations, in which MHV3 replication was identical to that found in the livers of control mice.

The study of the effect of interferon preparation on MHV3 multiplication demonstrates that the hepatocytes from HC mice are less susceptible to interferon, probably on account of alterations in the membrane receptors for interferon; in Kupffer cells the same effect has been observed (Pereira *et al.*, 1987). Consequently, the resistance to viral infection may be modified by an alimentary diet and this modification may also be found in primary hepatocyte cultures.

The administration of an HC diet to mice led to an increase in the total lipid and cholesterol content of the liver (Campbell *et al.*, 1982; Pereira *et al.*, 1986); it may therefore be postulated that modifications in the lipid and cholesterol content of the hepatocyte membrane occurred. An increase in cholesterol, which alters the membrane cholesterol-to-phospholipid molar ratio, interacts with the phospholipid membrane to form complexes and decreases the fluidity of the bilayers (Demel and de Kruyff, 1976; Shinitzky and Inbar, 1976). This increase in membrane microviscosity changes the membrane properties and reorganizes the functional proteins, making them more readily available at the surface membrane (Borochov and Shinitzky, 1976; Borochov *et al.*, 1979) as membrane receptors, enzymes and transport proteins.

Under these conditions, it is possible that viral receptors become more accessible to the virus, in particular to viral constituents such as the S glycoprotein which play an essential role in cell fusion (Collins *et al.*, 1982; Sturman and Holmes, 1983). Since our data show disparities in the size and number of syncytia in MHV3-infected liver and hepatocyte cultures from NR as compared to HC mice, differences in the cellular processing of the S glycoprotein may be a possibility. Sturman *et al.* (1985) and Frana *et al.* (1985) have demonstrated that cleavage of 180-kDa S to 90-kDa species by host cell enzymes is required for stimulating the cell-fusing activity of S glycoprotein, and thus determines the extent of MHV-A59-induced fusion of various cell lines. If this cleavage is more important or effective in HC cells than in control hepatocytes, the difference in the size of the syncytia observed can thereby be explained. The outcome of MHV3 infection may also be affected by the intracellular transportation of the S glycoprotein to the cell membrane which may be more rapid and effective in HC cells. The modification in membrane composition produced by the HC diet would thus render the cell membrane more sensitive to the fusing effects of S glycoprotein; a similar observation has been made by Daya *et al.* (1988) who noticed a dramatic increase in cell fusion after MHV infection (strain A59) of L2 and LM-K cells in which the membrane cholesterol content was increased.

These results show that fusion participates in MHV3 dissemination *in vivo* and *in vitro:* cell-cell fusion between infected and uninfected cells favours the spread of infection. If the membrane composition is modified by cholesterol enrichment, the fusion mechanism would seem to be accentuated and the spread of infection enhanced, but this supposition needs to be confirmed by further investigation, such as cholesterol enrichment of hepatocyte cultures from control mice to see whether MHV3 growth and the formation of syncytia are actually increased.

Acknowledgements

We wish to thank C. Royer for preparation of the photographs, J.-P. Martin for helpful discussion and V. Risch for typing the manuscript. We are grateful to T. Kirn for translating the manuscript into English.

The research was supported by INSERM (Institut National de la Santé et de la Recherche Médicale) and a grant from the DRET (Direction des Recherches, Etudes et Techniques) n° 87-125.

Relation entre une sensibilité accrue de la souris au virus de l'hépatite murine type 3 (MHV3) par administration d'un régime hypercholestérolémique et une augmentation de la permissivité au virus des hépatocytes en culture

L'administration d'un régime hypercholestérolémique (HC) entraîne une perte de résistance au virus de l'hépatite murine type 3 (MHV3) chez des souris génétiquement résistantes à ce virus. Elles meurent d'une hépatite aiguë avec un titre élevé en particules infectieuses dans le foie associé à de nombreux foyers de nécrose et un taux élevé des transaminases dans le sérum. Si après trois semaines de régime HC on remet les animaux au régime normal, l'état de résistance peut être rétabli au bout de 15 jours.

Cette modification du pouvoir pathogène s'accompagne d'une augmentation de la permissivité au MHV3 des hépatocytes isolés des souris HC qui s'expliquerait par une meilleure adsorption.

Nous avons émis l'hypothèse que le cholestérol incorporé dans les membranes des hépatocytes des souris HC en produisant une diminution de la fluidité membranaire entraînerait une meilleure accessibilité des récepteurs viraux.

Mots-clés: MHV3, Cholestérol, Hépatocyte; Membrane, Régime hypercholestérolémique, Souris, Culture cellulaire, Pathogenèse.

References

Arnheiter, H. (1980), Primary monolayer culture of adult mouse hepatocytes. A model for the study of hepatotropic viruses. *Arch. Virol.*, 63, 11-22.

Arnheiter, H., Baechi, T. & Haller, O. (1982), Adult mouse hepatocytes in primary monolayer culture express genetic resistance to mouse hepatitis virus type 3. *J. Immunol.*, 129, 1275-1281.

Berry, M.N. & Friend, D.S. (1969), High-yield preparation of isolated rat liver parenchymal cells. *J. Cell. Biol.*, 43, 506-520.

Bonner, W.M. & Laskey, R.A. (1974), A film detection method of tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Europ. J. Biochem.*, 46, 83-88.

Borochov, H. & Shinitzky, M. (1976), Vertical displacement of membrane proteins mediated by changes in microviscosity. *Proc. nat. Acad. Sci.* (Wash.), 73, 4526-4530.

Borochov, H., Abbott, R.E., Schachter, D. & Shinitzky, M. (1979), Modulation of erythrocyte membrane proteins by membrane cholesterol and lipid fluidity. *Biochemistry*, 18, 251-255.

Campbell, A.E., Loria, R.M., Madge, G.E. & Kaplan, A.M. (1982), Dietary hepatic cholesterol elevation: effects on coxsackievirus B infection and inflammation. *Infect. Immun.*, 37, 307-317.

Collins, A.R., Knobler, R.L., Powell, H. & Buchmeier, M.J. (1982), Monoclonal antibodies to murine hepatitis virus 4 (strain JHM) define the viral glycoprotein responsible for attachment and cell-cell fusion. *Virology*, 119, 358-371.

Daya, M., Cervin, M. & Anderson, R. (1988), Cholesterol enhances mouse hepatitis-virus-mediated cell fusion. *Virology*, 163, 276-283.

Demel, R.A. & De Kruyff, B. (1976), The function of sterols in membranes. *Biochem. biophys. Acta* (Amst.), 457, 109-132.

Deschenes, J., Valet, J.P. & Marceau, N. (1980), Hepatocytes from newborn and weanling rats in monolayer culture: isolation by perfusion, fibronectin-mediated adhesion, spreading, and functional activities. *In Vitro*, 16, 722-730.

Frana, M.F., Behnke, J.N., Sturman, L.S. & Holmes, K.V. (1985), Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: host-dependent differences in proteolytic cleavage and cell fusion. *J. Virol.*, 56, 912-920.

Guguen-Guillouzo, C. & Guillouzo, A. (1986), Méthodes de préparation d'hépatocytes adultes et fœtaux, *in* "Recherches en... Hépatocytes isolés et en culture" (A. Guillouzo & C. Guguen-Guillouzo) (pp. 1-12), INSERM, John Libbey Eurotext, Montrouge, France.

Kirn, A., Gut, J.P. & Gendrault, J.L. (1982), Interaction of viruses with sinusoidal cells, *in* "Progress in liver diseases", vol. 7 (H. Popper & F. Schaffner) (pp. 377-392). Grune and Stratton, New York.

Klaunig, J.E., Goldblatt, P.J., Hinton, D.E., Lipsky, M.M., Chacko, J. & Trump, B.F. (1981), Mouse liver cell culture. — I. Hepatocyte isolation. *In Vitro*, 17, 913-925.

Laemmli, U.K. (1970), Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (Lond.), 227, 680-685.

Lamontagne, L.M. & Dupuy, J.M. (1984), Natural resistance of mice to mouse hepatitis virus type 3 infection is expressed in embryonic fibroblast cells. *J. gen. Virol.*, 65, 1165-1171.

Le Prévost, C., Levy-Leblond, E., Virelizier, J.L. & Dupuy, J.M. (1975a), Immunopathology of mouse hepatitis virus type 3 infection. — I. Role of humoral and cell-mediated immunity in resistance mechanisms. *J. Immunol.*, 114, 221-225.

Le Prévost, C., Virelizier, J.L. & Dupuy, J.M. (1975b), Immunopathology of mouse hepatitis virus type 3 infection. — III. Clinical and virologic observation of a persistent viral infection. *J. Immunol.*, 115, 640-643.

Loria, R.M., Kibrick, S. & Madge, G.E. (1976), Infection of hypercholesterolemic mice with coxsackievirus B. *J. infect. Dis.*, 133, 655-662.

Martin, J.P., Koehren, F., Rannou, J.J. & Kirn, A. (1988), Temperature-sensitive mutants of mouse hepatitis virus type 3 (MHV3): isolation, biochemical and genetic characterization. *Arch. Virol.*, 100, 147-160.

Martin, J.P., Chen, W., Obert, G. & Koehren, F. (1990),

Characterization of attenuated mutants of MHV3: importance of E2 protein in organ tropism and infection of isolated liver cells, *in* "Molecular biology and pathogenicity of coronaviruses" (D. Cavanagh & D. Brown). Plenum Press, New York (in press).

Maslansky, C.J. & Williams, G.M. (1982), Primary cultures and the levels of cytochrome P450 in hepatocytes from mouse, rat, hamster and rabbit liver. *In Vitro,* 18, 683-693.

Pereira, C.A., Steffan, A.M. & Kirn, A. (1984a), Interaction between mouse hepatitis viruses and primary cultures of Kupffer and endothelial liver cells from resistant and susceptible inbred mouse strains. *J. gen. Virol.,* 65, 1617-1620.

Pereira, C.A., Steffan, A.M. & Kirn, A. (1984b), Kupffer and endothelial liver cell damage renders A/J mice susceptible to mouse hepatitis virus type 3. *Virus Res.,* 1, 557-563.

Pereira, C.A., Steffan, A.M., Klein, F., Rebel, G. & Kirn, A. (1986), Kupffer cells (KC) from mice fed a hypercholesterolemic diet (HC) have lost their ability to be activated by endotoxin *in vitro, in* "Cells of the hepatic sinusoid", vol. I (A. Kirn, D.L. Knook & E. Wisse) (pp. 365-370). The Kupffer Cell Foundation, Rijswijk, The Netherlands.

Pereira, C.A., Steffan, A.M., Koehren, F., Douglas, C.R. & Kirn, A. (1987), Increased susceptibility of mice to MHV3 infection induced by hypercholesterolemic diet: impairment of Kupffer cell function. *Immunobiol.,* 174, 253-265.

Renton, K.W., Deloria, L.B. & Mannering, G.J. (1978), Effects of polyriboinosinic acid-polyribocytidylic acid and a mouse interferon preparation on cytochrome P 450-dependent monooxygenase systems in cultures of primary mouse hepatocytes. *Mol. Pharmacol.,* 14, 672-681.

Seglen, P.O. (1976), Preparation of isolated rat liver cells, *in* "Methods in cell biology", vol. 13 (D.M. Prescott) (pp. 29-83). Academic Press, New-York, London.

Shinitzky, M. & Inbar, M. (1976), Microviscosity parameters and protein mobility in biological membranes. *Biochem. biophys. Acta* (Amst.), 433, 133-149.

Steffan, A.M. & Kirn, A. (1979), Multiplication of vaccinia virus in the livers of mice after frog virus 3-induced damage to sinusoidal cells. *J. Reticuloendoth. Soc.,* 26, 531-538.

Steffan, A.M., Pereira, C.A. & Kirn, A. (1986), Role of sinusoidal cells in the course of the hepatitis induced by mouse hepatitis virus type 3 (MHV3) in mice, *in* "Cells of the hepatic sinusoid", vol. I (A. Kirn, D.L. Knook & E. Wisse) (pp. 377-378). The Kupffer Cell Foundation, Rijswijk, The Netherlands.

Sturman, L.S. & Holmes, K.V. (1983), The molecular biology of coronaviruses. *Advanc. Virus Res.,* 28, 35-112.

Sturman, L.S., Ricard, C.S. & Holmes, K.V. (1985), Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: activation of cell-fusing activity of virus by trypsin and separation of two different 90 K cleavage fragments. *J. Virol.,* 56, 904-911.

Virelizier, J.L. (1980), Role of macrophages and interferon in natural resistance to mouse hepatitis virus infection. *Curr. Top. Microbiol. Immunol.,* 92, 53-64.

Virelizier, J.L. & Allison, A.C. (1976), Correlation of persistent mouse hepatitis virus (MHV3) infection with its effect on mouse macrophage cultures. *Arch. Virol.,* 50, 279-285.

Wege, H., Dorries, R. & Wege, H. (1984), Hybridoma antibodies to the murine coronavirus JHM: characterization of epitopes on the peplomer protein (E2). *J. gen. Virol.,* 65, 1931-1942.