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Mouse Hepatitis Virus Type 3 Infection Provokes a Decrease in the Number of Sinusoidal Endothelial Cell Fenestrae Both *In Vivo* and *In Vitro*

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Fenestrations of hepatic endothelial cells play an active role as a sieving barrier allowing extensive exchange between the blood and liver parenchyma. Alteration of these structures may be induced in the course of various pathological events and provoke important perturbations of liver function. We demonstrate here that sinusoidal endothelial cells are permissive for mouse hepatitis virus 3 (MHV3) *in vivo* and *in vitro* and that this infection leads to a striking decrease in the number of fenestrae. The disappearance of these structures observed under scanning electron microscopy or in cryofracture preparations *in vivo* and *in vitro* cannot be reversed by the action of cytochalasin B on the microfilament network. The decrease in the porosity seems to be related directly to the productive infection of the endothelial cells, because it was not observed in A/J mice resistant to the virus and in susceptible BALB/c mice immunized with a thermosensitive mutant in which no viral replication occurs. In conclusion, a viral infection of liver endothelial cells may cause extensive loss of the fenestrations and thus lead to important functional pertubations. (HEPATOLOGY 1995;22:395-401.)

The role of the endothelial cells (EC) of the hepatic sinusoid as a sieving barrier between the sinusoid and the space of Disse has been evoked by several authors.[1-11] We demonstrated that the damage to the sinusoidal lining allows viruses that are normally nonhepatotropic to infect hepatocytes.[12,13] Although the experimental evidence is still limited, there are some arguments to support the view that EC may control the entry of chylomicrons into the space of Disse[1-3] and thus play a role in the lipoprotein metabolism of the liver.[6-8] Moreover, it appears that the filtering capacity of EC may change according to the animal species[14] and may be modified under the influence of various factors.[2,5,7,8,15-34] We have shown that cytochalasin B (CB), which alters the microfilaments, produces a considerable increase in the porosity of EC.[35]

Because several viruses provoke an alteration of the cellular cytoskeleton[36-41] and because cultured liver EC may be infected by viruses,[42-44] we found it interesting to study the effect of the infection of EC on the fenestration pattern. This question is of particular importance, because (1) defenestration of hepatic sinusoids has been evoked as a cause of hyperlipoproteinemia[14]; (2) several authors have speculated about an eventual role of viruses in atherogenesis.[8]

In this study we demonstrate that mouse hepatitis virus 3 (MHV3), which infects EC,[43,44] produces a considerable reduction in the number of their fenestrae, thus leading to a drastic diminution in the porosity of the cells.

MATERIALS AND METHODS

Mice. A/J and BALB/c mice (20 to 25 g body weight) were obtained from the Centre de Sélection et d'Elevage d'Animaux de Laboratoire (Orléans, France) and IFFA CREDO (L'Arbresle, France), respectively, and kept under conventional conditions. Care of animals followed the appropriate guidelines. Six- to 8-week-old animals were infected intraperitoneally with 2×10^3 pfu (plaque-forming units) of MHV3 for 24 (5 animals) and 48 (5 animals) hours.

Immunization. A nonpathogen temperature-sensitive mutant of MHV3, D222[45] was injected three times intraperitoneally at 2-week intervals into BALB/c mice (10^3 pfu/injection).

Chemicals. Collagenase CLS from *Clostridium histolyticum* was purchased from Worthington (Millipore Corp., Bedford, MA). CB, obtained from Sigma Chemical Co. (St Louis, MO), was dissolved with dimethylsulfoxide (2.5 mg/mL) and then diluted with Dulbecco's modified Eagle medium to obtain a final concentration of 10 μg/mL.

***Liver Perfusion* In Situ.** Mice were anesthetized with ether and their livers perfused *in situ* through the portal vein with oxygenated Dulbecco's modified Eagle medium with or without 10 μg/mL CB, at a flow rate of 2 mL/min for 1 hour at 37°C. The livers were then fixed by perfusion for electron microscopy.

Isolation of Liver EC. EC from the liver were obtained by collagenase perfusion and centrifugal elutriation as described before.[46,47]

Abbreviations: EC, endothelial cells; CB, cytochalasin B; MHV3, mouse hepatitis virus 3.

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Morphological Examinations. The cells were fixed with 2.5% glutaraldehyde in 0.075 mol/L cacodylate buffer, pH 7.4, containing 4.5% sucrose, 1 mmol/L $MgCl_2$ and 1 mmol/L $CaCl_2$. The livers were fixed by perfusion through the portal vein with the same fixative. For transmission electron microscopy, the cells and small liver blocks were postfixed with 1% phosphate-buffered OsO_4, dehydrated with ethanol, and embedded in Lx 112 (Ladd, Burlington, VT). For scanning electron microscopy, the cells and the liver samples were dehydrated with ethanol, dried with hexamethyldisilazane,[48] and coated with gold.

For freeze-fracture, small blocks of livers were immersed in 30% glycerol in cacodylate buffer for at least 1 hour at 4°C then cut to fit in the specimen holder and frozen in subcooled nitrogen. Freeze-fracture replicas obtained in a Reichert-Jung 190 Cryofract freeze-fracture device (Cambridge Instruments, Villepinte, France) were cleaned with sodium hypochloride, washed in three changes of distilled water, and mounted on hexagonal 700-mesh gold grids. The samples were observed either in a Philips EM 410 or in a Philips SEM 501 B (Eindhoven, The Netherlands) electron microscope, respectively.

Morphometry. The measurements of the different parameters were performed on scanning electron microscopy photographs with a Kontron semiautomatic MOP videoplan analyzer (Eching-Munich, Germany). To determine the diameter and the porosity (which is the percentage of the cell surface opened by fenestrae), approximately 1,000 μm^2 was measured at a magnification of ×20,000.

RESULTS

Infection of BALB/c Mice. Twenty-four hours after infection, most of the liver tissue of BALB/c mice is well preserved: there are no necrotic foci, no obvious cellular alterations, and a few Kupffer cells and EC appear to be infected by the virus under electron microscopy (results not shown). However, as compared with EC from a control mouse, which exhibit typical sieve plates (Fig. 1A), all the EC of infected mice appear rather devoid of pores in the whole liver after 48 hours (Fig. 1B). This reduction in the number of fenestrae was restricted to the periportal area 24 hours after infection. MHV3 particles on the way to, or that have already been released by, the cell could also be observed (Fig. 1C). The cellular surface displays an irregular aspect because of the presence of numerous small bulging craters (Fig. 1C), which may represent the stage preceding the disappearance of the fenestrae.

Morphometric examinations have demonstrated a drop in the porosity of the endothelial lining from 10.0 ± 3% in controls, to 1.0 ± 0.2% in 48-hour-infected mice, the diameter of the fenestrae being 100 ± 16 nm and 73 ± 13 nm, respectively. The decrease in the porosity of the endothelial lining was also observed in cryofracture preparations. In control animals, clusters of rounded structures containing ice and therefore representing fenestrae were disseminated all over the surface of the sinusoid (Fig. 2A). In contrast, the surface of sinusoids of infected mice displayed a reduced number of fenestrae (Fig. 2B). It must be noticed that besides this lack of pores, the membrane of the EC seemed normal, as far as the number and appearance of the intramembrane particles are concerned.

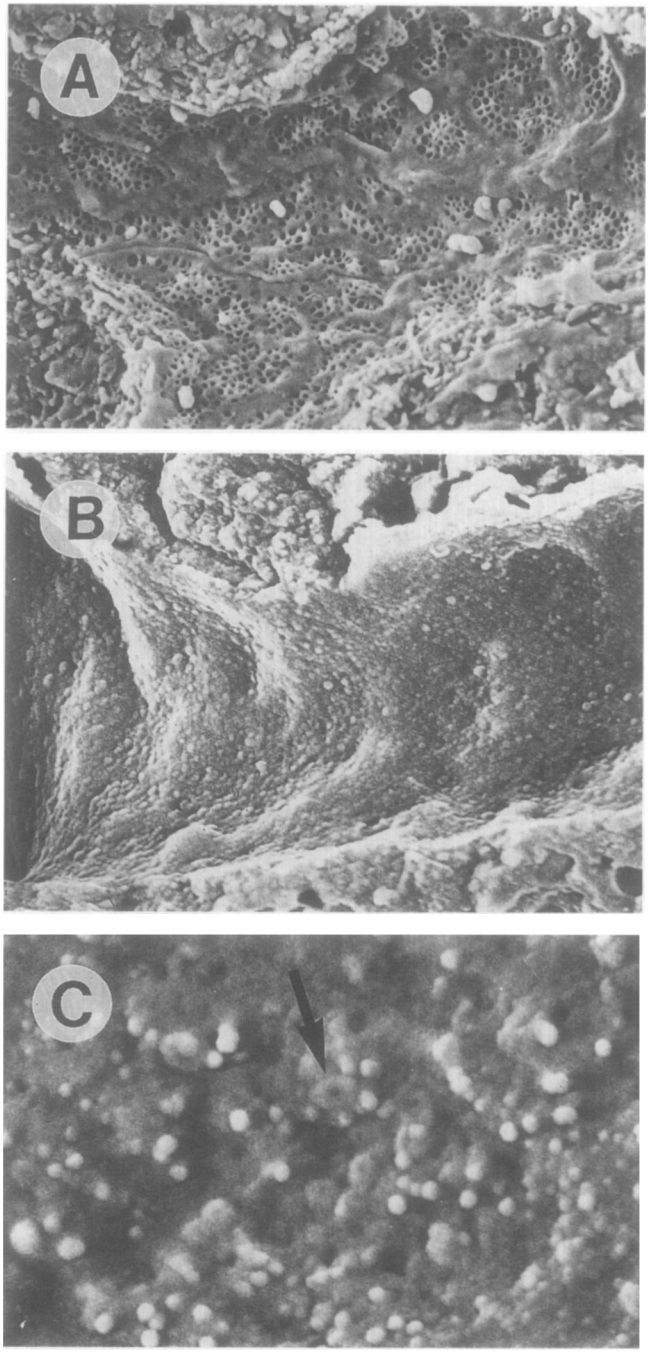

FIG. 1. Effect of MHV3 infection on the fenestration pattern of susceptible BALB/c mouse liver EC. Sinusoid in a control animal, displaying well-preserved fenestrae (A). (Original magnification ×7,000.) Sinusoid of a mouse infected for 48 hours appearing devoid of pores (B). (Original magnification ×7,000.) A high magnification suggests the budding of MHV3 particles at the cellular membrane. The surface of the cell is rough owing to the presence of small bulging craters (arrow) (C). (Original magnification ×30,000.)

The reduction in the number of pores was also confirmed by the observation of thin sections from 48-hour-infected mice livers. At the same time, numerous virus particles have been produced and may be found either in the EC (Fig. 3A) or in the space of Disse on the

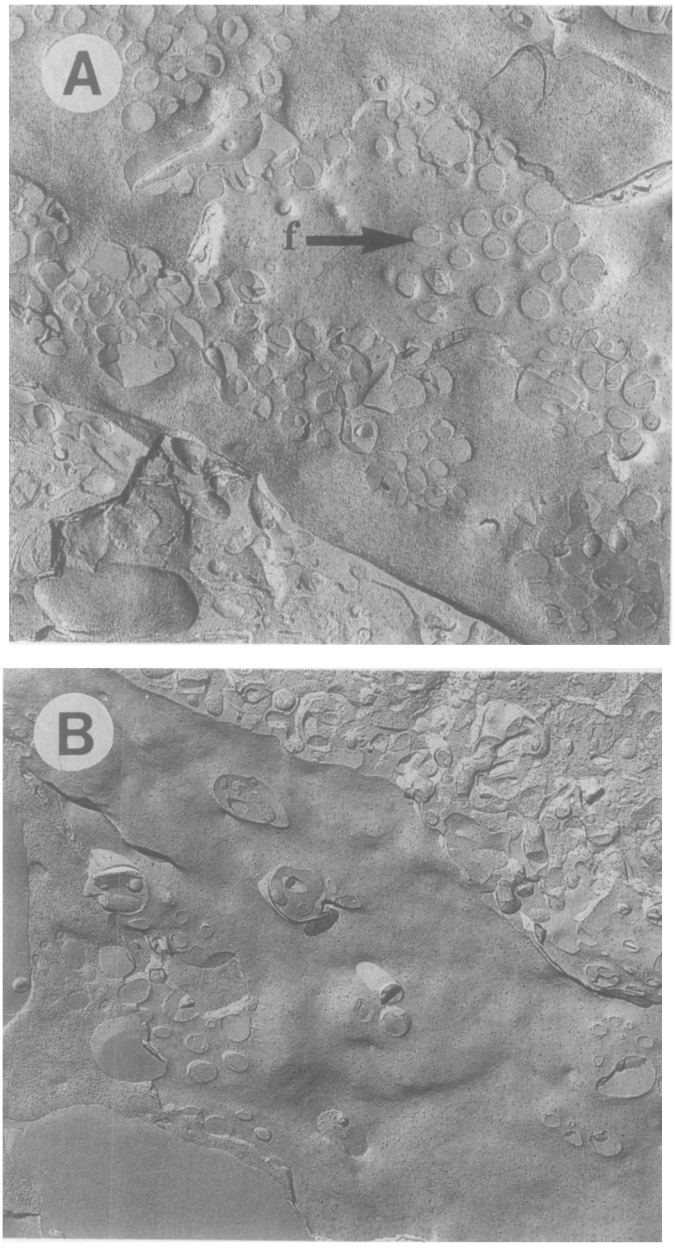

FIG. 2. Cryofracture study of the sinusoids of infected or non-infected BALB/c mice. In the control numerous fenestrae (f) are present (A). (Original magnification ×25,000.) In the animals infected for 48 hours, their number is notably reduced and the regular distribution of intramembrane particles suggests that fenestrae were not only shrunken, but completely disappeared (B). (Original magnification ×25,000.)

way to infect the hepatocytes (Fig. 3B). MHV3 antigens shown by immunofluorescence were frequently associated with the endothelial lining at that time of infection (results not shown).

Infection of Immunized BALB/c Mice and Genetically Resistant A/J Mice. To know if the alteration of the fenestration pattern is related to virus multiplication, experiments were carried out in immunized susceptible BALB/c mice and in A/J mice genetically resistant to MHV3, in which the replication of the virus is restricted. BALB/c mice were immunized with a thermosensitive mutant of MHV3 conferring protection against a lethal challenge with the wild strain. When immunized animals were infected with MHV3, neither viral antigens nor necrotic foci could be detected. Examination under scanning electron microscopy of liver specimens of animals infected for 48 hours showed well-preserved fenestrae (Fig. 4A) with a porosity similar to controls of around 10%.

In A/J mice, viral replication in the liver is restricted. Forty-eight hours after infection, only a few inflammatory foci may be found. At the same time, the fenestration pattern of the EC appears similar to that of uninfected animals (Fig. 4B), with a porosity of approximately 10%.

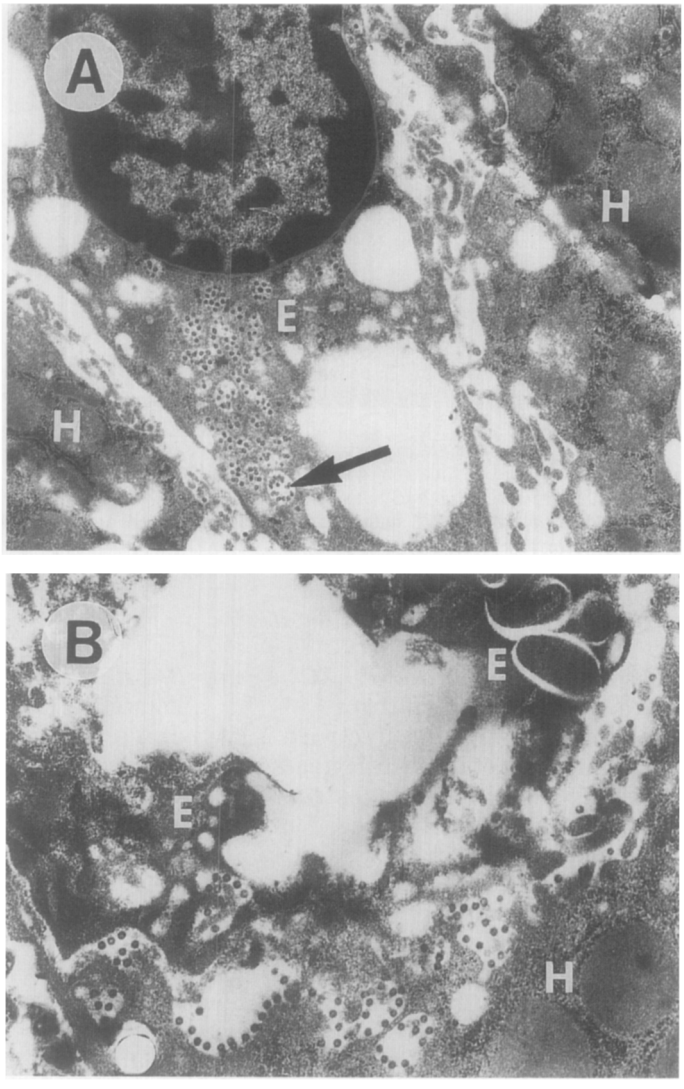

FIG. 3. TEM of EC (E) from the liver of a BALB/c mouse infected for 48 hours with MHV3. Numerous viral particles (arrow) are lying inside cytoplasmic vacuoles (A). (Original magnification ×12,300.) The MHV3 particles are released inside the space of Disse from which they are taken up by the hepatocytes (H) (B). (Original magnification ×17,400.)

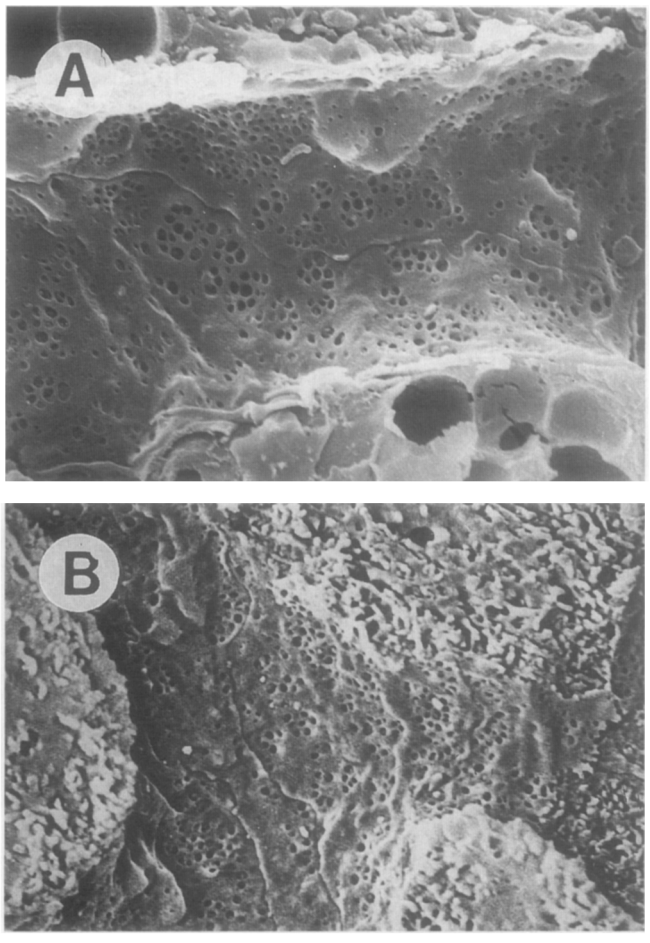

FIG. 4. Effect of MHV3 infection on the fenestration pattern in mice in which viral replication is restricted. Sinusoids from the liver of a BALB/c mouse that had been immunized with a temperature-sensitive mutant of MHV3 before being infected for 48 hours with the wild strain (A) (original magnification ×9,000), and from a liver of a genetically resistant A/J mouse after the same time of infection (B). (Original magnification ×6,000.)

Infection of Cultured EC From BALB/c Mice. Twenty-four-hour cultures of EC isolated from BALB/c mice were infected with MHV3 at a multiplicity of infection of 0.01 and fixed 24 hours later. As may be seen in Fig. 5A, the fenestrae in uninfected cells are well preserved and arranged in sieve plates. In the syncytia resulting from the infection (Fig. 5B) the fenestrae have almost completely disappeared (Fig. 5C), and numerous newly formed virus particles may be observed at the cell surface (Fig. 5C and 5D). It should also be noted that single cells whose infection with MHV3 was not obvious were also devoid of fenestrae (results not shown).

Effect of the Treatment With Cytochalasin B. In a recent study we reported that the treatment of EC with CB (10 μg/mL) induces a 3 to 4 times increase in the number of fenestrae.[35] In the current work we wondered if infected EC were still responsive to that drug. Therefore, livers of mice infected with MHV3 for 48 hours were perfused with CB during 1 hour, but no fenestrae could be induced (Fig. 6A) as compared with livers of uninfected mice treated in the same way (Fig. 6B). The porosity of these CB-perfused livers reached less than 1% in infected mice versus about 20% in controls. The same experiment done with cultured EC led to similar conclusions. If a 2-hour treatment of control cells resulted in a striking increase in the number of fenestrae (Fig. 6C), infected EC demonstrated only a few enlarged pores (Fig. 6D).

DISCUSSION

The first target for MHV3 infection in the liver is Kupffer and endothelial cells in which the virus multiplies before being released into the space of Disse, from where it may infect the hepatocytes.[44] We have already stressed the role of the sinusoidal cells in the pathogenesis of MHV3 by demonstrating that genetically resistant mice become susceptible to the virus if their sinusoidal lining is damaged.[13] Levy et al found more viral antigen in "reticuloendothelial cells" of susceptible BALB/c mice than in those of resistant A/J mice.[49]

The decrease in the number of fenestrae seems to be the first visible alteration of hepatic EC infected with MHV3. This change affects the EC in the liver of infected animals as well as EC infected *in vitro*. The decrease in the number of fenestrae starts in the vicinity of the periportal area as early as 24 hours after infection and affects the whole sinusoid after 48 hours as demonstrated by a decrease in the porosity. Levy et al, who studied the perturbations in the microcirculation during MHV3 infection, found that microthrombi in the sinusoids, which we observed also in our study, after 24 hours of infection were most frequently localized in the periportal zone.[49]

The fact that MHV3 does not induce a modification in the pattern of the fenestration in immunized BALB/c mice or in genetically resistant A/J mice demonstrates that viral multiplication is necessary for this phenomenon to occur. Moreover, the defenestration is due to the direct action of the virus and not to a mediator because it may be produced in cultured infected cells. In cells infected *in vitro* with MHV3, the fenestrae disappear not only in multinucleated cells but also in single cells, and there is direct relationship between the amount of viral particles or viral antigen and the intensity of defenestration. This argues for an early step of the viral cycle to be involved. Two factors may be responsible for the change in the fenestration pattern: (1) a fusion process of the cellular membrane, because MHV3 is a fusion-inducing virus; (2) an alteration of the cytoskeleton. High amounts of MHV3 may lead to the fusion of cells within 45 minutes, when glycoprotein E2, the fusion protein, is cleaved in two 90 K fragments.[50,51] One may thus speculate that disappearance of fenestrae by membrane fusion may occur as early as MHV3 released from lyzed KC enters in contact with the membrane of the EC. Conversely, fusion of cellular membrane occurs in the course of the infection: newly synthesized virus-specific proteins that mediate the appearance of the typical syncytia are

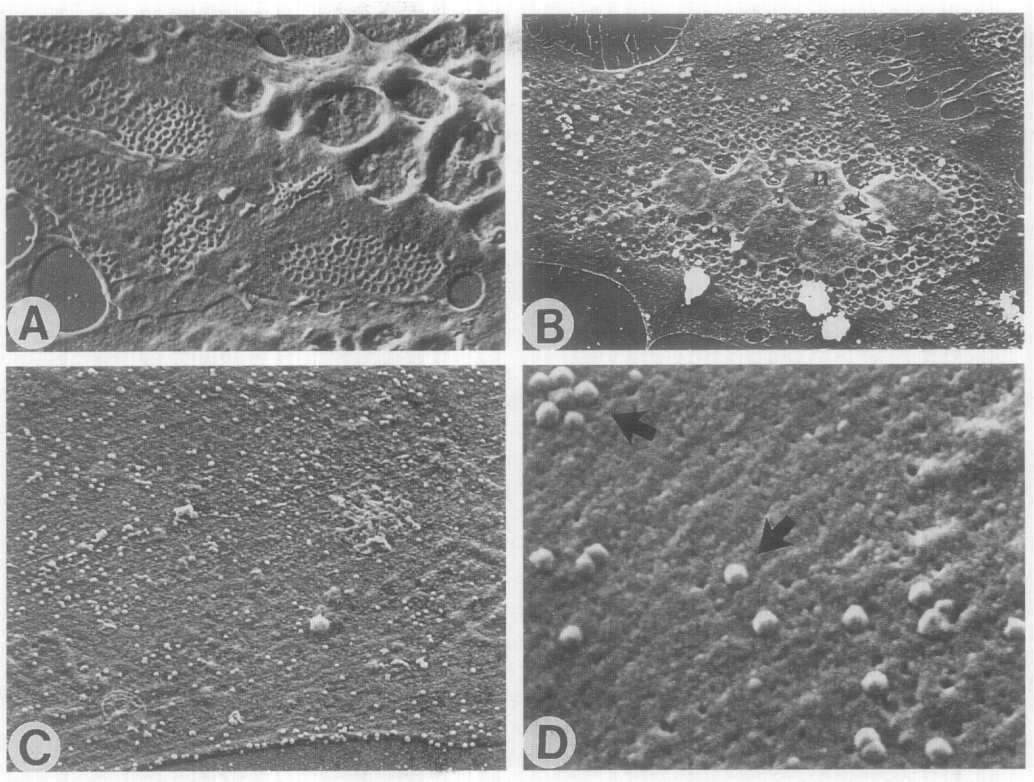

FIG. 5. Effect of MHV3 infection on cultured liver EC from BALB/c mice. Noninfected controls display typical fenestrae arranged in sieve plates (A). (Original magnification ×7,500.) Appearance of a syncytium with 6 nuclei (n) 24 hours after infection (B). (Original magnification ×1,400.) At higher magnifications the absence of pores becomes evident and viral particles (arrows) are distinguished at the cell membrane (C, D). (Original magnification ×10,000, ×40,000.)

associated with the plasma membrane.[52] The absence of small pores in the cryofracture samples of infected cells gives evidence that a fusion of the plasma membrane occurred. However, experiments in which fusion is inhibited either by monoclonal antibodies directed against glycoprotein E2 or by protein inhibitors preventing its cleavage would be interesting and help demonstrate the involvement of fusion. The second factor that may be responsible for the defenestration, the alteration of the cytoskeleton, fits with fusion processes, because microfilaments are involved in the formation of fenestrae and in the modulation of their number.[35] The fact that CB fails to induce fenestrae in infected cells strongly suggests that cytoskeleton function is im-

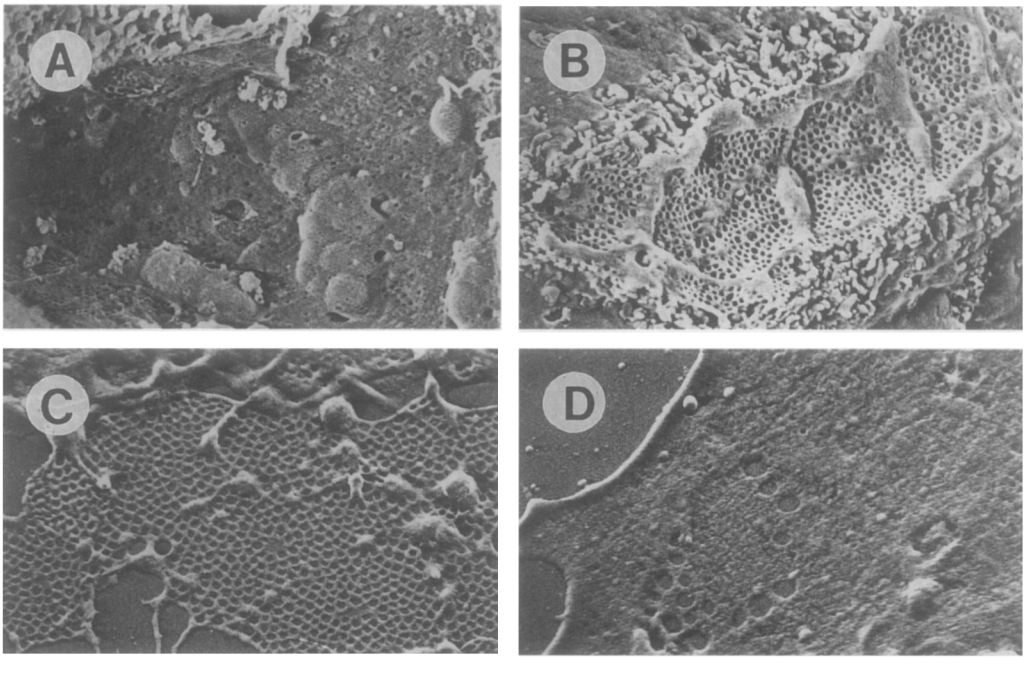

FIG. 6. Effect of cytochalasin B (CB) on the fenestration pattern of noninfected and MHV3-infected EC *in vivo* (A, B) and *in vitro* (C, D). Rare structures recalling fenestrae are observed in BALB/c mice infected for 48 hours that were perfused for 1 hour with CB before fixation (A) (original magnification ×7,500), whereas in controls numerous fenestrae are almost uniformly scattered all over the cellular surface (B). (Original magnification ×7,500.) In cultured EC treated with CB for 2 hours, the cytoplasm is highly fenestrated (C) (original magnification ×7,000.) in contrast to 24-hour-infected cells in which only a few enlarged pores could be induced (D). (Original magnification ×16,000.)

paired. Interaction of viruses with the cytoskeleton leads to very different effects on the cells.[36-41] Whereas cytomegalovirus produces the disruption of microfilaments[39] and measles virus leads to a diminution in the amount of actin,[36] herpesvirus induces an increase in the actin content of the cell.[41] The interaction of MHV3 with the cytoskeleton is only poorly documented. Mallucci and Edwards[53] have shown that there is a relationship between cell fusion and the actin myosin system. An alteration of the cytoskeleton may already occur in MHV3 infection during the first stages when the viral particles penetrate into the cells by fusion, which may lead to changes in the permeability of the membrane, allowing small ions to destabilize the cytoskeleton.

We are studying the interactions between primary cultures of human liver sinusoidal cells with human immunodeficiency virus HIV-1.[54] This retrovirus known to induce also cellular fusions leads to no obvious modifications of the fenestration pattern in the liver of acquired immunodeficiency syndrome patients. Moreover, *in vitro,* fenestrae are still present in virus-producing human liver EC.[54] We observed the same phenomenon in cat liver endothelial cells infected with FIV, the feline immunodeficiency virus (manuscript in preparation). It must be stressed that in the EC infected with these two retroviruses we could not detect any alterations of the microfilaments (Unpublished results).

A decrease in the number of fenestrae has been reported in diverse pathological situations like tumor invasion of the liver[19,23,34] and ethanol absorption.[17,20,25,27] A loss of sieve plates was also observed in chronic active hepatitis[55] but, because the liver biopsy specimens were fixed by immersion, caution must be exerted to interpret these results. One may wonder what the consequences of the defenestration *in vivo* may be. It has been postulated that the decreased porosity of the liver sieve hinders the passage of cholesterol-rich chylomicron remnants from the blood to the hepatocytes and induces perturbation of the lipoprotein metabolism, thus favoring accumulation of fat in the hepatocytes, which could be a factor in atherogenesis.[8] The steatosis characterized by an increased amount of neutral lipids described in the livers of MHV3-infected mice by others in our laboratory[56] has also been observed in the current study, but the relationship between the decrease in the porosity and steatosis could not be established until now. Moreover, the infection of mice with a thermosensitive mutant of MHV3, which does not change the porosity of the EC, nevertheless produces an acute steatosis.[57]

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