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The virulence of mouse hepatitis virus 3, as evidenced by permissivity of cultured hepatic cells toward escape mutants

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It has been shown that the spike (S) glycoprotein from the *Coronaviridae* is a determining factor in the cell fusion properties as well as in the neuropathogenic effects of some isolated murine hepatitis viruses (MHV) (Fleming *et al.*, 1986; Makino *et al.*, 1987; Daziel *et al.*, 1988; Wege *et al.*, 1988). It therefore seems to play a major role in the pathogenicity of the virus and influences its replication in tissue cultures and its ability to invade various organs in the animal (Daziel *et al.*, 1986; Wege *et al.*, 1988). The role of S glycoprotein in determining MHV3 virulence has been studied superficially, although the nucleotide fingerprints of hepatotropic MHV3 and neurotropic MHVA59 were very similar (Lai *et al.*, 1981).

We prepared monoclonal antibodies (mAb) for the S glycoprotein and used them to select attenuated MHV3 escape mutants for further investigations into the viral pathogenicity and hepatotropism of MHV3, which produces an acute fulminating hepatitis with extensive necrosis of the liver in susceptible BALB/c mice (Lucchiari *et al.*, 1991; Martin *et al.*, 1988; 1990).

Among the 60 mAb obtained by the method of Fazekas de St Groth and Scheidegger (1980), 15

were able to neutralize the subcloned MHV3 (MHV3 wt) at a dilution of over 1/5,000 (table I). All fifteen of them recognized the S protein in its precursor form (120 kDa), its glycosylated uncleaved form (180 kDa) and its cleaved mature form (90 kDa). Among these antibodies, 12 were also able to inhibit cell fusion, if they were added 2 h after viral infection. The 15 above mentioned mAb were used to select escape mutants. In each, 1.5×10^5 infectious particles were incubated with an individual mAb at a 1/100 dilution. The number of mutants isolated varied for each mAb. The mutation frequency was about 10^{-4} to 10^{-5} . Initially, 283 escape mutants were isolated and, after recloning in presence of the specific mAb, 100 PFU of each were injected by the intraperitoneal route into five 8- to 12-week-old sensitive BALB/c mice (IFFA Credo, L'Arbresle, France) free of coronavirus antibodies. With MHV3 wt, the mice died 3 to 4 days after infection. The infection of mice with MHV3 51.6 and MHV3 Cl12 obtained with two different mAb (A51 and A37, respectively) identified by agar gel diffusion as being an IgG1 and an IgA isotype, provoked a delay in mortality (fig. 1A). The MHV3 51.6 was of medium virulence, with mice dying 5 to 9 days

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Table I. Biochemical and biological characterization of mAb.

mAb	Neutralization	Inhibition	Protection of mice	Isotype	Number of mutants
A2	>10,000	1,000	9/10	IgG1	2
A6	5,000	0	6/10	IgG2b	23
A15	10,000	0	6/10	IgG1	29
A34	5,000	0	4/10	IgG2a	35
A37	>10,000	400	0/10	IgA	21
A42	10,000	400	3/10	IgG2a	23
A45	>10,000	1,000	7/10	IgG3	1
A46	5,000	100	10/10	IgG2a	19
A48	10,000	100	7/10	IgG2a	26
A50	>10,000	1,000	5/10	IgG1	0
A51	10,000	100	0/10	IgG1	24
A53	>10,000	1,000	9/10	IgG1	1
A55	>10,000	400	7/10	IgG2a	15
A56	5,000	0	5/10	IgG2a	19
A60	>10,000	400	4/10	IgG2a	45

The titre of neutralization and inhibition of cell fusion was the highest dilution able to halve the number of plaques. Protection *in vivo* was tested by injection of 0.2 ml of an ascites fluid into mice 1 day before the injection of 100 PFU of MHV3 wt.

after infection; MHV3 C112 was less virulent, with mice dying 8 to 10 days post-infection.

Virus replication in the livers of BALB/c mice infected with MHV3 wt, MHV3 51.6 or MHV3 C112 (fig. 1B) corresponded to the degree of virulence shown by these viruses in BALB/c mice. In MHV3-wt-infected mice, the virus titres reached a peak 2 days after infection. In MHV3-51.6-infected mice, the virus titre increased gradually to reach a peak 8 days after infection. In MHV3-C112-infected mice, virus titres were not detectable until 4 days after infection, when they started to increase.

To correlate these *in vivo* data with *in vitro* experiments, we isolated four types of hepatic

cells. The predominant cells in the liver are hepatocytes (Hc), which numerically represent about 60% of the total liver cell population. Hc were isolated according to Berry and Friend (1969) and Seglen (1976), partially modified by Braunwald *et al.* (1991). The Kupffer cells (Kc), liver endothelial cells (Ec) and Ito cells (Ic) make up the sinusoidal wall. The Kc, which are macrophages in the liver and have immunological functions, are found inside the lumen of the hepatic sinusoid at strategic positions; the Ec form a barrier which separates the perisinusoidal space from the sinusoid and possesses characteristic fenestrae which act as filtration barriers and also provide a means of communication between the sinusoid and the space of Disse; the Ic, for fat

Ec = endothelial cell.
Hc = hepatocyte.
Ic = Ito cell.
i.p. = intraperitoneally.
Kc = Kupffer cell.
mAb = monoclonal antibody.

MHV = mouse hepatitis virus.
m.o.i. = multiplicity of infection.
PFU = plaque-forming unit.
S = spike.
wt = wild type.

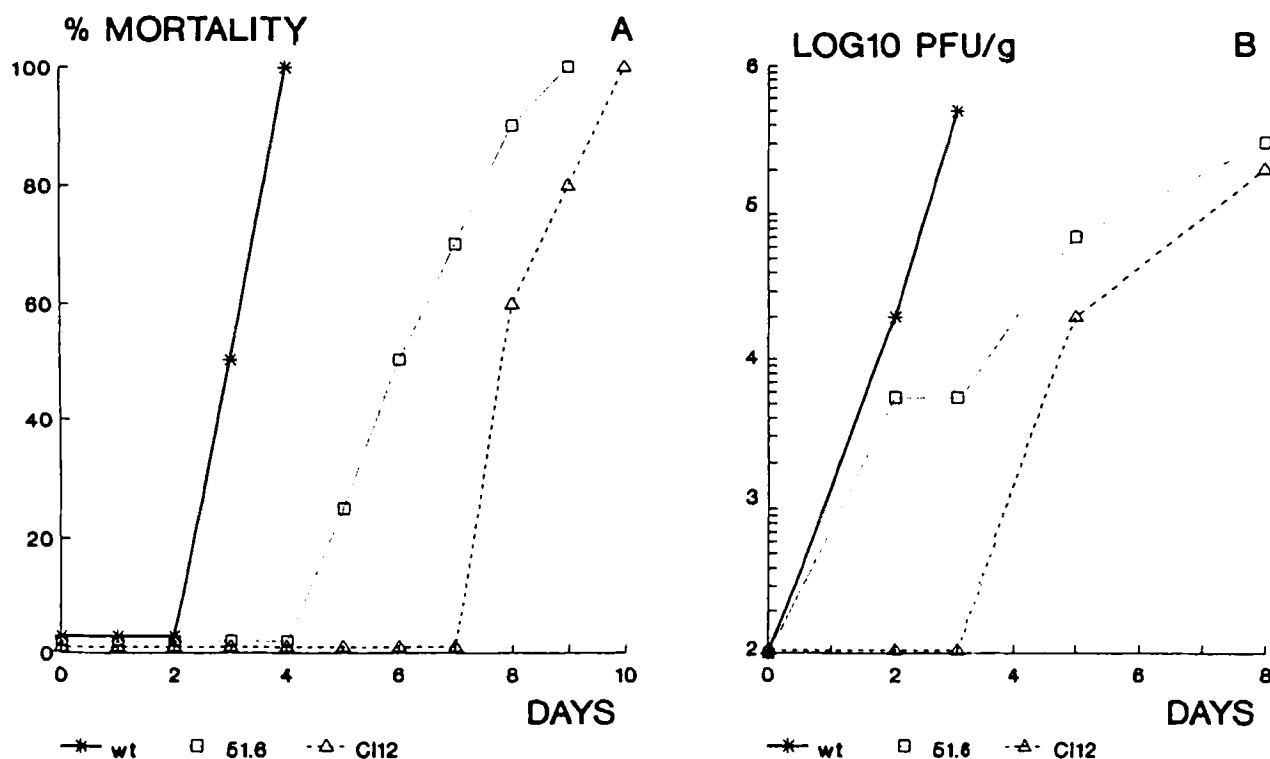


Fig. 1. Mortality (A) of mice infected with MHV3 and kinetics of virus growth in the liver (B).

Groups of 40 BALB/c mice were inoculated i.p. with 100 PFU of MHV3 wt (*), MHV3 51.6 (□) or MHV3 C112 (Δ). The animals were observed and the mortality recorded. At subsequent intervals, groups of 5 mice were sacrificed, the livers removed and the virus titrated. The virus titres reported as log₁₀ PFU/g of tissue are the average of 5 different determinations.

storage, are usually located in the sinusoidal recesses within the space of Disse and are known to play a role in storing vitamin A and maintaining the sinusoidal vascular structure. The technique for isolation of Kc and Ec has already been described (Steffan *et al.*, 1986), and Ic were prepared according to a previously described procedure (Chen *et al.*, 1989).

Virus multiplication in cultured hepatic cells is shown in figure 2. In all cell cultures, MHV3 wt, the most virulent, was capable of replicating faster than the other isolates, consistently reaching higher titres. MHV3 51.6, of medium virulence, replicated in the cultures, except in Ec where basal titres were found throughout the experiment. The less virulent MHV3 C112 was capable of replicating, reach-

ing low titres only in Ic and showing basal titres in the other cells throughout the experiment.

In this work, we compared the *in vivo* and *in vitro* virulence of two MHV3 mutants which escaped neutralization by one of two different anti-mAb. The procedure that we used for isolating the mutants led to the obtaining of a virus with a mutation in a determinant of the S glycoprotein, which is limited enough to enable replication in the L929 cell line used to propagate the virus. The data obtained indicate that the modified *in vivo* virulence observed for two MHV3 mutants could reflect an impairment of the replication in hepatic cells. Indeed, only basic viral titres were detected in some cultured hepatic cells. The basic titres found throughout the

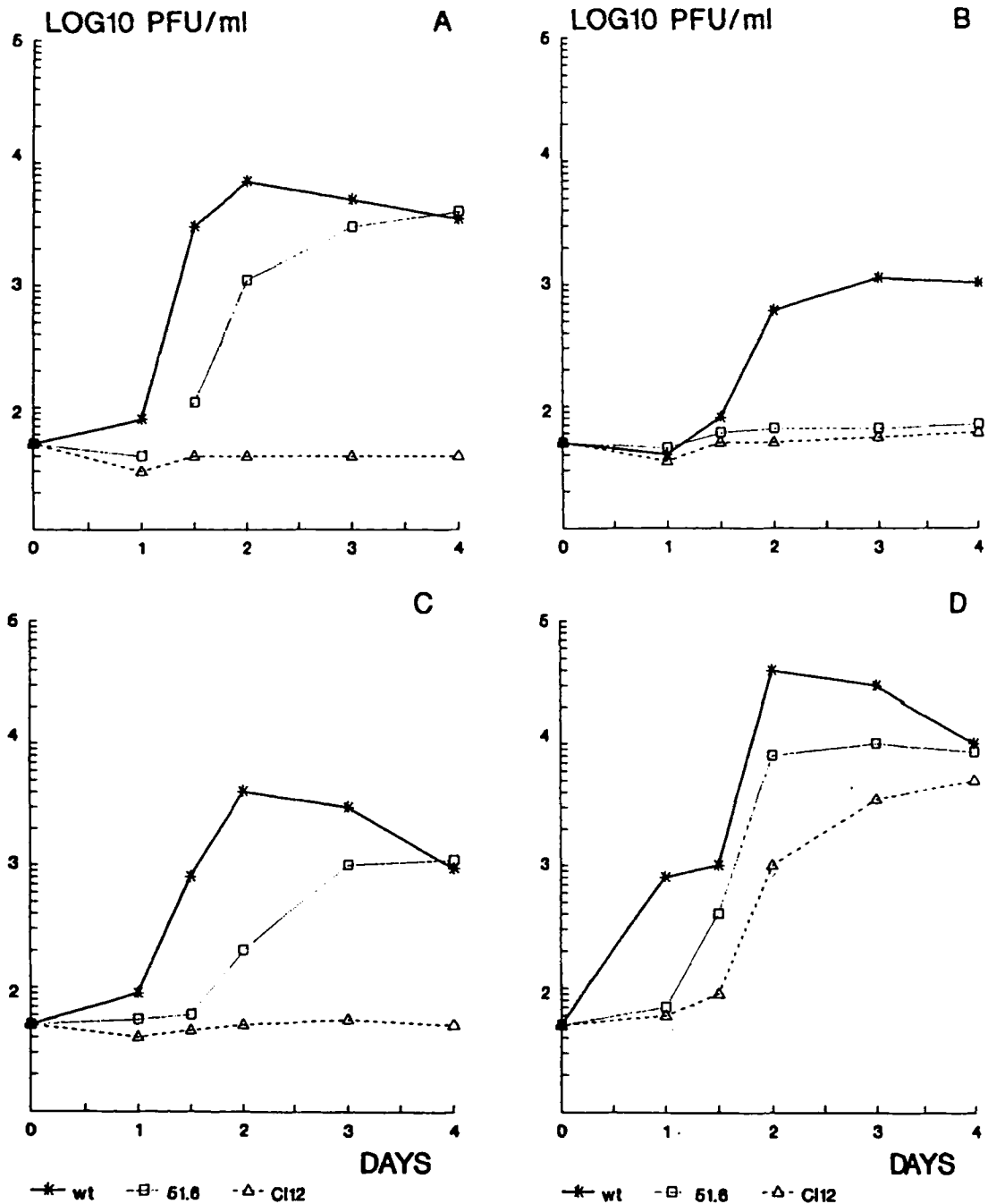


Fig. 2. Kinetics of MHV3 multiplication in cultured hepatic cells.

Cultures of Kupffer cells (A), endothelial liver cells (B), hepatocytes (C) or Ito cells (D) were infected with 0.1 m.o.i. of MHV3 wt (*), MHV3 51.6 (□) or MHV3 CI12 (Δ). Supernatants were collected at different times after infection and the virus titrated. The virus titres, reported as log₁₀ PFU/ml, is the average of 5 different experiments.

experiments, in Ec infected with MHV3 51.6 and in Kc, Ec and Hc infected with MHV3 C112, indicate that, although these cells are permissive for the virus, its replication is very limited, though sufficient to maintain virus titre for a long period of time.

The first hepatic cells in contact with the blood are Kc and Ec. After virus multiplication and lysis of these cells, the virus reaches the parenchymal cells, where its multiplication leads to focal necrosis that can be responsible for physiological dysfunction associated with fatal hepatitis. It should be borne in mind that impairment in adsorption of a given virus to strategically placed cells, such as Ec or Kc, may be a limiting factor in viral multiplication. This has important implications for the outcome of infection.

An alteration in the immune functions of BALB/c mice consecutive to the infection, as well as the selection of revertant viruses possessing a wild phenotype, could account for the observed results. However, the latter hypothesis has been excluded, since viruses isolated from the livers of mice infected by each of the mutants were still resistant to neutralization by relevant mAb. Our results are consistent with the notion that our MV3 escape mutants display at least one single mutation in a determinant of the S glycoprotein. These mutations could be responsible for impairment in recognition of a cellular receptor by the virus in hepatic cells (Martin *et al.*, 1990), thus hindering its multiplication in the liver and delaying the evolution of the disease.

Key-words: Hepatitis, MHV3, Replication, Permissivity; Hepatic cells, Escape mutants, Virulence.

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Corrélation entre le pouvoir pathogène de deux mutants d'échappement du virus de l'hépatite murine 3 et la permissivité des cellules hépatiques en culture primaire

Deux mutants d'échappement à la neutralisation du MHV3 (MHV 51.6 et MHV C112) montrent, en comparaison au MHV3 sauvage (wt), un ralentissement de leur multiplication chez des souris sensibles BALB/c et une atténuation du pouvoir pathogène provoquant un retard considérable de la mortalité. Le MHV3 wt se réplique à des titres élevés dans toutes les cellules hépatiques en culture. Le MHV3 51.6, de virulence intermédiaire, se multiplie dans toutes les cellules hépatiques excepté dans les cellules endothéliales. Le mutant qui tue le plus tardivement les souris (MHV C112) se multiplie uniquement dans les cellules de Ito, les autres étant non permissives. Ces résultats montrent une corrélation directe entre la virulence du MHV3 et son aptitude à se répliquer de manière efficace dans les cellules hépatiques *in vitro*.

Mots-clés: Hépatite, MHV3, Réplication, Permissivité; Cellules hépatiques, Mutants d'échappement, Virulence.

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