CHARACTERIZATION OF A NON-PATHOGENIC MHV3 VARIANT DERIVED FROM A PERSISTENTLY INFECTED LYMPHOID CELL LINE

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INTRODUCTION

Mouse hepatitis virus type 3 infection leads to various types of evolution according to strain, age and immune status of animals[1,2]. Three types of viral sensitivity were observed: resistance, full susceptibility and semisusceptibility. The latter is characterized by a chronic disease with occurrence of paralysis, immunodepression, and viral persistency since MHV3 can be recovered during the first three months postinfection in most animals from brain, liver, spleen and lymph nodes[1]. During the first four days of infection, virus was recovered from the liver of resistant as well as susceptible strain mice. In resistant A/J strain mice, viral titers were consistently low whereas titers greater than 10^4 were always found in susceptible C57BL/6 animals. In the resistant mouse strain, virus was cleared from liver, brain and serum within seven days. In contrast, virus continued to replicate until death in susceptible animals[1]. Immunosuppressive treatment, however, can abogate the resistance displayed by adult A/J mice, leading to an acute disease and to death. Protection of susceptible newborn mice against MHV3 infection requires the transfer of several cell populations originating from syngenic adult donors: adherent spleen cells, T lymphocytes and a third population present in the non-adherent spleen cell fraction as well as in peritoneal exudate and in bone marrow cells[2,3]. Resistance to the acute disease was also correlated with viral replication in hepatocytes[4], in embryonic fibroblasts[5] and in peritoneal exudate cells[2,6,7]. Recent work suggested that at least two complementary mechanisms were required to confer resistance to MHV3: resistance genes operating at the macrophage level and cells capable to elicit an efficient immune response[8].

In previous works, we showed that persistent infections can be carried out in vitro in lymphoid cell lines originating from semisusceptible mouse strain. Virus progeny derived from such persistent infections has lost the ability to induce a lethal desease when injected into susceptible mice. The absence of pathogenicity was related to the induction of a subclinical infection which elicited defense mechanisms[9].

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The aim of the present work was to determine the mechanism(s) involved in the loss of viral pathogenicity of YAC-MHV3 virus by studying the level of viral replication in various organs and thus the permissivity of lymphoid cells and macrophages. We found that the YAC-MHV3 variant could not replicate in thymocytes but could replicate at a low level in macrophages. YAC-MHV3 virus displayed, however, a normal replication titers in mitogen-stimulated thymocytes. An additionnal intracellular viral protein of 72kD was detected in the YAC-MHV3 variant.

RESULTS AND DISCUSSION

Pathogenicity of YAC-MHV3 and L2-MHV3 according to mouse strains and sites of inoculation

In order to analyze the nonpathogenic properties of YAC-MHV3, compared to pathogenic L2-MHV3, resistant A/J and susceptible C57BL/6 mice were infected intraperitoneally (i.p.) or intracerebrally (i.c.) with 1000 $TCID_{50}$/0.1 ml of either viruses. As shown in Table 1, YAC-MHV3 variant did not induce any clinical disease in animals injected i.p. or i.c. In contrast, only resistant A/J mice injected i.p. with pathogenic L2-MHV3 survived. C57BL/6 mice, inoculated i.c. or i.p., as well as A/J mice inoculated i.c. died of an acute disease.

In both susceptible and resistant mouse strains, the liver is the main target organ of MHV3 infection and is a priviliged site for viral replication[1]. After i.p. injection of A/J mice, virus titers remained low and liver cellular lesions scarce, suggesting that the target cell population, likely macrophages, may constitute the first line of defense during i.p. infection. Such a cell population may be deficient in the brain, so that dissemination of infection with fully pathogenic particles can occur in the absence of a prior attenuation by the macrophage barrier. In addition, the absence of in vivo pathogenicity of YAC-MHV3 following i.c. inoculation suggested a marked attenuation of the virus.

Table 1. In vivo pathogenicity of YAC-MHV3 and L2-MHV3 in genetically resistant A/J and susceptible C57BL/6 mice according to sites of inoculation.

Virus	Mouse strain	No surviving / No tested	
		Intraperitoneal	Intracerebral
YAC-MHV3	A/J	12/12	12/12
	C57BL/6	12/12	12/12
L2-MHV3	A/J	12/12	0/12
	C57BL/6	0/12	0/12

Adult mice were infected with 1000 $TCID_{50}$/0.1 ml of YAC-MHV3 or L2-MHV3.

Table 2. Viral titers in various organs at 48 hours postinfection, following intraperitoneal or intracerebral inoculation of YAC-MHV3 and L2-MHV3 in resistant A/J and susceptible C57BL/6 mice.

Virus	Organ	Virus titers[a]			
		Intraperitoneal		Intracerebral	
		A/J	C57BL/6	A/J	C57BL/6
YAC-MHV3	Brain	6.5	2.8	5.1	4.8
	Liver	3.1	6.1	7.1	3.1
	Spleen	3.1	2.8	2.8	3.9
	Thymus	<1.6	<1.6	<1.6	<1.6
L2-MHV3	Brain	5.6	5.1	4.1	>7.1
	Liver	2.0	3.1	2.8	3.1
	Spleen	5.1	4.1	5.1	3.1
	Thymus	2.4	3.1	3.1	2.5

[a] $\log_{10}TCID_{50}$/ml. Adult mice were infected with 1000 $TCID_{50}$/0.1 ml. of YAC-MHV3 or L2-MHV3.

In order to determine the mechanism of attenuation of YAC-MHV3 variant, YAC-MHV3 and L2-MHV3 virus titers, at 48 hr postinfection, were recorded in brain, liver, spleen and thymus after i.c. or i.p. inoculation or resistant A/J or susceptible C57BL/6 mice. As shown in Table 2, no significant differences between both mouse strains were observed in viral titers recorded in organs of animals infected with L2-MHV3. Following infection with YAC-MHV3, similar titers were observed in brain and liver of resistant or susceptible animals. Lower viral titers, however, were displayed in spleen and no virus was detected in thymus of resistant or susceptible animals following either i.c. or i.p. inoculation. This suggests that the non-pathogenicity of YAC-MHV3, following i.c. or i.p. inoculation, may be related to the inability of YAC-MHV3 to replicate in thymic cells and, to a lesser extent, in spleen cells. These results are in agreement with previous work showing that normal T cell functions were required for the phenotypic expression of resistance and that resistance could be conferred by thymocytes and adherent spleen cells[2,8].

Restriction of YAC-MHV3 and L2-MHV replication in carrier state infections carried out in peritoneal exudate (PE), non-adherent spleen (NAS) cells and thymocytes (THY) originating from various mouse strains

Expression of in vivo pathogenicity of MHV3 in macrophages and lymphoid cells was evaluated by induction of carrier state infections in PE, NAS and THY cells collected from resistant A/J, susceptible C57BL/6 and semisusceptible A/Sn mice. Cell populations were cultured for 24 hr prior to infection with YAC-MHV3 or L2-MHV3 at a multiplicity of infection (m.o.i.) of 0.01 to 0.1. Carrier state infections were maintained by successive transfers of supernatants, collected every other day from viral infected cells, and used as virus inoculum 1:10 (v/v) for infection of newly collected cells, originating from the same mouse strain. Cytopathic effects, characterized by focus formation, and virus titers in supernatants were evaluated every other day. As shown in Table 3, infectious virus was not detected after 4 days of culture when cell populations from A/J, C57BL/6 or A/Sn mice were infected with YAC-MHV3. A similar restriction

Table 3. YAC-MHV3 or L2-MHV3 infections produced into peritoneal exudate (PE), non-adherent spleen (NAS) and thymic (THY) cells from resistant A/J and susceptible C57BL/6.

Virus	Mouse strain	Cells	Virus titers[a] Time postinfection (days)[b]				
			2	4	6	8	10
YAC-MHV3	A/J	PE	4.3	<1.6	<1.6	<1.6	<1.6
		NAS	<1.6	<1.6	<1.6	<1.6	<1.6
		THY	<1.6	<1.6	<1.6	<1.6	<1.6
	C57BL/6	PE	2.6	<1.6	<1.6	<1.6	<1.6
		NAS	<1.6	<1.6	<1.6	<1.6	<1.6
		THY	<1.6	<1.6	<1.6	<1.6	<1.6
	A/Sn	PE	3.4	<1.6	<1.6	<1.6	<1.6
		NAS	<1.6	<1.6	<1.6	<1.6	<1.6
		THY	<1.6	<1.6	<1.6	<1.6	<1.6
L2-MHV3	A/J	PE	4.3	<1.6	<1.6	<1.6	<1.6
		NAS	3.7	2.2	<1.6	<1.6	<1.6
		THY	2.8	<1.6	<1.6	<1.6	<1.6
	C57BL/6	PE	5.8	6.4	4.3	7.1	4.5
		NAS	3.8	3.8	2.8	2.0	2.9
		THY	5.0	4.3	2.8	3.2	5.0
	A/Sn	PE	3.4	5.5	4.3	5.2	4.5
		NAS	3.4	2.3	2.7	2.2	<1.6
		THY	4.3	3.4	2.2	2.0	<1.6

[a] $\log_{10}TCID_{50}$/ml.

[b] Cells were cultured for 24 hr and infected with 0.01 to 0.1 m.o.i. of YAC-MHV3 or L2-MHV3. Carrier state infections were maintained by successive transfer of supernatants collected every other day from infected cells and used as virus inoculum (1:10) for infection of newly collected cells originating from the same strain of mice.

Table 4. YAC-MHV-MHV3 replication in phytohemagglutinin (PHA)- stimulated thymocytes (THY) from various strains.

Doses of PHA (ug)	Virus titers[a] in THY from:		
	A/J	C57BL/6	A/Sn
10	3.8	4.8	4.1
20	3.1	4.3	4.1
40	3.1	3.3	4.8
0	<1.6	<1.6	<1.6

[a] $\log_{10}TCID_{50}$/ml at 48 hr postinfection.

was observed with A/J cells infected with L2-MHV3. However, no or delayed restriction occurred in susceptible (C57BL/6) or semisusceptible (A/Sn) cells infected with L2-MHV3. These results suggest that the non-pathogenicity of YAC-MHV3 may be associated with the loss of ability to infect lymphoid cells in spleen and thymus. The restriction of YAC-MHV3 in macrophages follows the same pattern as that of the pathogenic L2-MHV3 in genetically resistant A/J. In addition, YAC-MHV3 which is a variant produced in a lymphoid T cell line, has lost, as shown herein, the ability to infect thymocytes.

In order to explain such apparent discrepant results, THY cells were stimulated with phytohemaglutinin (PHA) and subsequently infected with YAC-MHV3. As shown in Table 4, the viral restriction observed in THY cells was abrogated when cells, collected from A/J, C57BL/6 or A/Sn mice, were stimulated with PHA. These results indicate that YAC-MHV3 has lost the ability to infect resting cells only, but not dividing THY cells.

Analysis of intracellular proteins of YAC-MHV3 and L2-MHV3

Modifications of intracellular viral proteins, may be related to the nonpathogenic property of YAC-MHV3, were studied by using PAGE-electrophoretic separation followed by immunoblotting[10] using various antisera. L2 cells were infected with 1 to 10 m.o.i. of L2-MHV3 or YAC-MHV3. Cells were harvested at 8 hr postinfection and prepared as previously described. Viral proteins were revealed by using a rabbit polyclonal antiserum directed against purified L2-MHV3 which was detected with a anti-rabbit antiserum conjugated to peroxidase, as well as with an antiserum prepared in A/J mice immunized twice with L2-MHV3 and revealed with peroxidase-conjugated anti-mouse IgG. The presence of three major groups of viral proteins at least can be visualized in Fig. 1: i) two high molecular weight proteins, around 180 Kd and 90 Kd, in L2-MHV3 (lane 3) and YAC-MHV3 (lane 4) corresponding to surface glycoproteins; ii) a nucleoprotein complex including 60, 57 and 54 Kd proteins in L2-MHV3 and of 60, 57 Kd and at a higher concentration, 54 and 52 Kd proteins, in YAC-MHV3; iii) a non-structural protein of about 44 Kd. The smaller glycoproteins were not detected in such experimental conditions.

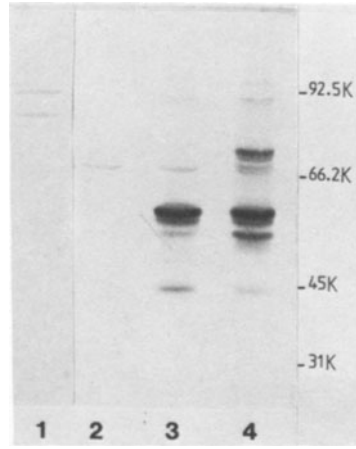

Fig. 1. Intracellular proteins of pathogenic L2-MHV3 and non-pathogenic YAC-MHV3 revealed by PAGE-electrophoresis followed by an immunoblotting using rabbit anti-L2-MHV3 antiserum and anti-IgG antisera conjugated to peroxidase. Lane 1: YAC cells; lane 2: L2 cells; lane 3: L2-MHV3 and lane 4: YAC-MHV3.

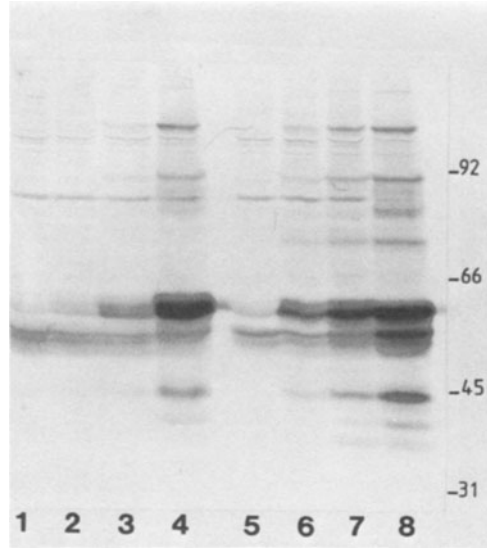

Fig. 2. Kinetic studies of intracellular L2-MHV3 (lanes 1 to 4) and YAC-MHV3 (lanes 5 to 8) revealed by PAGE-electrophoresis followed by an immunoblotting using mouse anti-L2-MHV3 antiserum and anti-IgG antiserum conjugated to peroxidase. Lanes 1 and 5: 4 hr p.i.; lanes 2 and 6: 6 hr p.i.; lanes 3 and 7: 7 hr p.i., and lanes 4 and 8: 9 hr p.i.

An additional viral protein of 72 Kd was detected in YAC-MHV3 only. No viral protein or virus-related protein was observed in YAC and L2 uninfected cells.

Kinetic studies were performed by L2-MHV3 or YAC-MHV3 infection of L2 cells which were harvested at 4, 6, 7 and 9 hr postinfection and treated as previously described. As shown in Fig. 2 in lanes 1 to 4, and lanes 5 to 8, corresponding to L2-MHV3 and YAC-MHV3 respectively, the occurrence of 52, 54 and 57 Kd proteins was observed at 4 hr postinfection. At 7 hr postinfection (lanes 3 and 7), surface glycoproteins of 90 and 180 Kd was detected in L2-MHV3 and YAC-MHV3 infected cells. Such proteins can be detect as soon as at 6 hr postinfection (lane 6) in YAC-MHV3 infected cells. The 60 Kd protein can be visualized at 7 hr postinfection but at a higher concentration in YAC-MHV3 (lane 7) than in L2-MHV3 (lane 3) infected cells. The 54 Kd protein (lanes 4 and 8) as well as the non-structural proteins of 45 Kd and 40 Kd were detected at 9 hr postinfection at an higher concentration in YAC-MHV3 than in L2-MHV3 infected cells. The 72 Kd protein was detected at 6 hr postinfection in YAC-MHV3 infected cell only (lane 6).

These observations suggest that the nucleoprotein of pathogenic L2-MHV3 can gradually mature to form the 60 Kd protein, but immature, lower molecular weight proteins, seem be conserved at a high concentration in YAC-MHV3. In addition, the 72 Kd protein was observed in YAC-MHV3 infected cells only and started to occur 6 hr after infection. This suggests that the nucleoprotein undergoes various steps of maturation before being functional and that any alteration in the maturation steps may result in modifications of the in vivo pathogenicity. The production of the 72 Kd protein during viral infection may be an observation of a major importance but no information is available as yet with regards to the role of this protein in viral pathogenicity. The rabbit or mouse antisera used in the experiments were prepared against L2-MHV3 and not against YAC-MHV3 and therefore contained antibodies against the 72 Kd protein. This may indicate that an antigenic 72 Kd protein was included or associated with the viral particles and or was generated during the in vivo infection in mice. This protein, however, was not produced, at a detectable level, during L2-MHV3 infection of L2 cells.

CONCLUSIONS

MHV3 persistent infection in YAC lymphoid cell line leads to the emergence of a virus a variant which can replicate in some target cells, since subclinical infection occurs in vivo, but which cannot induce a lethal acute disease in susceptible mice. The work presented here shows that this variant is highly attenuated since, even after i.c. inoculation, YAC-MHV3 virus cannot induce a lethal disease in susceptible mice. In addition, YAC-MHV3 variant has lost the ability to replicate in thymocytes and in non-adherent spleen cells derived from resistant as well as from susceptible mouse strains. The role of such target cells appeared to be important in the outcome of the disease since it was observed that whole spleen cells mixed with a mixture of spleen cell populations, or adherent spleen cells mixed with thymocytes, can protect x-irradiated susceptible A/J recipient against MHV3 infection[8]. Such cell preparations contained T lymphocytes and macrophages. Since YAC-MHV3 variant cannot infect or kill T lymphocytes and macrophages, this suggests that the integrity of these cells is essential to block the development of clinical manifestations and subsequent death. The lymphotropism of MHV3 appears therefore to play a major role in the outcome of the disease. The YAC-MHV3 variant, although produced by a T cell line, was not able, however, to replicate in resting cells but only in stimulated or dividing cells, indicating that the target cells are different for the variant and for the wild virus.

The YAC-MHV3 variant was also different in induction of viral protein synthesis in L2 cells. In YAC-MHV3 preparation, an additional protein of 72 Kd and a higher concentration of low molecular weight nucleoproteins were observed. Such results are in agreement with the work of Fleming et al.[11] indicating that the 60 Kd protein may be one of gene product which mediates the distinct disease patterns manifested by different murine coronaviruses. The sequencing by oligonucleotide mapping of mouse coronaviruses exhibiting various pathogenicities suggested that genetic sequences associated with viral pathogenicity were present in mRNA species 1 and 3, which corresponded to genes coding for the major glycoproteins and for RNA polymerase[12]. Work is in progress to identify the origin and the role of the 72 Kd protein and the cell mechanisms involved in the maturation steps of the nucleoprotein complex in order to understand the expression of such viral products in the MHV3 in vivo pathogenicity.

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