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USING A DEFECTIVE-INTERFERING RNA SYSTEM TO EXPRESS THE HE PROTEIN OF MOUSE HEPATITIS VIRUS FOR STUDYING VIRAL PATHOGENESIS

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1. ABSTRACT

We have developed a defective-interfering (DI) RNA of mouse hepatitis virus (MHV) as a vector for expressing a variety of cellular and viral genes including the chloramphenicol acetyltransferase (CAT), hemagglutinin'esterase (HE), and gamma interferon. Here, we used the HE-expressing DI RNA for examining the role of HE protein in viral pathogenesis. The pseudorecombinant virus containing an expressed HE protein was generated by infecting cells with MHV-A59, which does not express HE, and transfecting the *in vitro*-transcribed DI RNA containing the HE gene. These pseudorecombinant viruses (DE-HE A59) were then inoculated intracerebrally into mice. Viruses recovered from cells infected with A59 and transfected with DI RNA expressing the CAT gene (DE-CAT A59) were used as a control. At various time points after inoculation, mice were observed for clinical symptoms. Tissues (brains and livers) were obtained for determining the replication of DI RNA by RT-PCR, virus replication by plaque assay, antigen expression by immunohistochemistry, and pathological changes. Results showed that all mice infected with DE-CAT A59 succumbed to infection by 9 days postinfection (d p.i). These

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data are identical to the pathogenesis of the parental A59 virus, demonstrating that inclusion of the DI RNA did not by itself alter pathogenesis. In contrast, only 40 % of mice infected with DE-HE A59 succumbed to infection. The subgenomic mRNAs transcribed from the DI vector were detected at 1 and 2 d p.i. but not at subsequent time points, indicating that the genes in the DI vector were expressed only at an early stage of viral infection. No significant difference in virus replication in the brains was detected between these two groups of mice, suggesting that virus replication in brains was not affected by the expression of the HE. Histopathological examination showed only a small increase in the extent of inflammatory cell infiltration and reduced viral antigen in the mice infected with DE-HE A59. There was no difference in virus replication in the livers at 2 and 4 d p.i., but a 3 $\log_{10}$ reduction was detected in the livers of mice infected with DE-HE A59 at 6 d p.i. Histological examination showed a significant reduction in viral antigen, inflammation and necrosis in mice infected with DE-HE A59. These results indicate that the expression of HE from the DI vector altered the viral pathogenesis. This study thus demonstrates the usefulness of this system in studying the role of viral or cellular genes expressed locally at the sites of viral infection in viral pathogenesis.

2. INTRODUCTION

Mouse hepatitis virus, the prototype murine coronavirus, is a member of the *Coronaviridae*. It contains a single-strand, positive-sense RNA genome of 31 kb (Lee et al., 1991). Due to the unusual large genome size, it has not been able to make an infectious cDNA clone of any coronaviruses to date. Thus, studies of coronavirus pathogenesis at the molecular level have been largely hampered. The development of a defective-interfering RNA as an expression system provides an alternative genetic approach for defining the roles of individual viral gene products in viral pathogenesis.

Viral defective-interfering (DI) particles are usually generated under certain evolutionary pressure from infected hosts or tissue cultures. They require helper virus for their replication; but they in turn interfere with the replication of a co-infected helper virus. Many DI particles of MHV have been isolated, two, which have been derived from MHV strain JHM, are well-characterized (Makino et al., 1988). DIssE contains an RNA genome of 2.2 kb and DIssF 3.5 kb. They contain three and five fragments derived from discontinuous regions of the parental viral genome, respectively. These DI RNAs also contain essential cis-acting signals for replication, thus behaving like a minigenome. Upon transfection, they replicate efficiently in the presence of a helper virus. However, sinve these DI RNAs do not contain intergenic (IG) sequences, which serve as transcription initiation sites or promotors, they are not able to transcribe subgenomic mRNAs. Insertion of an IG sequence into the DI RNA allows transcription of a subgenomic mRNA from the IG site (Makino et al., 1991). Because of the small size as well as the presence of all *cis*-acting replication signals, DI RNAs have become an alternative yet powerful genetic approach for studying replication, transcription, recombination and pathogenesis of coronaviruses (Lai, 1992; Lin et al., 1993; Liao and Lai, 1992; Liao and Lai, 1994; Zhang et al., 1994).

MHV contains four or five structural proteins. The spike (S) protein, which forms the characteristic peplomers, is involves in virus attachment to the receptors of permissive cells, elicitation of neutralizing antibodies, and cell-fusion. The membrane (M) protein and the small envelope (E) protein are essential for virion assembly, morphogenesis, and formation of the viral envelope. The nucleocapsid (N) protein is associated with viral genomic RNA to

form the nucleocapsid. The hemagglutinin/esterase (HE) protein is an optional glycoprotein, present only in certain MHV strains (Yokomori et al., 1989). It contains both the receptor-binding (binding to sialic acid-containing receptor) and receptor-destroying (acetylesterase) activities (Yokomori and Lai, 1989), similar to the HEF protein of influenza C virus (Herrler et al., 1985a,b). In bovine coronavirus, which is from the same antigenic subgroup as MHV, HE appears to be important for virus infection, as monoclonal antibodies against the HE have been shown to neutralize bovine coronavirus infectivity (Deregt and Babiuk, 1987; Deregt et al., 1989). This is in contrast to MHV-DVIM, in which the S protein was prevented from interacting with its cell receptor but which had a functional HE protein, which was unable to initiate a productive infection (Gagneten et al., 1995). However, the presence of HE in the neuropathogenic strain JHM and the preservation of the function of HE known in other viruses raise an interesting question: Does the HE play a role in pathogenesis, particularly in neuropathogenesis, of the neuropathogenic MHV strain JHM in mice? Passive immunization of mice with HE monoclonal antibodies protected mice from lethal infection and altered the pathogenicity (Yokomori et al., 1992). JHM variant, which expresses abundant HE, exhibited more neurovirulent in C57BL/6 mice than one which expresses little HE (Yokomori et al., 1995). These findings suggest that the HE protein contributes to viral neuropathogenicity by influencing either the rate of virus spread and/or cell tropism (Yokomori et al., 1995). However, the contribution of other viral gene products cannot be rigorously ruled out. Thus, the exact role of the HE protein in MHV neuropathogenicity remains to be illucidated.

Recently, we developed the DI RNA expression system, in which the bacterial chloramphenicol acetyltransferase (CAT) gene (Liao and Lai, 1994), the MHV HE gene (Liao et al., 1995) and the mammalian cellular gene interferon gamma (Zhang et al., 1997) were placed behind an IG sequence as a promoter and were expressed in cell culture. In the present study, we used the HE gene as an example to to test the idea of whether a viral structural gene can be efficiently expressed locally in the central nervous system (CNS) using this expression system. Our results showed that the HE gene was expressed in the CNS, and that the expression of HE altered viral pathogenesis. This study thus demonstrates the feasibility of using the DI expression system to express a foreign gene in the loci of virus replication *in vivo* and provides a model for studying viral pathogenesis.

3. MATERIALS AND METHODS

3.1. Mice

C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, MA) at 6–7 weeks of age. Mice were housed locally in micro isolator cages and were used within 7 days of receipt. None was found to have preexisting anti-MHV antibodies prior to use. Mice were infected intracranially (i.c.) with $1x10^5$ plaque forming units (PFU) of virus contained in 30 μl of phosphate buffered saline (PBS).

3.2. Viruses and Cells

MHV strain A59 was used throughout the study. It was plaque-purified twice and further passaged twice in cell cultures to enrich its infectivity before further use. The murine astrocytoma cell line DBT (Hirano et al., 1974) was used for virus propagation, plaque assay and preparation of DI stocks.

3.3. Construction of DI Vector

The HE gene was cloned into the DI cDNA of p25CAT to replace the CAT gene, resulting in pDE-HE, as described previously (Liao et al., 1995).

3.4. Preparation of DI Stock Viruses

For making DI stock viruses, plasmid DNAs of pDE-HE and p25CAT were linearized with restriction enzyme *Xba*I and subjected to *in vitro* transcription as described (Zhang et al., 1994). The *in vitro* transcribed RNA was then transfected into MHV A59-infected DBT cells with the DOTAP method (Boehringer-Mannheim). At 12 to 14 h posttransfection, culture medium was harvested and cell debri were clarified by low speed centrifugation. Clarified supernatants were used as DI stocks, designated DE-HE A59 or DE-CAT A59.

3.5. Determination of Virus Titers in Tissues

Virus titers in tissue were determined by homogenization of 1/2 the brain or 1/2 lobe of liver in 4.0 ml of Dulbecco's PBS, pH7.4 using Tenbrock tissue homogenizers. The remainder was processed for histopathology (see below). Following centrifugation at 1,500 *g* for 20 min at 4 °C supernatants were assayed immediately, or frozen at -70 °C. Virus titers were determined by plaque assay using monolayers of DBT cells as previously described (Yokomori et al., 1992). Data presented are the average titer per gram of tissue for groups of three or more mice.

3.6. RNA Isolation and RT-PCR Analysis

For detection of the expression of HE and CAT genes in mice following infection with DI viruses, RNAs were isolated from tissues with Trizol Reagent according to the manufacturer's instruction (Life Technologies), and were subjected to reverse transcription (RT) and polymerase chain reaction (PCR) using primer pairs 5'-L9 (specific to the leader RNA)/3'-HE74 for HE gene (Zhang and Lai, 1994) and 5'-L9/3'CAT542 (Zhang et al., 1994) for the CAT gene.

3.7. Histopathology

For routine histopathological analysis mice were sacrificed by CO_2 asphyxiation. Brains were removed and dissected in the midcoronal plane. Brains, spinal cords and livers were fixed for 3 h in Clark's soloution (75% ethanol and 25% glacial acetic acid) and embedded in paraffin. Sections were stained with hematoxylin and eosin for routine examination. The distribution of viral antigen was examined using immunoperoxidase staining (Vectastain-ABC kit, Vector Laboratories, Burlingame, CA) and anti-JHMV mAb J.3.3 specific for the carboxy terminus of the N protein (Fleming et al., 1983). All slides were read in a blinded manner.

4. RESULTS AND DISCUSSION

4.1. Expression of the HE Gene in the CNS Via an MHV DI Vector

To determine the expression of the HE gene in the CNS, RNA was extracted from the brains of DE-HE A59 infected mice at various time points and analyzed by RT-PCR.

The RT-PCR specifically detects HE-containing subgenomic mRNA which is transcribed from the IG site and contains a leader sequence at the 5'-end. Thus, subgenomic mRNA can be distinguished from the original input DI RNA. The subgenomic HE-containing mRNA was undetectable at 12 h p.i. However, expression of the HE mRNA could be detected at 24 and 48 h p.i., but not at subsequent time points. Similarly, in the control experiment, in which mice were infected with DE-CAT A59, the CAT mRNA could also be detected only at these time points. These data suggest that the expression of DI RNA following infection is limited to the first 2 days of the infection.

4.2. Expression of the HE Gene Alters Viral Pathogenesis

To determine if the expression of the HE gene from a DI vector could influence the pathogenesis of A59 virus infection, mice were infected i.c. with $1x10^5$ PFU of DE-HE A59, DE-CAT A59, and parental A59. All mice infected with DE-CAT A59 succumbed to infection by day 9 p.i, similar to mice infected with parental A59 alone. These data demonstrate that inclusion of the DI RNA in the virus pool did not by itself alter the outcome of A59 infection. In contrast to these groups of mice, mice infected with the identical dose of DE-HE A59 showed identical signs of encephalitis by day 6 p.i.; however, 60% of these mice survived infection. Clinical symptoms resolved in all surviving mice by 12 to 14 d p.i. and no mice succumbed during the 30 days of observation. These data demonstrate that expression of the HE gene from the DI RNA alters the pathogenesis of A59 infection.

4.3. Viral Replication and Pathological Changes in the CNS of Infected Mice

To determine the basis for the reduction in mortality, virus titers in the brains of mice infected with DE-HE A59 were compared to mice infected with DE-CAT A59. As shown in Fig. 1A, a slight, but not statistically significant reduction in virus titers within the CNS was found at day 6 p.i. in the mice infected with DE-HE A59 compared to mice infected with DE-CAT A59 or parental A59 (data not shown). No difference was detected at either day 1 or day 2 p.i. when HE gene expression was detected in the CNS. Brains and spinal cords were also examined for histopathological changes at days 1, 2, 4, and 6 p.i. No differences were detected in either the extent or distribution of either mononuclear cell infilrates or viral antigen at days 1, 2 and 6 p.i (data not shown). At day 4 p.i. there was a small increase in the extent of inflammatory cell infiltration and reduced viral antigen in the mice infected with DE-HE A59 (data not shown). These data suggest that expression of the HE gene within the CNS results in a transient increase in CNS inflammation and reduction of viral antigen within the CNS.

To examine whether the altered pathogenesis of DE-HE A59 was due to the transient expression of the HE gene, virus was recovered from the brains of DE-HE A59 infected mice at 6 d p.i. This time point was chosen based on the absence of detectable HE gene expression in the CNS. All of the mice infected with this recovered virus (A59-R) with 1×10^5 PFU succumbed to infection by 9 d p.i.. The amount of virus present in the CNS of these mice was similar to that in mice infected with DE-HE A59, DE-CAT A59 or parental A59. These data suggest that the difference in mortality following infection with DE-HE A59 is not due to alterations in either virus replication within the CNS, cellular tropism or extent of inflammatory changes. However, the pathogenesis of the recovered A59 suggests that the reduced mortality is due to the transient expression of the HE gene from the DI RNA.

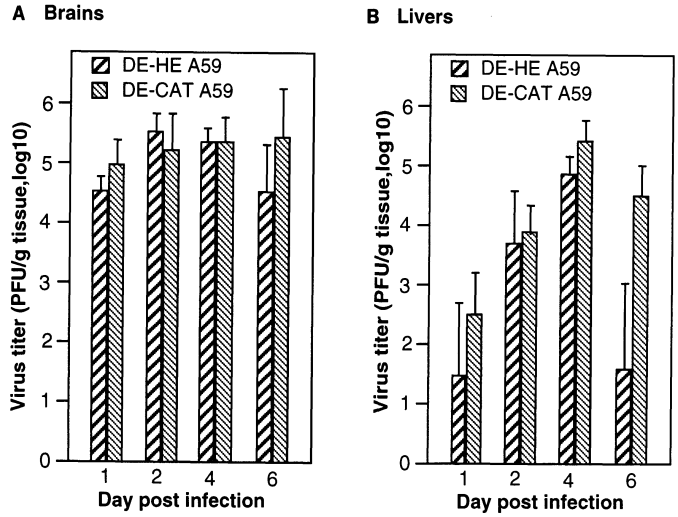

Figure 1. Viral replication in mice after intracrainial infection. C57BL/6 mice were inoculated intracraniallly with 1×10^5 PFU of DE-HE A59 and DE-CAT A59. There were at least 3 mice in each group. At various time points after infection, mice were sacrificed, and brains and livers were collected for virus isolation. Virus titer was determined by plaque-assay and is indicated as PFU/gram tissue. (A) Virus titers in the brains. (B) Virus titers in the livers.

4.4. Viral Replication and Pathological Changes in the Livers of Infected Mice

The A59 strain of MHV is both neurotropic and hepatotropic (Lavi et al., 1986). To explore the possibility that the decreased mortality of mice infected with DE-HE A59 was due to altered hepatotropism, virus replication and pathological changes in the livers of mice infected with DE-HE A59 and DE-CAT A59 were compared. Fig. 1B shows that at day 1 p.i. there was approximately 1 log less infectious virus recovered from the livers of mice infected with DE-HE A59 compared to mice infected with DE-CAT A59. This initial difference was transient, however, since there was no difference in virus titer at day 2 or 4 p.i. In contrast, by day 6 p.i., when the DE-CAT A59 infected mice were beginning to succumb to infection, there was a substantial 3 $\log_{10}$ difference in infectious virus as compared to mice infected with DE-CAT A59, parental A59, or A59-R (data not shown). Histological examination of the livers of the mice infected with DE-CAT A59 showed increased viral antigen and inflammatory changes beginning at day 2 p.i. By day 6 p.i., the livers of these mice showed evidence of extensive necrosis with prominent viral antigen. No differences were noted in the extent of inflammatory changes, necrosis or viral antigen in the livers of the mice infected with DE-HE A59 at day 1 or 2 p.i. However, by day 4 p.i., less viral antigen and less necrosis was noted in the mice infected with DE-HE A59. By day 6 p.i., there was a significant reduction in the amount of viral antigen, consistent with the decrease in recovery of infectious virus and substantially less necrosis compared to the almost confluent necrosis found in the mice infected with either parental A59 or DE-CAT A59. Similar to the data presented above, mice infected i.c. with virus recovered

from mice initially infected with DE-HE A59 showed extensive liver necrosis by day 6 p.i. Taken together, these data indicate that the reduced mortality found in mice infected with DE-HE A59 is due to reduced hepatitis and is a consequence of the transient expression of the HE gene.

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REFERENCES

Deregt, D., and Babiuk, L. A., 1987, Monoclonal antibodies to bovine coronavirus: Characteristics and topographical mapping of neutralizing epitopes on the E2 and E3 glycoproteins, *Virology* **161**: 410–420.

Deregt, D., Gifford, G.A., Khalid Ijaz, M., Watts, T.C., Gilchrist, J.E., Haines, D.M., and Babiuk, L.A., 1989, Monoclonal antibodies to bovine coronavirus glycoproteins E2 and E3: demonstation of in vivo neutralizing activity, *J. Gen. Virol.* **70**:993–998.

Fleming, J.O., Stohlman, S.A., Harmon, R.C., Lai, M.M.C., Frelinger, J.A., and Weiner, L.P., 1983, Antigenic relationship of murine coronaviruses: analysis using monoclonal antibodies to JHM (MHV-4) virus, *Virology* **131**: 296–307.

Gagnetan, S., Gout, O., Dubois-Dalcq, M., Rottier, P., Rossen, J., and Holmes, K.V., 1995, Interaction of mouse hepatitis virus (MHV) spike glycoprotein with receptor glycoprotein MHVR is required for infection with an MHV strain that expresses the hemagglutinin-esterase glycoprotein, *J. Virol.* **69**: 889–895.

Herrler, G., Rott, R., Klenk, H.D., Müller, H.P., Shukda, A.K., and Schauer, R., 1985, The receptor-destroying enzyme of influenza C is virus neuraminidate-O-acetyl-esterase, EMBO J. **4**:1503–1506.

Herrler, G., Rott, R., and Klenk, H.D., 1985, Neuraminic acid is involved in the binding of influenza C virus to erythrocytes, *Virology* **141**: 144–147.

Hirano, N., Fujiwara, K., Hino, S., and Matsumoto, M., 1974, Replication and plaque formation of mouse hepatitis virus (MHV-2) in mouse cell line DBT culture, *Arch. Gesamte Virusforsch.* **44**, 298–302.

Lai, M.M.C., 1992, RNA recombination in animal and plant viruses, *Microbiol. Rev.* **56**: 61–79.

Lavi, E., Gilden, D.H., Highkin, M.K., Weiss, S.R., 1986, The organ tropism of mouse hepatitis virus A59 in mice is dependent on dose and route of inoculation, *Lab. Ani. Sci.* **36**:130–135.

Lee, H.-J., Shieh, C.-K., Gorbalenya, A. E., Koonin, E. V., La Monica, N., Tuler, J., Bagdzyahdzhyan, A., and Lai, M. M. C., 1991, The complete sequence (22 kilobases) of murine coronavirus gene 1 encoding the putative proteases and RNA polymerase, *Virology* **180**: 567–582.

Liao, C.-L., and Lai, M. M. C., 1994, Requirement of the 5'-end genomic sequence as an upstream cis-acting element for coronavirus subgenomic mRNA transcription, *J. Virol.* **68**: 4727–4737.

Liao, C.-L., Zhang, X. M., and Lai, M. M. C., 1995, Coronavirus defective-interfering RNA as an expression vector: the generation of a pseudorecombinant mouse hepatitis virus expressing hemagglutinin-esterase, *Virology* **208**: 319–327.

Lin, Y.-J., and Lai, M. M. C, 1993, Deletion mapping of a mouse hepatitis virus defective interfering RNA reveals the requirement of an internal and discontiguous sequence for replication, *J. Virol.* **67**: 6110–6118.

Makino, S., Shieh, C.-K., Soe, L. H., Baker, S. C., and Lai, M. M. C., 1988, Primary structure and translation of a defective-interfering RNA of murine coronavirus, *Virology* **166:** 550–560.

Makino, S., Joo, M., and Makino, J. K., 1991, A system for study of coronavirus mRNA synthesis: A regulated, expressed subgenomic defective-interfering RNA results from intergenic site insertion, *J. Virol.* **65**: 6031–6041.

Yokomori, K., La Monica, N., Makino, S., Shieh, C.-K., and Lai, M.M.C., 1989, Biosynthesis, structure, and biological activities of envelope protein gp65 of murine coronavirus, *Virology* **173**: 683–691.

Yokomori, K., Baker, S.C., Stohlman, S.A., and Lai, M.M.C., 1992, Hemagglutinin-esterase (HE)-specific monoclonal antibodies alter the neuropathogenicity of mouse hepatitis virus, *J. Virol.* **66**:2865–2874.

Yokomori, K., Asanaka, M., Stohlman, S.A., Makino, S., Shubin, R.A., Gilmore, W., Weiner, L.P., Wang, F.I., and Lai, M.M.C., 1995, Neuropathogenicity of mouse hepatitis virus JHM isolates differing in hemagglutinin-esterase protein expression, *J. Neurovirol.* **1**:330–339.

Zhang, X. M., Liao, C.-L., and Lai, M. M. C., 1994, Coronavirus leader RNA regulates and initiates subgenomic mRNA transcription both in *trans* and in cis, *J. Virol.* **68**: 4738–4746.

Zhang, X.M., Hinton, D.R., Cue, D., Stohlman, S., and Lai, M.M.C., 1997: Expression of gamma interferon by a coronavirus defective-interfering RNA vector and its effect on viral replication, spread and pathogenicity, *Virology.* In press.