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## Expression of Hemagglutinin/Esterase by a Mouse Hepatitis Virus Coronavirus Defective–Interfering RNA Alters Viral Pathogenesis

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A defective-interfering (DI) RNA of mouse hepatitis virus (MHV) was developed as a vector for expressing MHV hemagglutinin/esterase (HE) protein. The virus containing an expressed HE protein (A59-DE-HE) 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. A similar virus (A59-DE-CAT) expressing the chloramphenicol acetyltransferase (CAT) was used as a control. These viruses were inoculated intracerebrally into mice, and the role of the HE protein in viral pathogenesis was evaluated. Results showed that all mice infected with parental A59 or A59-DE-CAT succumbed to infection by 9 days postinfection (p.i.), demonstrating that inclusion of the DI did not by itself alter pathogenesis. In contrast, 60% of mice infected with A59-DE-HE survived infection. HE- or CAT-specific subgenomic mRNAs were detected in the brains at days 1 and 2 p.i. but not later, indicating that the genes in the DI vector were expressed only in the early stage of viral infection. No significant difference in virus titer or viral antigen expression in brains was observed between A59-DE-HE- and A59-DE-CAT-infected mice, suggesting that virus replication in brain was not affected by the expression of HE. However, at day 3 p.i. there was a slight increase in the extent of inflammatory cell infiltration in the brains of the A59-DE-HE-infected mice. Surprisingly, virus titers in the livers of A59-DE-HE-infected mice were 3 log<sub>10</sub> lower than that of the A59-DE-CAT-infected mice at day 6 p.i. Also, substantially less necrosis and viral antigen were detected in the livers of the A59-DE-HE-infected mice. This may account for the reduced mortality of these mice. The possible contribution of the host immune system to this difference in pathogenesis was analyzed by comparing the expression of four cytokines. Results showed that both tumor necrosis factor- $\alpha$  and interleukin-6 mRNAs increased in the brains of the A59-DE-HE-infected mice at day 2 p.i., whereas interferon- $\gamma$  and interleukin-1 $\alpha$  mRNAs were similar between A59-DE-HE- and A59-DE-CAT-infected mice. These data suggest that the transient expression of HE protein enhances an early innate immune response, possibly contributing to the eventual clearance of virus from the liver. This study indicates the feasibility of the DI expression system for studying roles of viral proteins during MHV infection. © 1998

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### INTRODUCTION

The development of infectious, full-length cDNA clones of viral RNA has provided a powerful tool for understanding the structure and function of individual viral genes and for studying their roles in viral pathogenesis. Unfortunately, an infectious cDNA clone is currently not available for some RNA viruses with a large genome, such as coronaviruses. We have recently developed an alternative approach, namely, a defective–interfering (DI) RNA (Makino *et al.* 1985, 1988, 1991), which functions like a minigenome, as an expression vector (Liao and Lai, 1994). Such a vector can express viral or foreign proteins,

including cytokines (Liao *et al.* 1995; Zhang *et al.* 1997), thus allowing more critical analysis of the functional role of these proteins in coronavirus pathogenesis.

Mouse hepatitis virus (MHV), a murine coronavirus, is a member of the Coronaviridae. It contains a single-strand, positive-sense RNA genome of 31 kb (Lee *et al.* 1991). In addition to the genomic-length RNA, six or seven subgenomic mRNAs are synthesized in infected cells. These RNAs are conested at the 3' end and extend toward the 5' end to different lengths at intergenic (IG) regions (Lai *et al.* 1981). Each mRNA contains a leader RNA of approximately 70 nt at the 5' end, which is identical to the leader of the genomic RNA (Lai *et al.* 1983, 1984). The IG sequence serves as an initiation site and is required for the synthesis of a subgenomic mRNA. In general, each mRNA translates only one protein from the 5'-most open reading frame (ORF) (Lai and Cavanagh, 1997).

MHV contains four or five structural proteins. The spike (S) protein is the major surface glycoprotein of the

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virion. It facilitates the attachment of virus to the cellular receptors, elicits the production of neutralizing antibodies, and induces cell fusion (Sturman and Holmes, 1983). The membrane (M) glycoprotein is also associated with the viral envelope and, together with another viral envelope (E) protein, is required for virus assembly (Bos *et al.* 1996; Vennema *et al.* 1996). The nucleocapsid (N) protein is a phosphorylated protein which together with the viral genomic RNA forms the nucleocapsid. The hemagglutinin/esterase (HE) protein is an optional envelope protein, present only in some MHV strains (Yokomori *et al.* 1992). It contains hemagglutination (receptor-binding) and acetyl-esterase (receptor-destroying) activities, similar to the HE protein of influenza C virus (Herrler *et al.* 1985, 1888; Yokomori *et al.* 1989). The HE protein binds sialic acid-containing cell surface molecules. The precise role of the HE protein in coronaviral infection is not clear. It has been shown that the HE protein of bovine coronavirus induced the production of neutralizing antibodies and that inhibition of the esterase activity resulted in a 100- to 400-fold reduction in viral infectivity, suggesting an important role of the HE protein in viral infectivity (Deregt and Babiuk, 1987; Vlasak *et al.* 1988). In contrast, no neutralizing antibody specific for the HE protein of MHV has been identified (Yokomori *et al.* 1992), and expression of the HE protein alone, without the S protein, is not sufficient to initiate MHV infection (Gagnetet *et al.* 1995). An MHV variant that expresses abundant HE appeared to be more neurovirulent than the one that expresses little HE (Yokomori *et al.* 1995). Consistent with these data, passive immunization with HE monoclonal antibodies protected mice from lethal infection and altered the pathogenicity (Yokomori *et al.* 1992). These findings suggest that the HE protein contributes to viral neuropathogenicity by influencing either the rate of virus spread, viral tropism, or both (Yokomori *et al.* 1995). Although five separate clonal populations expressing either high or low levels of HE protein yielded identical results, no precise role of the HE protein could be unequivocally determined because these virus variants may contain additional mutations in other viral genes.

In a previous study (Liao *et al.* 1995), we expressed the HE protein of MHV in cell culture using the MHV-DI RNA expression vector. In this system, the expression of the HE-containing DI RNA requires the presence of a helper MHV, and the expressed HE protein was shown to be at least in part incorporated into the virions. However, due to the lack of a specific packaging signal, the DI RNA cannot be efficiently packaged into virus particles; it can only be copackaged nonspecifically with the helper virus particles for a limited number of cell culture passages. The DI RNA eventually loses its association with the helper virus and its ability to replicate during serial passages in cell culture. Therefore, the expression of the DI RNA is transient and does not involve a recombination between the helper virus genome and the DI RNA. In the

present study, we used this DI RNA vector system to express the viral HE gene in the CNS of mice in the presence of an HE-deficient helper virus. We found that the transient expression of the HE protein by this vector altered viral pathogenesis. This study thus demonstrates unequivocally that HE protein plays a role in MHV pathogenesis and further demonstrates the feasibility of using this DI RNA vector for *in vivo* expression of a foreign protein.

## RESULTS

### Expression of the HE gene alters virulence

It has previously been shown that MHV-A59 virus does not express HE protein due to mutations in the transcription start signal and coding sequence (Luytjes *et al.* 1988; Shieh *et al.* 1989). To assess the function of HE protein, we were interested in determining if the expression of a functional HE gene derived from JHM virus by the DI RNA vector could influence the pathogenesis of A59 virus (Fig. 1). Mice were infected ic with  $1 \times 10^5$  PFU each of A59-DE-HE, A59-DE-CAT, or parental A59. Figure 2 shows that 100% of mice infected with A59-DE-CAT succumbed to infection by day 9 postinfection (p.i.), similar to mice infected with the parental A59 alone. These data demonstrate that inclusion of DI RNA that expresses a CAT gene did not by itself alter the outcome of A59 infection. In contrast, 60% of mice infected with A59-DE-HE virus survived infection. Mice infected with all three virus preparations showed similar signs of encephalitis (hunched back and ruffled fur) for the first 5 to 6 days p.i. Subsequently, clinical symptoms gradually improved in those A59-DE-HE-infected mice that had survived infection; by 12 to 14 days p.i., the clinical signs were completely resolved and no mice subsequently succumbed to infection. These data demonstrate that the expression of the JHM virus HE gene by the DI RNA vector substantially alters the pathogenesis of A59 virus infection.

### Expression of the HE gene in the CNS

We have previously shown that the expression of genes from the DI vector can be maintained for only a few passages *in vitro* (Liao *et al.* 1995). Thus, it was not clear whether the HE protein could be expressed from the DI vector within the CNS. To address this issue, RNA was extracted from brains of infected mice at various times p.i. and analyzed by RT-PCR using primers which specifically detect HE-containing RNA (see Materials and Methods). Since the HE-containing subgenomic mRNA is transcribed from the IG site within the vector RNA and contains a leader sequence at the 5' end, the PCR product corresponding to the subgenomic HE mRNA is distinguishable from that of the input DI RNA by their size (Fig. 1). The result shows that the subgenomic

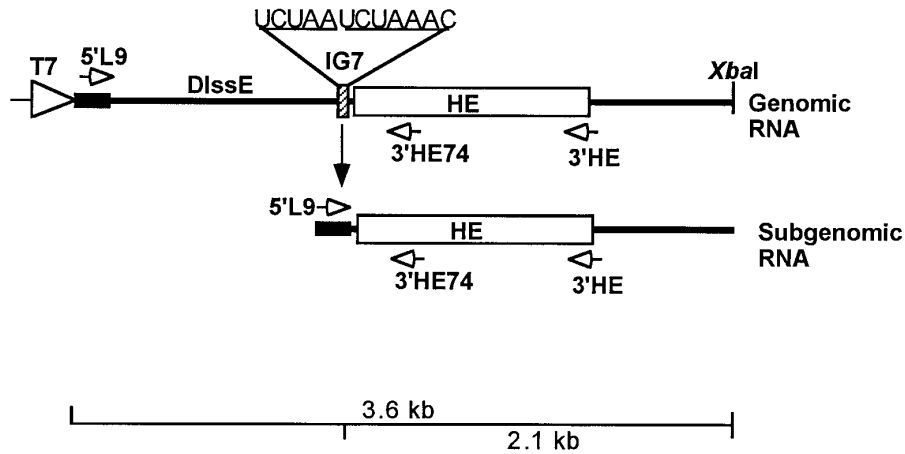


FIG. 1. Diagram of the structure of pDE-HE. Only the genomic and subgenomic DI sequences are shown. The HE gene was placed behind the coronavirus transcription promoter IG7. Positions and names of the primers (5'-L9 and 3'-HE or 3'-HE74) used in RT-PCR to amplify genomic and subgenomic mRNAs (shown on the right) in tissues of mice infected with DE-HE-containing MHV-A59. The sizes of the HE-containing DI genomic and subgenomic RNAs are indicated in kilobases below the sequences. The solid arrow indicates the transcription start site from the IG7 sequence.

HE mRNA was undetectable in RNA extracted from the brains of infected mice at 12 h p.i. (data not shown); however, it became detectable in RNA samples prepared from the brains of A59-DE-HE-infected mice at 24, 36, and 48 h p.i. (Table 1 shows data at 24 and 48 h p.i. only). No HE mRNA was detected in RNA samples obtained on day 4 or 6 p.i. Similarly, the mRNA encoding CAT could be detected in A59-DE-CAT-infected mice only at 24, 36, and 48 h p.i. (Table 1). These data suggest that the HE or the CAT gene indeed could be expressed from the DI RNA in the brains during *in vivo* infection but that their expression was limited to the first 2 days p.i., consistent with its limited ability to be serially passaged *in vitro* (Liao *et al.* 1995). Because of the low level of mRNA expression, the amounts of HE- or CAT-specific mRNA were not quantitatively compared.

#### Pathogenesis in the CNS following infection with A59-DE-HE

To determine the basis for the reduction in mortality following A59-DE-HE infection compared to other viruses (Fig. 2), virus titers in the brains of mice infected with A59-DE-HE were initially compared to mice infected with the parental A59 or A59-DE-CAT at 6 days p.i. Based on the survival of most mice infected with A59-DE-HE, reduced viral replication in the CNS was anticipated. However, only a slight (approximately 1 log<sub>10</sub>) reduction in virus titer within the CNS was noted at 6 days p.i. in A59-DE-HE-infected mice compared to mice infected with either A59-DE-CAT or parental A59 (Table 1). We reasoned that, since HE protein expression was detectable only early in infection, a more significant reduction of virus titer in CNS might be detectable at an earlier time of infection; thus, virus titers in the CNS of mice infected with A59-DE-HE and A59-DE-CAT were compared at 1, 2, and 4 days p.i. Similar to the titers found at 6 days p.i., no significant difference was detected at any of the early time points (Table 1). Therefore, although HE expression in the CNS was detected for the first 2 days p.i., there appeared to be no significant difference in virus replication in the CNS between A59-DE-HE- and A59-DE-CAT-infected mice.

The pathogenicity of A59-DE-HE and A59-DE-CAT in the CNS was then examined to determine the basis for the difference in mortality between the two groups. Brains and spinal cords were examined at 1, 2, 3, 4, and 6 days p.i. for mononuclear cell infiltrates and distribution of virus-infected cells. Consistent with the similar amount of virus replication, no differences were detected in either the extent or the distribution of either mononuclear cell infiltration or viral antigen at 1 or 2 days p.i. in two separate experiments. However, at

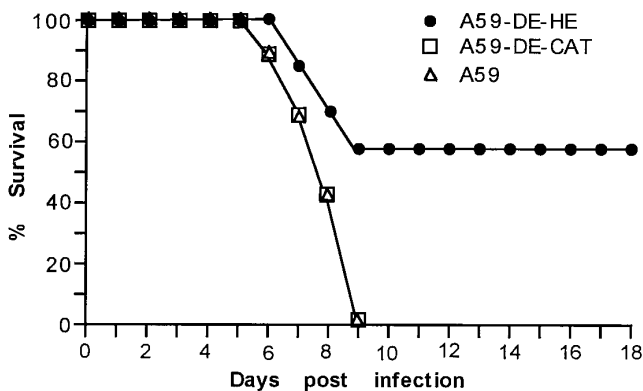


FIG. 2. Survival rate of mice following infection with A59-DE-HE, A59-DE-CAT, or A59. Eight-week-old C57BL/6 mice (7 mice per group) were intracerebrally inoculated with A59-DE-HE or A59-DE-CAT or A59 (10<sup>5</sup> PFU/mouse) and observed daily for clinical symptoms and survival.

TABLE 1

Virus Replication in the CNS Following Intracerebral Inoculation of Mice with the HE-Expressing and Control Viruses

Day <sup>a</sup>	A59-DE-HE		A59-DE-CAT		A59	A59-R <sup>d</sup>
	PCR <sup>b</sup>	Titer <sup>c</sup>	PCR	Titer	Titer	Titer
1	+	4.59 ± 0.16 (5)	+	5.05 ± 0.44 (5)	ND <sup>e</sup>	ND
2	+	5.66 ± 0.34 (5)	+	5.27 ± 0.66 (5)	ND	ND
4	–	5.45 ± 0.14 (3)	–	5.43 ± 0.45 (3)	ND	ND
6	–	4.58 ± 0.98 (9)	–	5.62 ± 1.01 (6)	5.12 ± 0.65 (3)	5.76 ± 0.35 (3)

<sup>a</sup> Day(s) postinfection. Mice were infected ic with  $1 \times 10^5$  PFU of various viruses.

<sup>b</sup> RT-PCR detecting the subgenomic mRNA expressed from the DI RNA.

<sup>c</sup> Mean titer in PFU/g brain, in  $\log_{10} \pm$  standard deviation (number/group).

<sup>d</sup> Virus recovered from the CNS of mice at day 6 following infection with A59-DE-HE.

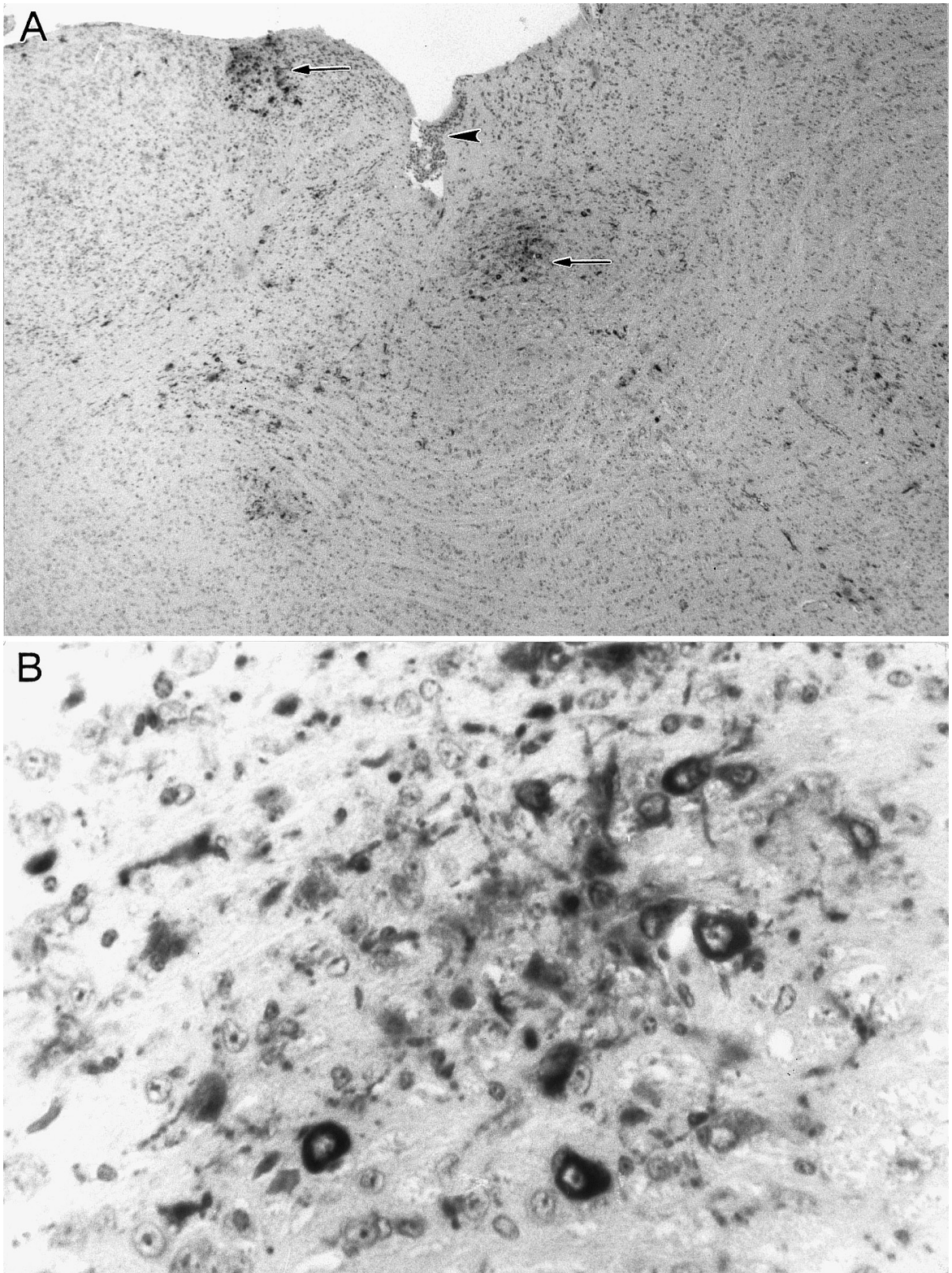
<sup>e</sup> Not done.

3 days p.i., there was a slight but reproducible increase in the extent of inflammatory cell infiltration and reduced viral antigen in the mice infected with A59-DE-HE compared to mice infected with A59-DE-CAT (Fig. 3). Quantitation of viral antigen-positive cells revealed approximately one-third fewer positive cells in the A59-DE-HE-infected mice than in those in A59-DE-CAT-infected mice. This difference was no longer noticeable by 6 days p.i. These data suggest that expression of the HE gene within the CNS resulted in a transient increase in CNS inflammation and reduction in viral antigen. This difference was transient and minor and did not appear sufficient to explain the increased survival of mice infected with A59-DE-HE. Survivors of A59-DE-HE infection examined at 21 days p.i. showed mononuclear cell infiltrations, presence of viral antigen, and extensive demyelination, consistent with the pathology reported for mice infected with sublethal doses of parental A59 (Lavi *et al.* 1984).

To ensure that the altered pathogenesis of A59-DE-HE was due to transient HE expression but not to the selection of a variant A59 virus, virus was recovered from A59-DE-HE-infected mice at 6 days p.i. This time point was chosen based on the absence of detectable HE gene expression in the CNS. Virus isolated (designated A59-R) was propagated once in DBT cells and used to infect mice using the same dose as that in the previous experiments ( $1 \times 10^5$  PFU). All mice infected with this virus succumbed to infection by day 9. Furthermore, the amount of virus present in the CNS of these mice at day 6 was equivalent to the virus detected in mice infected with A59-DE-HE, A59-DE-CAT, or parental A59 (Table 1). These combined data suggest that the difference in mortality following ic infection with A59-DE-HE was not due to alterations in virus replication within the CNS, altered cellular tropism, or selection of an attenuated variant.

### Viral replication and pathology in the liver in A59-DE-HE infection

The A59 strain of MHV is both neurotropic and hepatotropic (Lavi *et al.* 1986). Since differences in virus replication and pathogenesis of A59-DE-HE in the CNS did not appear to be sufficient to account for the differences in mortality, we explored the possibility that the decreased mortality in A59-DE-HE-infected mice was due to altered hepatotropism. Therefore, virus replication and pathological changes in the livers of mice infected with A59-DE-HE and A59-DE-CAT were compared. Table 2 shows that at day 1 p.i., there was approximately 1  $\log_{10}$  less infectious virus recovered from the liver of mice infected with A59-DE-HE than from mice infected with A59-DE-CAT. However, no differences in virus replication were found at either day 2 or 4 p.i. Surprisingly, by day 6 p.i., when the A59-DE-CAT-infected mice were beginning to succumb to infection, there was a substantial reduction (3  $\log_{10}$ ) in the amount of infectious virus in the liver of A59-DE-HE-infected mice (Table 2). This difference was reproducible in three independent experiments. Histological examination of the livers of mice infected with A59-DE-CAT showed increasing viral antigen and inflammatory changes with time throughout the infection. By day 6 p.i., the livers of these mice showed evidence of extensive necrosis with prominent viral antigen (Figs. 4A and 4B). Similar hepatic necrosis was found following ic infection with both parental A59 and A59-R (data not shown). A59-DE-HE-infected mice also showed similar inflammatory changes, hepatic necrosis, and viral antigen in the livers at day 1 or 2 p.i., compared to A59-DE-CAT-infected mice. However, by day 3 p.i., fewer viral antigen-positive cells and reduced necrosis were noted in mice infected with A59-DE-HE. By day 6 p.i., there was a significant reduction in the amount of viral antigen (Fig. 4D), consistent with decreased titer of infectious virus (Table 2) and substantially less necrosis (Fig. 4C), in contrast to the almost confluent necrosis



**FIG. 3.** Detection of viral antigen and histopathologic changes in the brains of mice infected with A59-DE-CAT or A59-DE-HE. In (A), 3 days after infection with A59-DE-CAT, sections of brain show small foci of chronic inflammation (arrowhead) with multiple foci of antigen-positive cells (arrows). At higher magnification (B), the foci demonstrate a mixed population of virus-positive cells. In (C), 3 days after infection with A59-DE-HE, sections of brain show increased subarachnoid and perivascular inflammatory infiltrates (arrowheads) but fewer virus-positive cells (arrows). At higher magnification (D), a mixed population of virus-positive cells is seen. Immunoperoxidase stain by the ABC method for viral antigen (using N-specific mAb J.3.3) with hematoxylin counterstain. Magnifications:  $\times 40$  (A, C),  $\times 440$  (B, D).

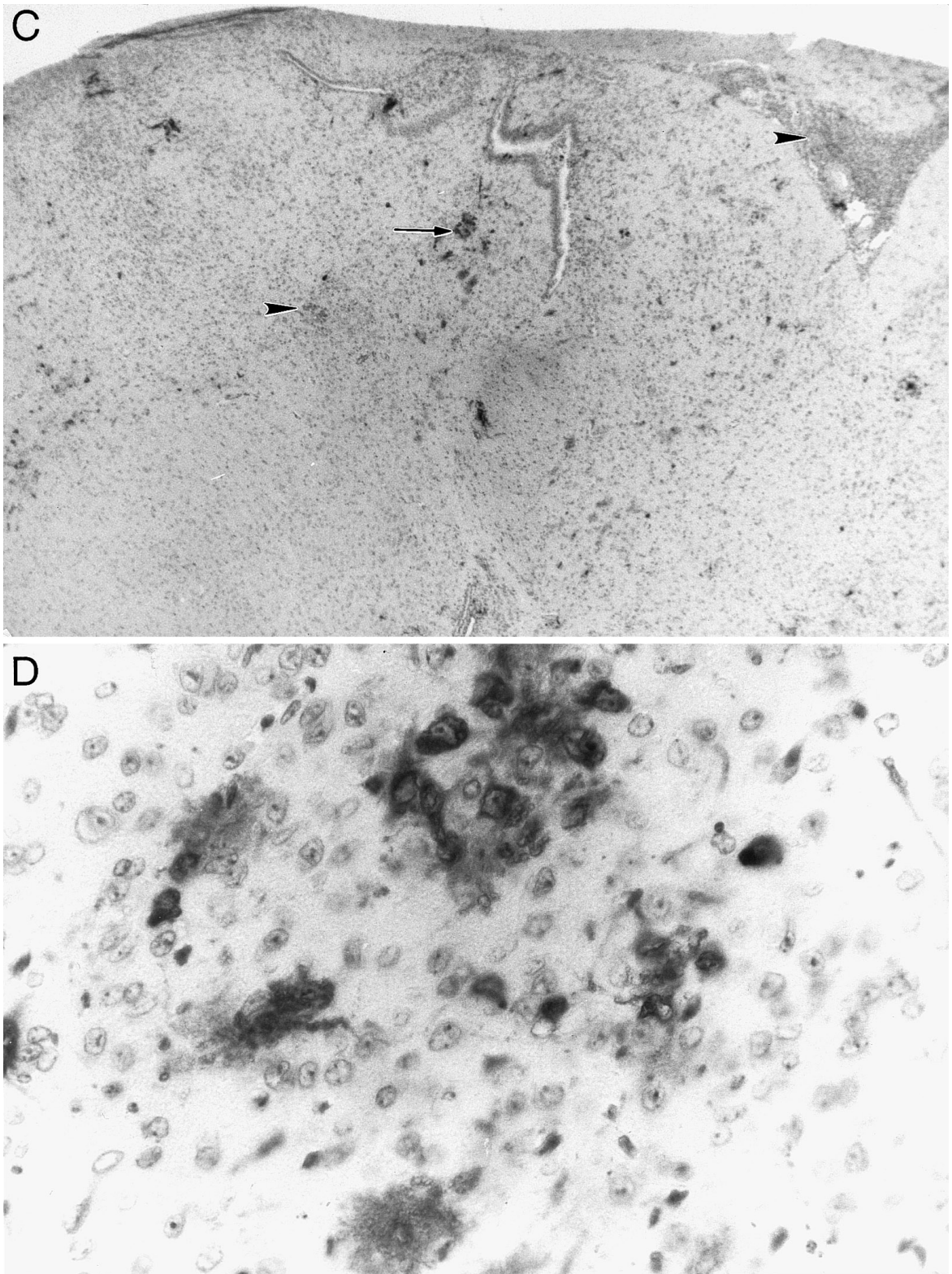


FIG. 3—Continued

found in the mice infected with either parental A59 or A59-DE-CAT (Figs. 4A and 4B). Again, the A59 virus (A59-R) recovered from the CNS at day 6 p.i. did not differ from the original A59 virus. These data suggest that the

transient expression of the HE gene in the CNS affected the virus replication and subsequent pathology in the liver. This is likely the basis for the reduced mortality of A59-DE-HE-infected mice.

TABLE 2

Virus Replication in the Liver Following Intracerebral Inoculation of Mice with the HE-Expressing and Control Viruses

Day <sup>a</sup>	Virus titer <sup>b</sup>			
	A59-DE-HE	A59-DE-CAT	A59	A59-R
1	1.32 ± 1.43 (5)	2.45 ± 0.82 (5)	ND <sup>c</sup>	ND
2	3.68 ± 1.06 (5)	3.98 ± 0.52 (5)	ND	ND
4	4.85 ± 0.28 (3)	5.41 ± 0.32 (3)	ND	ND
6	1.51 ± 1.57 (9)	4.64 ± 0.40 (6)	5.31 ± 0.40 (3)	5.81 ± 0.12 (3)

<sup>a</sup> Day(s) postinfection. Mice were infected ic on day 0 with  $1 \times 10^5$  PFU.

<sup>b</sup> Mean titer in PFU/g liver, in  $\log_{10} \pm$  standard deviation (number/group).

<sup>c</sup> Not done.

To determine if the activation of the mouse fibrinogen-like protein (*fgl-2*) gene was associated with the fulminant hepatitis induced by ic infection with the A59-DE-CAT virus, as has been shown to be the case in the hepatitis induced by MHV-3 (Parr *et al.* 1995), livers were examined for the presence of *fgl-2* gene mRNA by RT-PCR. No difference in expression of *fgl-2* mRNA was detected between A59-DE-HE- and A59-DE-CAT-infected mice (data not shown). Histologic examination of the livers did not reveal coagulopathy typically induced by MHV-3 infection (Parr *et al.* 1995).

We next examined the virus titers in the spleen following ic infection to determine if the difference in virus titer in the livers of A59-DE-HE-infected mice was due to a difference in virus spread from the CNS. A slight reduction in virus titer in the spleens of mice infected with A59-DE-HE on day 1 p.i. was found compared to mice infected with A59-DE-CAT (Table 3). However, no difference was detected at day 2 or 4 p.i., and no infectious virus could be recovered from the spleens of either group by day 6 p.i. (Table 3). These data suggest that A59 replicated poorly and only transiently in the spleen following ic infection; nevertheless, this small amount of virus in the spleen may have spread to the liver via a hematogenous route. Therefore, A59-DE-HE and A59-DE-CAT appeared to spread similarly from the CNS to spleen and liver.

To determine if A59-DE-HE exhibited reduced hepatotropism, compared to A59-DE-CAT and parental A59, we examined virus replication in the liver following intraperitoneal (ip) infection. Groups of four mice were infected with  $1 \times 10^5$  PFU of each virus ip. Surprisingly, at 6 days p.i., no infectious virus was recovered from the livers or CNS of any of the three groups and no evidence of hepatitis or viral antigen was detected (data not shown). These data indicate that in contrast to previous data (Lavi *et al.* 1986), none of these three virus preparations are directly hepatotropic following ip infection.

### Cytokine mRNA expression

To correlate early, transient HE protein expression with the observed alterations in mononuclear cell infiltrates, expression of various cytokine mRNAs in the CNS and liver were examined at 2 days p.i. This time point was chosen since it was the last time point p.i. when HE mRNA could be detected (Table 1). Increased expression of the mRNAs encoding TNF- $\alpha$  was detected in the CNS of A59-DE-HE-infected mice compared to samples from mice infected with the A59-DE-CAT virus (Fig. 5A). The TNF- $\alpha$  mRNA expression in the liver showed a similar trend, although the level of this mRNA in the liver was lower than that in the CNS (Fig. 5A). IL-6 mRNA was also increased in the CNS of mice infected with A59-DE-HE virus (Fig. 5B); however, no differences were detected in the liver between the two groups. Although the level of IFN- $\gamma$  mRNA was very low in the tissues from both groups, a slight decrease in IFN- $\gamma$  mRNA was detected in the CNS but not in the livers of mice infected with the A59-DE-HE virus (Fig. 5C). No difference was found in the expression of IL-1 $\alpha$  mRNA in either CNS or liver (Fig. 5D). These data indicate that the transient expression of the HE protein resulted in the early induction of both TNF- $\alpha$  and IL-6 mRNA in the CNS.

### DISCUSSION

The HE protein is expressed only by some strains of MHV (Yokomori *et al.* 1991); thus, it is not required for viral replication. During virus passage in tissue culture or in animals, the HE gene often undergoes mutation so that the virus loses the ability to synthesize HE protein (Yokomori *et al.* 1991). However, reverse changes have also occurred; for example, a JHM variant, which has three repeats of the UCUAA pentanucleotide at the 3' end of the leader sequence (Makino and Lai, 1989) and synthesized a very small amount of HE, generated, upon passage, a JHM variant containing two repeats of the UCUAA pentanucleotide, resulting in expression of a large amount of HE protein (Makino and Lai, 1989; Shieh *et al.* 1989; Yokomori *et al.* 1989; La Monica *et al.* 1992).



Initial demonstration that HE may play a role in viral neuropathogenesis was obtained from studies showing that passive transfer of anti-HE monoclonal antibodies protected mice from lethal infection by JHM viruses expressing various amounts of HE, but not a JHM variant which expressed no HE (Yokomori *et al.* 1992). Histopathological studies of the HE-protected mice suggested that an early mononuclear infiltration into the CNS was associated with the protection by this monoclonal antibody. Subsequent comparisons of the pathogenicity of the JHM variants expressing different levels of HE demonstrated that the JHM variant which expresses high levels of HE replicated less efficiently in glial cells *in vitro* and preferentially infected neurons *in vivo* (Yokomori *et al.* 1995). Together, these data suggested that HE protein is at least one of the factors influencing the neuropathogenesis of JHM in the CNS. However, the effect of HE protein on viral pathogenesis may depend on the genetic background of the virus. This question was difficult to assess in the previous studies since JHM variants used in those studies may differ not only in HE gene expression but also in other unscrutinized viral genes as well. In this report, we examined the influence of HE protein on neuropathogenesis of the A59 strain of MHV using a DI vector approach, so that the presence or the absence of HE can be studied on the same virus background. A59 was chosen because its pathogenesis in mice has been well described (Lavi *et al.* 1984, 1986) and it does not produce any background level of the HE protein due to a mutation in the transcription start signal and a premature stop codon in the HE gene (Luytjes *et al.* 1988; Shieh *et al.* 1989). Our data showed that expression of the HE gene from the DI vector did indeed alter the pathogenesis of A59 infection by increasing the survival rate of the infected mice when compared to either parental A59 or A59 containing a CAT-expressing DI RNA (A59-DE-CAT). However, this higher survival rate was apparently due to the reduction in liver pathology. In the CNS, the expression of HE by DI RNA resulted in only a slight and transient increase of mononuclear cell infiltration and no alteration in cellular tropism. The apparent differences between this DI-expressed HE and the HE expressed directly from the virus was probably due to the transient nature of HE expression by the DI RNA, while the latter was expressed continuously throughout the infection. Nevertheless, this study shows that even transient expression of the HE protein can alter viral pathogenicity.

These data removed two constraints upon the use of DI vector for expressing foreign proteins during viral infections. First, expression of an irrelevant gene from the DI RNA during infection did not alter pathogenesis of the virus. This suggests that the weak interference activity exhibited by DI RNA *in vitro* (Makino *et al.* 1985) plays very little role during virus infection *in vivo*. Second, the conservation of wild-type virus after the DI RNA had

been lost suggested that the presence of DI RNA did not result in the selection of a variant virus, which potentially could have been derived by RNA recombination, either during *in vitro* preparation of the DI RNA-containing virus or during subsequent *in vivo* replication.

The A59 strain of MHV is a dual tropic virus, infecting both the CNS and liver following ic or intranasal infection (Lavi *et al.* 1986). At low infectious doses, the hepatitis usually resolves and survivors develop extensive chronic demyelination within the CNS (Lavi *et al.* 1984). Our data using a relatively large infecting dose show that mice infected with the parental A59 or A59-DE-CAT or A59-DE-HE virus succumb to the infection most likely due to hepatic necrosis, since there was only relatively mild CNS pathology at the time of death of the infected mice. Because virus replication in the liver and spleen early in infection was similar between A59-DE-HE- and A59-DE-CAT-infected mice, it is unlikely that there was a fundamental difference in hepatotropism between these two viruses. Therefore, the striking resolution of hepatitis and the reduction in infectious virus in mice infected with A59-DE-HE at day 6 p.i. suggests that an early function of the innate immune response is most likely responsible for this phenomenon. It is worthy of note that no CD4<sup>+</sup> or CD8<sup>+</sup> T cell epitopes have been detected within the HE gene (Bergmann *et al.* 1996; Van der Veen, 1996). In addition, there is no detectable anti-MHV antibody in the serum at the time when the hepatitis resolves (Parra *et al.* 1997).

Together, these data suggest that HE gene expression may activate or enhance a function of nonspecific innate immunity. Consistent with this notion, increased levels of TNF- $\alpha$  and IL-6 mRNA were detected in the CNS of mice infected with A59-DE-HE. Although TNF- $\alpha$  mRNA has previously been demonstrated to increase in the CNS following infection with the JHM strain of MHV (Pearce *et al.* 1994; Parra *et al.* 1997), inhibition of TNF has no effect on either virus clearance or CNS mononuclear cell infiltration (Stohlman *et al.* 1995). Therefore, the potential role of increased TNF- $\alpha$  mRNA expression following expression of the HE protein is not clear. Increased IL-6 mRNA, another pleiotropic cytokine (Van Snick, 1990), was also detected in the CNS of A59-DE-HE-infected mice. IL-6 is secreted from both endothelial cells and astrocytes following MHV infection (Joseph *et al.* 1993) and IL-6 mRNA peaks rapidly in the CNS following MHV infection as a direct consequence of the immune response to infection (Parra *et al.* 1997). Based on a kinetic analysis of IL-6 mRNA induction in the CNS following MHV infection (Parra *et al.* 1997), IL-6 has been suggested to play a role in the inflammatory response via enhanced recruitment of mononuclear cells and activation of acute phase responses in addition to its role as a cofactor for CTL induction (Romano *et al.* 1997; Kopf *et al.* 1994). Increased IL-6 mRNA present in the CNS of mice infected with A59-DE-HE virus is consistent with the

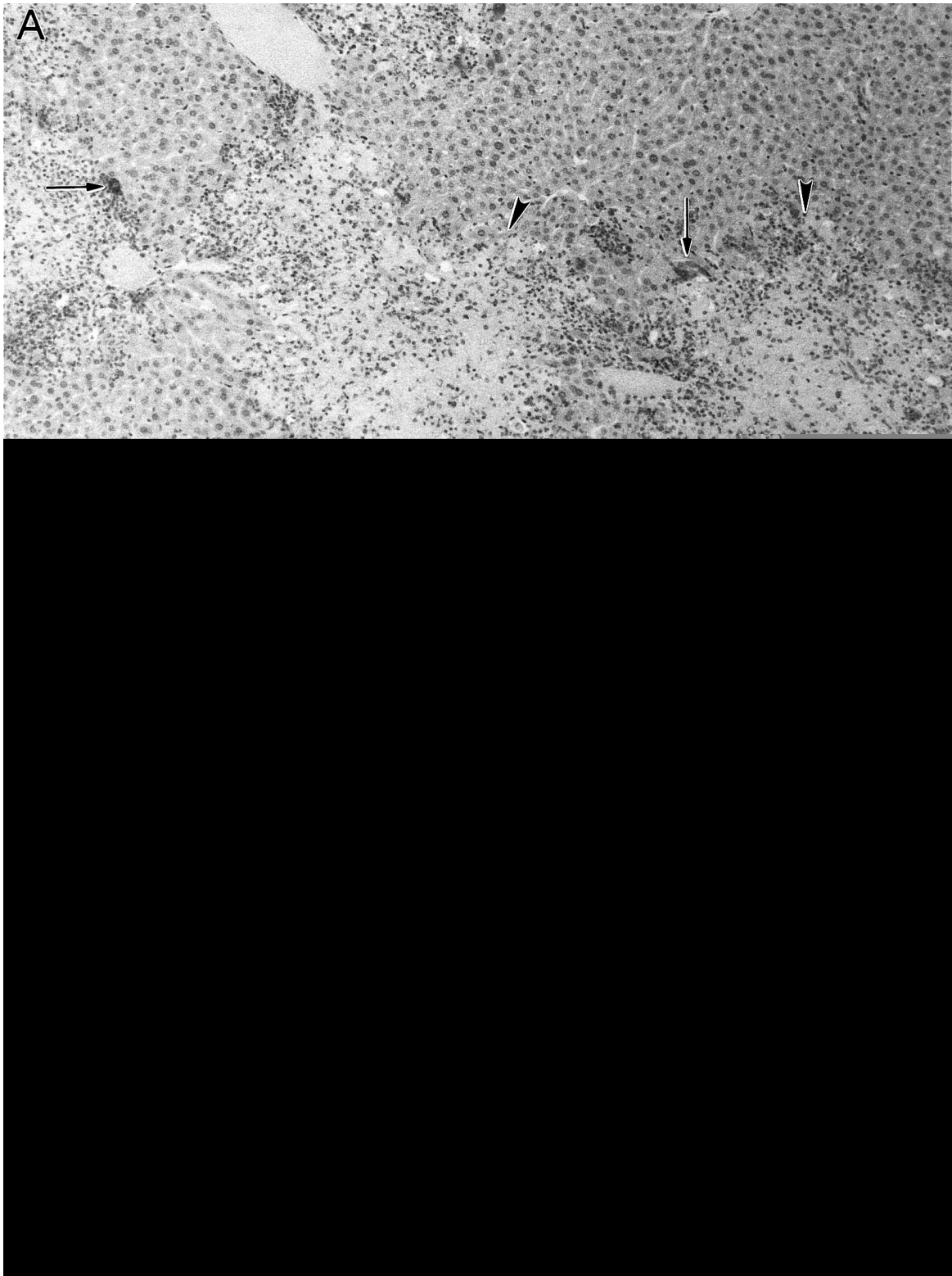


FIG. 4. Detection of viral antigen and histopathologic changes in the livers of mice infected with A59-DE-CAT or A59-DE-HE. In (A), 6 days after infection with A59-DE-CAT, sections of liver show large confluent foci of necrosis (arrowheads) with many virus-positive hepatocytes (arrows), seen in (B) at higher magnification. In (C), 6 days after infection with A59-DE-HE, sections of liver show only rare foci of hepatic necrosis. While some foci are antigen-negative, other foci show rare antigen-positive cells (arrow) (D). Immunoperoxidase stain by the ABC method for viral antigen (using N-specific mAb J.3.3) with hematoxylin counterstain. Magnifications:  $\times 110$  (A, C);  $\times 440$  (B, D).

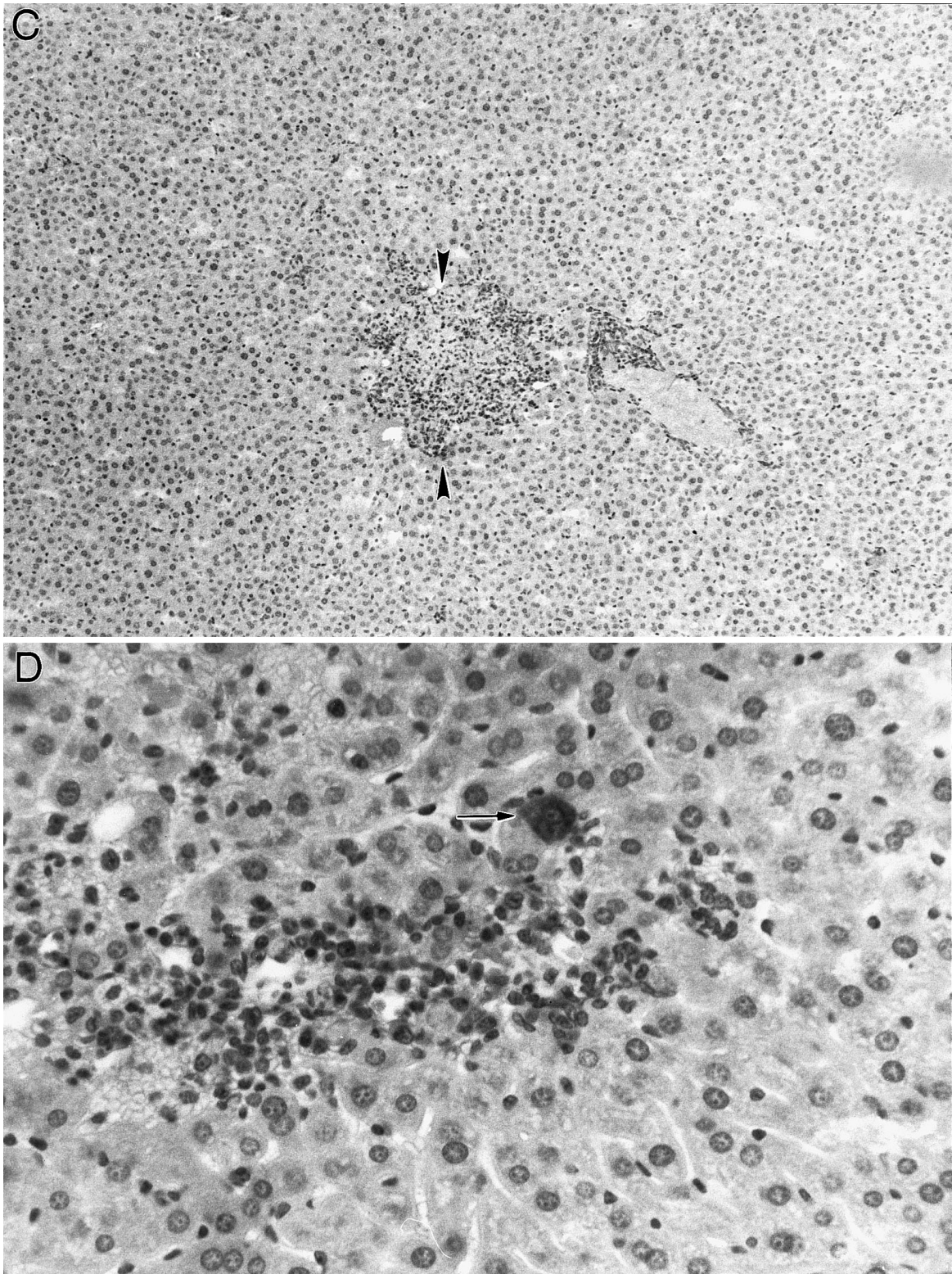


FIG. 4—Continued

increased mononuclear cell infiltration at a time during infection (day 3 p.i.) prior to detectable virus-specific immunity. These data support the suggestion that HE protein expression enhances the innate immune re-

sponse, contributing to the eventual clearance of virus from the liver.

This study and the previous report of the expression of IFN- $\gamma$  (Zhang *et al.* 1997) using a MHV-DI RNA vector

TABLE 3

Virus Replication in the Spleen Following Intracerebral Inoculation of Mice with the HE-Expressing and Control Viruses

Day <sup>a</sup>	Virus titer <sup>b</sup>	
	A59-DE-HE	A59-DE-CAT
1	0.19 ± 0.42 (5)	1.08 ± 1.00 (5)
2	1.28 ± 1.55 (5)	0.44 ± 0.62 (3)
4	1.33 ± 0.65 (5)	1.03 ± 1.08 (3)
6	Not detectable	Not detectable

<sup>a</sup> Day(s) postinfection. Mice were infected ic with  $1 \times 10^5$  PFU of various viruses on day 0.

<sup>b</sup> Mean titer in PFU/g spleen, in  $\log_{10} \pm$  standard deviation (number/group).

system indicate the feasibility of this system for studying roles of viral proteins or cytokines during MHV infection. Although expression of proteins is transient and localized at the sites of viral infection, these features may actually offer advantages in studying viral pathogenesis.

## MATERIALS AND METHODS

### Mice

C57BL/6 mice were purchased from The Jackson Laboratories (Bar Harbor, ME) at 6–7 weeks of age. Mice were housed locally in microisolator cages and were used within 7 days of receipt. None were found to have preexisting anti-MHV antibodies prior to use. Mice were inoculated intracranially (ic) with  $1 \times 10^5$  plaque-forming units (PFU) of virus in 30  $\mu$ l of Dulbecco's phosphate-buffered saline (DPBS).

### Viruses and cells

MHV strain A59 was used throughout the study. It was originally obtained from Dr. Cliff Bond of University of

California, San Diego (Leibowitz *et al.* 1981), plaque-purified four times, and used within eight passages of purification. The murine astrocytoma cell line DBT (Hirano *et al.* 1974) was used for virus propagation, plaque assay, and preparation of DI stocks.

### Construction of DI vector

To construct a DI vector containing the HE gene, we used p25CAT (Liao and Lai, 1994), which contains a CAT gene behind the (IG) sequence of gene 7 (IG7) in the cDNA of DIssE RNA (Makino *et al.* 1988) as a starting plasmid. The cDNA of the HE gene was generated by reverse transcription–polymerase chain reaction (RT–PCR) amplification using the cytoplasmic RNAs from MHV-JHM-infected cells as templates and two primers, 5'-HE (5'-GGG ACT AGT ATT GTT GAG AAT CTA ATC TAA ACT TTA AGG AAT GGG CAG TAC GTG C-3') and 3'-HE (5'-GGG CTG CAG TTA TGC CTC ATG CAA TCT-3'), corresponding to the ends of the HE gene plus *SpeI* and *PstI* (italicized sequences). Following restriction enzyme digestion with *SpeI* and *PstI*, a 1.4-kb cDNA containing the complete HE gene sequence was purified from low-melting-point agarose gel and cloned into the *SpeI* and *PstI* sites of p25CAT to replace the CAT gene (Liao and Lai, 1994), resulting in pDE-HE (Fig. 1).

### Preparation of DI stock viruses

For making DI stock viruses, plasmid DNAs of pDE-HE and p25CAT were linearized with restriction enzyme *XbaI* and subjected to *in vitro* transcription. The *in vitro* transcription was carried out in a reaction containing 1 $\times$  transcription buffer (Promega, Madison, WI), 10 mM dithiothreitol (DTT), 1 U/ $\mu$ l RNasin, 0.5 mM each ATP, CTP, UTP, and GTP, 5  $\mu$ g linearized DNA of pDE-HE or p25CAT, and 40 U T7 RNA polymerase at 37°C for 2 h according to the procedure recommended by the manu-

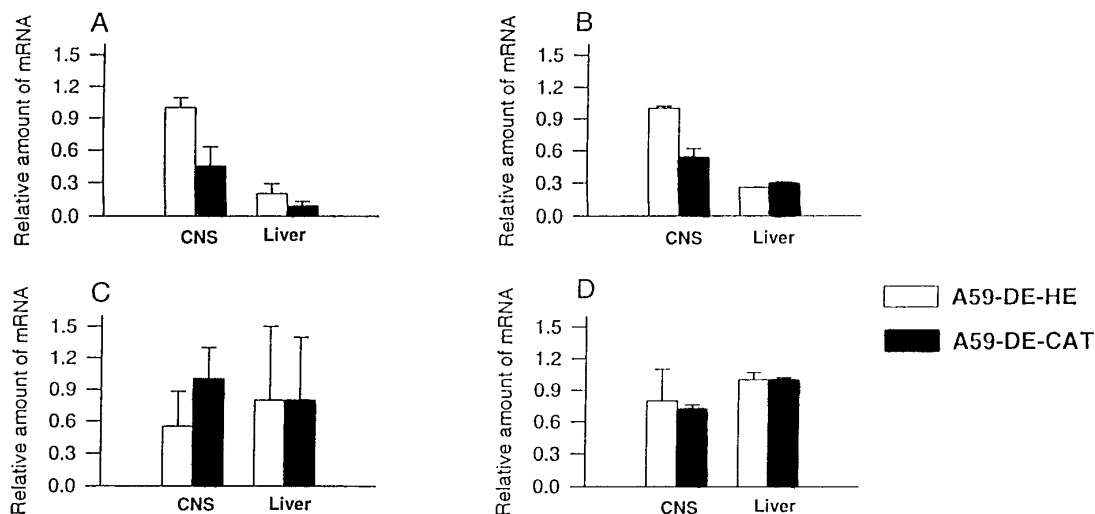


FIG. 5. Expression of TNF- $\alpha$  (A), IL-6 (B), IFN- $\gamma$  (C), and IL-1 $\alpha$  (D) mRNAs in the CNS and livers of mice infected with A59-DE-HE or A59-DE-CAT at 2 days p.i.

facturer (Promega). The *in vitro*-transcribed RNA was transfected into MHV-A59-infected DBT cells using the DOTAP method (Boehringer-Mannheim, Indianapolis, IN). Briefly, monolayers of DBT cells at approximately 80% confluence in 60-mm petri dishes were infected with A59 at a m.o.i. of 5. At 1 h postinfection (p.i.), virus-infected cells were washed once with serum-free Eagle's minimal essential medium (MEM) and then covered with 2 ml of MEM containing 1% newborn calf serum. Ten micrograms of *in vitro*-transcribed RNA was mixed with DOTAP (Boehringer-Mannheim) in 1× HBS buffer (20 mM HEPES; 150 mM NaCl; pH 7.4). The RNA-DOTAP mixture was incubated at room temperature for 10 min and then added slowly to the cell culture. At 12 to 14 h posttransfection, culture medium was harvested and cell debris removed by low-speed centrifugation. Supernatants were used as DI stocks. Each DI stock consisted of a mixture of virus populations (helper virus alone, helper virus containing HE protein on the surface, and helper virus containing a copackaged DI genomic RNA); only the virus with a copackaged DI RNA could synthesize the HE protein. Prior to inoculation into animals, virus titers of the DI stocks were determined by plaque assay using DBT cells as previously described (Fleming *et al.* 1983). The titers were  $7.5 \times 10^7$  for A59-DE-CAT and  $8.0 \times 10^7$  for A59-DE-HE. The replication of the DI RNAs of these stock viruses in DBT cells was determined by RT-PCR (Zhang *et al.* 1997) and was shown to be similar. The expression of the HE and CAT proteins in DBT cells was confirmed by immunoprecipitation using an anti-HE antibody (Yokomori *et al.* 1992) and CAT assay (Zhang *et al.* 1994), respectively. These DI stocks were then used to inoculate mice intracerebrally at  $1 \times 10^5$  PFU per animal.

### Determination of virus titers in tissues

Virus titers in mouse tissues were determined by homogenization of one half of the brain or liver lobe in 4.0 ml of DPBS, pH 7.4, using Tenbrock tissue homogenizers. The remainder was processed for histopathology (see below). Following centrifugation of the tissue homogenates at 1500 *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 (Fleming *et al.* 1983). Data were presented as the average  $\log_{10}$  titer per gram of tissue for groups of three or more mice.

### RNA isolation and RT-PCR analysis

RNAs were isolated from tissues with the Trizol Reagent according to the manufacturer's instruction (Life Technologies, Grand Island, NY). Briefly, approximately 0.1 g of tissue was homogenized in 1 ml Trizol Reagent using a glass-Teflon homogenizer. Following incubation of the homogenized sample for 5 min at room temperature, 0.2 ml of chloroform was added to each sample.

RNAs were separated by centrifugation at 12,000 *g* for 15 min at 4°C, precipitated with ethanol, and resuspended in water.

For detection of the mRNA species representing HE and CAT genes, RNA samples were subjected to reverse transcription (RT) using primers 3'-HE and 3'-CAT542 (Liao and Lai, 1994), respectively. cDNAs were amplified by nested PCR (95°C, 1 min for denaturing; 59°C, 30 s for annealing; 72°C, 2 min for extension). The primer pairs 5'-L9 (specific to the leader RNA, Zhang *et al.* 1994)/3'-HE for the HE gene and 5'-L9/3'-CAT542 for the CAT gene were used for the first PCR and 5'-L9/3'-HE74 (Zhang and Lai, 1994) or 5'-L9/3'-CAT106 (Zhang *et al.* 1994), respectively, for the second PCR. Each PCR was carried out for 25 cycles. These PCRs would amplify both the full-length DI RNA (3.6 kb for DE-HE and 2.9 kb for DE-CAT) and the subgenomic mRNAs representing HE and CAT genes (2.1 kb for DE-HE and 1.4 kb for DE-CAT). The PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. The nature of the PCR product representing the subgenomic mRNA was confirmed by the size and the restriction patterns of the PCR products.

For detection of the gene coding for the mouse fibrinogen-like protein (*fgl-2*, formerly named *musfiblp*), RT-PCR was carried out using the antisense primer 1423B (5'-TAA ACA GTA AGT GAA TTA CCA C-3', complementary to nucleotide positions 2063-2084) and the sense primer 1423A (5'-ATG GGT GGA GGC TGG ACG GT-3', corresponding to nucleotide positions 861-880) (Parr *et al.* 1995). Primers were kindly provided by Dr. Julian Leibowitz (University of Texas A & M). For cytokine mRNA expression, RNAs were reverse-transcribed using AMV reverse transcriptase and oligo(dT) primers (Promega, Madison, WI). The cDNAs were amplified by PCR using oligonucleotide primers specific for interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-6, and IL-1 $\alpha$  (Cua *et al.* 1995; Parra *et al.* 1997). PCR products were quantitated by dot blot assays of serial dilution using specific [ $\gamma$ -<sup>32</sup>P]ATP-labeled oligonucleotide probes as previously described (Cua *et al.* 1995; Parra *et al.* 1997). Signal intensities were measured using a Phosphor Imager scanner (Molecular Dynamics, Sunnyvale, CA). Values obtained for the cytokine mRNAs were normalized to the housekeeping enzyme hypoxanthine phosphoribosyltransferase (HPRT) mRNA values obtained in parallel experiments to compensate for variations in input cDNA. Data are expressed as the relative values of the maximum obtained for individual samples.

### Histopathology

For routine histopathological analysis, mice were sacrificed by CO<sub>2</sub> asphyxiation. Brains were removed and dissected in the midcoronal plane. The side of the brain opposite the needle track was taken for immunohisto-

chemical study in all cases. Brain hemispheres, spinal cords, and livers were fixed for 3 h in Clark's solution (75% ethanol and 25% glacial acetic acid) and embedded in paraffin. For routine analysis of brain sections a single coronal section including cerebellum, brain stem, hippocampus, and olfactory cortex was used. The same region of the brain was examined in all animals. At critical time points (e.g., day 3 p.i.) five step sections were taken through each hemisphere and stained for viral antigen. All viral antigen-positive cells were counted in each section and summed for each animal. Sections were stained with hematoxylin and eosin for microscopic examination. The distribution of viral antigen was examined using immunoperoxidase staining (Vectastain-ABC kit, Vector Laboratories, Burlingame, CA) and anti-JHMV monoclonal antibody 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.

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