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NOTES

Murine Coronavirus Spike Protein Determines the Ability of the Virus To Replicate in the Liver and Cause Hepatitis

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Recombinant mouse hepatitis viruses (MHV) differing only in the spike gene, containing A59, MHV-4, and MHV-2 spike genes in the background of the A59 genome, were compared for their ability to replicate in the liver and induce hepatitis in weanling C57BL/6 mice infected with 500 PFU of each virus by intrahepatic injection. Penn98-1, expressing the MHV-2 spike gene, replicated to high titer in the liver, similar to MHV-2, and induced severe hepatitis with extensive hepatocellular necrosis. $S_{A59}R13$, expressing the A59 spike gene, replicated to a somewhat lower titer and induced moderate to severe hepatitis with zonal necrosis, similar to MHV-A59. S_4R21, expressing the MHV-4 spike gene, replicated to a minimal extent and induced few if any pathological changes, similar to MHV-4. Thus, the extent of replication and the degree of hepatitis in the liver induced by these recombinant viruses were determined largely by the spike protein.

Various strains of mouse hepatitis virus (MHV) induce different patterns of pathogenesis, including enteritis, hepatitis, encephalitis, and demyelination in the mouse (20, 21). We are considering three strains here, MHV-A59, MHV-2, and MHV-4 (an isolate of MHV-JHM). The MHV-A59 strain is dualtropic, producing moderate to severe hepatitis as well as mild to moderate acute meningoencephalitis and chronic demyelination in C57BL/6 weanling mice (29, 30). The MHV-4 strain causes severe acute encephalitis, chronic demyelination, and only minimal levels of hepatitis (6, 23). The MHV-2 strain causes severe hepatitis and meningitis but is unable to cause encephalitis (7, 20, 42). There are previous studies demonstrating a relationship between attenuation of neurovirulence (6, 10, 13, 42) or hepatitis (14, 28) and the presence of mutations and variations in the spike (S) gene. The S protein, found on the virion envelope and on the plasma membrane of infected cells, is responsible for attachment to viral receptor and virus-cell fusion during viral entry and for cell-to-cell fusion later during infection. S is a 180-kDa glycoprotein, which (in the case of most MHV spike proteins) is cleaved into two noncovalently associated 90-kDa subunits, the amino-terminal S1 and carboxy-terminal S2 subunits (14, 33). It is speculated that the S1 subunit forms the globular head of the spike and the S2 subunit forms the membrane-bound stalk (8). Recently, a receptor-binding activity has been demonstrated using a recombinant protein containing the amino-terminal 330 residues of the S1 subunit of MHV-JHM (25, 41). S2 is believed to contain the domain that mediates fusion of viral and cell membranes

(5, 8). The MHV-2 spike, while highly homologous in sequence to the spike proteins of other MHV strains, remains uncleaved and does not mediate fusion (44, 45).

Using targeted recombination technology (11, 12, 35), we have directly demonstrated that the spike protein is a major determinant of the neuropathogenic properties of MHV (39). When the S gene of MHV-4 was introduced into the background of MHV-A59, the resulting recombinant viruses (S_4R21 and S_4R22) were highly neurotropic, displaying similar pathogenic properties to parental MHV-4 after intracerebral inoculation into mice. These experiments did not address the question of whether the hepatitis phenotype of the recombinant viruses is also determined by the spike protein.

In order to more completely explore the role of the spike protein in controlling the ability to induce hepatitis, we compared three isogenic recombinant viruses that differ only in the spike gene, expressing the spike protein of A59 ($S_{A59}R13$), MHV-2 (Penn98-1), or MHV-4 (S_4R21) in the background of the A59 genome. This study shows that the level of viral replication in the liver is determined by the spike gene and that the amount of antigen staining and necrosis in the liver correlates with the level of viral replication. Thus, the ability to induce hepatitis is largely determined by the spike gene.

We have previously described the targeted recombination technology used to select recombinant viruses differing only in the spike gene, including the wild-type A59 recombinants and the MHV-4 spike-containing recombinant viruses that we have compared for neuropathogenesis (32, 39). In this study, we are using, in addition, a third type of recombinant virus only containing the MHV-2 spike gene, as described by Das Sarma et al. (7). Briefly, recombinant viruses were selected using the Alb4 mutant of MHV-A59 and synthetic capped RNA transcribed from pMH54, a plasmid containing a portion of the

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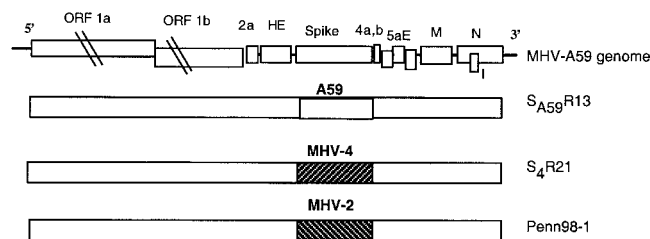
FIG. 1. Schematic diagram of MHV-A59 genome and generated recombinant viruses. The MHV genome is 31 kb. The sizes of the genes are drawn approximately to scale except for gene 1. Viral genes encoding nonstructural proteins (1a, 1b, 2a, 4a and b, and 5a) and structural proteins (HE, spike, E, M, N, and the internal gene I) are indicated. $S_{A59}R13$, used as a control recombinant virus, has the A59 spike reintroduced in the A59 background genome. S_4R21 and Penn98-1 recombinant viruses present the MHV-4 and the MHV-2 spike genes in the MHV-A59 background, respectively.

A59 HE pseudogene through the 3′ end of the genome (26) with the MHV-4 or MHV-2 spike gene substituted for the MHV-A59 spike gene (7, 39). In the experiments described below, we used $S_{A59}R13$, a wild-type recombinant expressing the MHV-A59 spike protein; S_4R21, expressing the MHV-4 spike protein; and Penn 98-1, expressing the MHV-2 spike protein. The spike genes of Penn98-1, S_4R21, and $S_{A59}R13$ were sequenced and shown to be identical to those from the parental viruses. In every case, a second independent recombinant had the identical spike gene sequence and displayed the same properties. Figure 1 shows a schematic diagram of these recombinant viruses.

We initially compared the replication in the liver of the parental MHV-A59, MHV-2, and MHV-4 (Fig. 2) by inoculating 500 PFU of each virus directly into the liver of weanling C57BL/6 mice (The Jackson Laboratory, Bar Harbor, Maine). We used direct intrahepatic rather than intracerebral inoculation because this eliminates the central nervous system (CNS) disease induced by MHV-A59 and MHV-4 and isolates the hepatitis. We have previously shown that this method delivers virus into the liver (17). Mice were sacrificed at 1, 3, 5, and 7 days postinfection (p.i.). After perfusion of animals with phosphate-buffered saline, livers were homogenized, and virus was titrated on L-2 cells, as previously described (17). While the nonhepatotropic MHV-4 replicated only slightly above the level of detection, both A59 and MHV-2 replicated efficiently, higher in the case of MHV-2, peaking at day 5, as previously observed for MHV-A59 (18, 31) (Fig. 2). This is consistent with previous results reported for these strains of virus (20).

Recombinant viruses $S_{A59}R13$, Penn98-1, and S_4R21 were inoculated intrahepatically into weanling mice, which were sacrificed at selected times postinfection, and viruses from liver homogenates were titrated as described above (Fig. 2). All of the recombinant viruses replicated at levels similar to those of the parental strains. At day 5 p.i., Penn98-1 virus replicated to one log higher than $S_{A59}R13$ and 3 logs over S_4R21 (Penn98-1 versus $S_{A59}R13$, $P < 0.03$; Penn98-1 versus S_4R21, $P < 0.004$; Wilcoxon's rank sum test). The MHV-4 spike-containing recombinant virus (S_4R21), like MHV-4 itself, replicated to a minimal extent above the level of detection (Fig. 2). Thus, since all the recombinants had the same MHV-A59 back-

ground, the level of replication was largely determined by the spike.

In order to further assess hepatitis induced by these viruses, histopathology studies on liver sections were performed. For histological diagnosis, formalin-fixed liver tissue was embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Hepatitis was scored as mild, moderate, or severe with the following criteria: (i) mild hepatitis (level 1): very few, small foci of inflammation and hepatocellular necrosis; (ii) moderate hepatitis (level 2): multiple foci of hepatocellular necrosis separated by normal liver; and (iii) severe hepatitis (level 3): either diffuse confluent (bridging) lesions or multiple foci. Replicate sections were stained immunohistochemically. All slides were read in a blinded manner. Briefly, sections were incubated with a monoclonal antibody (MAb) against the nucleocapsid protein (N) of MHV-JHM (MAb clone 1-16-1, kindly provided by J. L. Leibowitz, Texas A&M University), and immunohistochemistry was performed by the avidin-biotin-immunoperoxidase technique (Vector Laboratories, Burlingame, Calif.) using diaminobenzidine tetrahydrochloride as the substrate and counterstained with methyl green (Dako, Carpinteria, Calif.). As controls, replicate sections of each sample were incubated with an unrelated antibody (mouse immunoglobulin G, 10 μg/ml) and with secondary antibody alone. Furthermore, liver sections from mock-infected mice were incubated with the anti-N (MHV) MAb. These data are shown in Figs. 3, 4, and 5. Only the data obtained with the recombinant viruses are shown, as representative of data also observed in their parental viruses.

First, we found that the spike protein determines the degree of hepatitis (Fig. 3A). The degree of hepatitis induced by
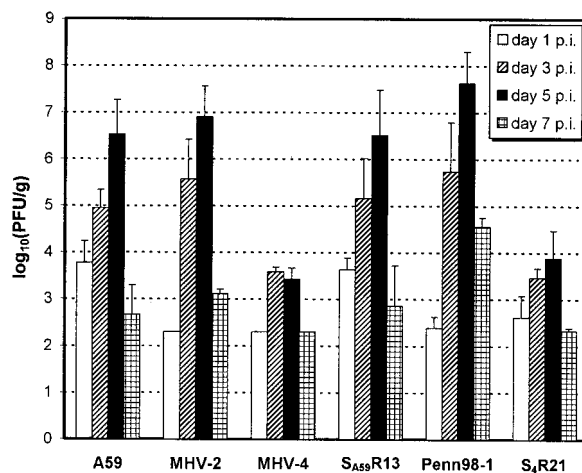

FIG. 2. Titers of MHV in livers of animals infected with parental and recombinant viruses. C57BL/6 mice were infected intrahepatically with 500 PFU of MHV-A59, MHV-2, MHV-4, and the recombinants $S_{A59}R13$, Penn98-1, and S_4R21, and sacrificed at days 1, 3, 5, and 7 p.i. Virus titers were determined by plaque assay. Virus titers are presented as logarithmic means of PFU per gram of liver. Error bars represent logarithmic standard deviation. The limit of detection was 300 PFU/g of liver. Numbers of mice examined were as follows: MHV-A59, 18; MHV-2, 15; MHV-4, 8; $S_{A59}R13$, 23; Penn98-1, 19; and S_4R21, 24. At day 5 p.i., replication of Penn98-1 was significantly higher than that of $S_{A59}R13$ and S_4R21 (Wilcoxon's rank sum test: $P < 0.03$ and $P < 0.004$, respectively).

$S_{A59}R13$ was less severe than the extreme hepatitis induced by Penn 98-1 ($P < 0.05$, Wilcoxon's rank sum test), whereas S_4R21 induced mild hepatitis ($S_{A59}R13$ versus S_4R21, $P < 0.05$; Penn98-1 versus S_4R21, $P < 0.01$; Wilcoxon's rank sum test). Second, we found a correlation between the viral replication titers and the degree of hepatitis induced by each virus (Spearman's correlation coefficient: $r = 0.85$, $P < 0.001$, Fig. 3B). Third, the extent of hepatocellular injury was also monitored by counting the number of nonconfluent necrotic foci per liver section, and this analysis also demonstrated a positive correlation between virus titers and the severity of hepatitis (Spearman's correlation coefficient: $r = 0.88$, $P < 0.001$) (Fig. 3C).

Figures 4 and 5 show immunohistochemistry staining of liver sections from $S_{A59}R13$-, Penn98-1-, and S_4R21-infected mice by day 5 p.i. At low magnification (Fig. 4), Penn98-1 showed evidence of extensive hepatocellular necrosis, whereas $S_{A59}R13$ showed several areas of focal necrosis. The antigen staining always colocalized with the necrotic areas. In contrast, in S_4R21-infected livers, the hepatic parenchyma appeared almost normal, with very low levels of antigen staining. Higher magnification (Fig. 5) revealed inflammatory cell infiltration associated with necrotic areas in the livers infected by $S_{A59}R13$ and Penn98-1. Livers from animals infected with either $S_{A59}R13$ or Penn98-1 also exhibited staining of sinusoidal lining cells (consistent with Kupfer or endothelial cells or both). In the case of Penn98-1-infected animals, hepatocellular degeneration and necrosis were so extreme that it was not possible to find isolated hepatocytes immunoreactive for viral antigen, while in the livers of $S_{A59}R13$-infected animals, staining was observed both in necrotic foci and in isolated hepatocytes. Finally, livers from animals infected with S_4R21 showed viral antigen staining in sinusoidal lining cells and small clusters of hepatocytes as well as in isolated hepatocytes. Little if any tissue destruction or inflammation was observed. This is consistent with the minimal level of virus replication observed with S_4R21.

Thus, our results indicate that substitution of the spike gene of either MHV-2 or MHV-4 for the MHV-A59 spike gene within the A59 genome is sufficient to produce the hepatitis phenotype of the strain from which the spike is derived. This is not surprising, since the spike is responsible for viral attachment to the receptor, entry, and cell-to-cell fusion, and thus it may be expected to play a crucial role in initiation of infection as well as in spread of the virus. It is noteworthy that the S protein of MHV has recently been exchanged by targeted RNA recombination for the S protein of feline infectious peritonitis virus. The resulting chimeric virus acquired the ability to infect feline cells and lost the ability to infect murine cells, demonstrating that receptor utilization is a major factor in determining the host range of coronavirus infections (26).

It is likely that interaction of the spike and the viral receptor may play a role in the outcome of infection in the liver. We have previously shown that a one-amino-acid substitution, Q159L, within the amino-terminal region of the MHV-A59 spike, a region that has been demonstrated to bind to receptor in an vitro assay, results in the inability of the virus to replicate in the liver and induce hepatitis (32). This suggests that the ability to induce hepatitis may be regulated at the receptor level. Indeed, there are differences in the interaction of the MHV-A59 and MHV-4 spikes with viral receptors (46). The primary cellular receptor for MHV is a transmembrane glyco-
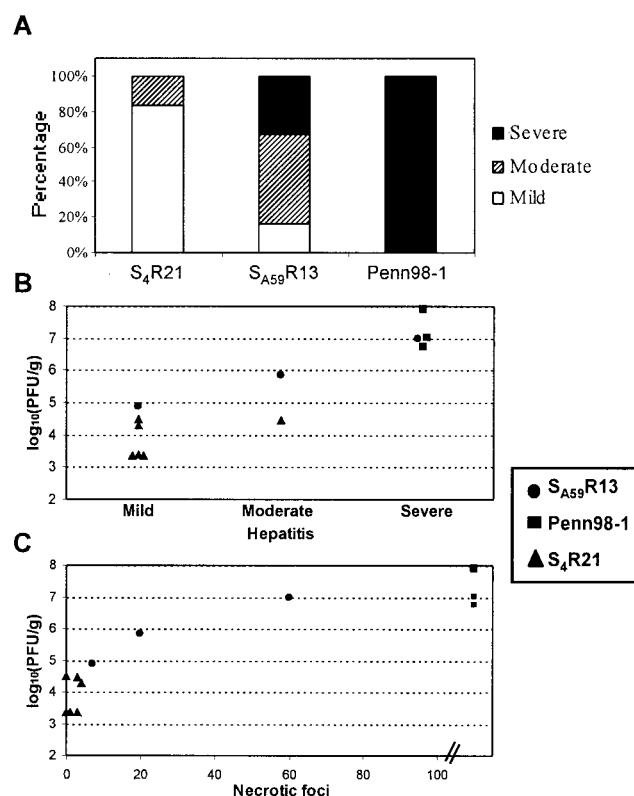

FIG. 3. Spike protein determines degree of hepatitis and level of replication in the liver. The extent of hepatitis (scored as described in the text) and virus titers of recombinant viruses in livers of C57BL/6 mice after intrahepatic inoculation with 500 PFU of $S_{A59}R13$, Penn98-1, and S_4R21 viruses were studied. (A) Hepatitis induced by $S_{A59}R13$ was milder than the severe hepatitis induced by Penn98-1 ($P < 0.05$, Wilcoxon's rank sum test), whereas S_4R21 induced a low extent of hepatitis ($S_{A59}R13$ versus S_4R21, $P < 0.05$; Penn98-1 versus S_4R21, $P < 0.01$; Wilcoxon's rank sum test). The results are shown as the percentage of mice with mild, moderate, and severe hepatitis, with six animals included per recombinant virus. Viral replication correlated both with the degree of hepatitis induced by each virus (B) and with the number of nonconfluent necrotic foci (C) (Spearman's correlation coefficients: $r = 0.85$, $P < 0.001$, and $r = 0.88$, $P < 0.001$, respectively). Each point represents viral replication and type of hepatitis or number of nonconfluent necrotic foci at day 5 p.i. of individual mice (three with $S_{A59}R13$, 3 with Penn98-1, and six with S_4R21).

protein of the murine biliary glycoprotein (Bgp) subfamily of the carcinoembryonic antigen family, also known as MHVR or Bgpla. Bgpla is found on epithelial and endothelial cells as well as on B lymphocytes and macrophages. Godfraind et al. (16) recently found that in the liver, Bgpla expression correlates with infection of hepatocytes and endothelial cells, leading to the development of hepatitis.

Both parenchyma cells (mostly hepatocytes) and sinusoidal lining cells (endothelial and/or Kupfer cells) express viral antigen after infection with all three viruses, and similar patterns of labeled sinusoidal lining vessel cells are found. The major difference among the strains lies in the hepatocellular degeneration and necrosis induced by each virus. Consistent with this is the staining of the hepatocytes. In liver sections from S_4R21-infected mice, a few isolated labeled hepatocytes are observed. In the case of $S_{A59}R13$-infected mice, there are many more
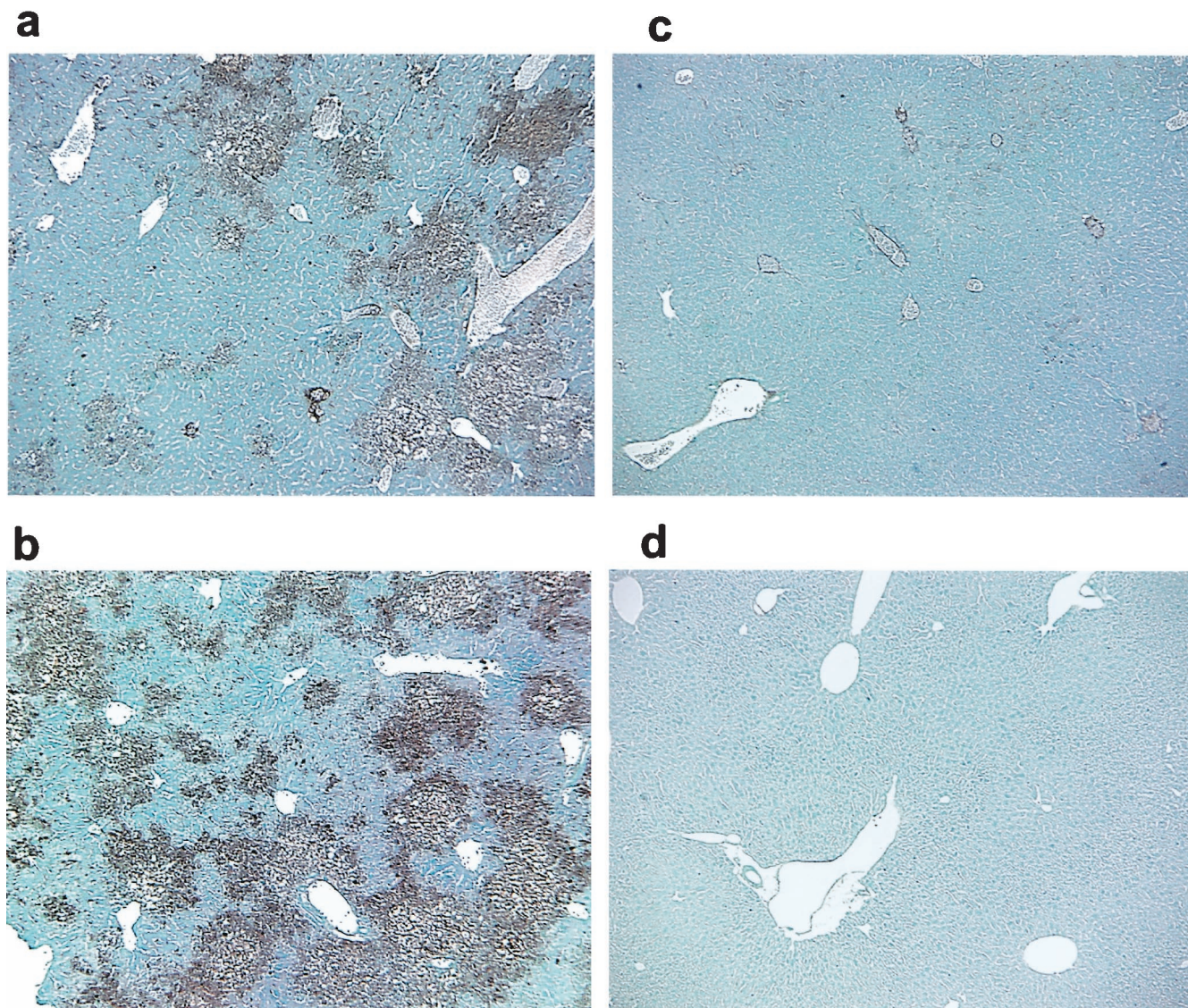
FIG. 4. Immunohistochemistry of liver sections of C57BL/6 mice infected with the recombinant viruses $S_{A59}R13$ (a), Penn98-1 (b), and S_4R21 (c) and a mock-infected mouse (d). Mice were infected as described for Fig. 2 and then sacrificed at day 5 p.i. Livers were removed, fixed and sectioned. MHV was detected by immunolabeling with an MAb against the nucleocapsid (N) protein of MHV, using the avidin-biotin-immunoperoxidase technique (Vector) as described in the text. Viral antigen was associated with areas of hepatocellular necrosis in $S_{A59}R13$ (a) and Penn98-1 (b). S_4R21-infected mice (c) showed low levels of viral antigen staining. No signs of pathology or viral antigen were found in a mock-infected control (d). Magnification, ×40.

labeled hepatocytes as well as antigen-positive necrotic foci. With Penn98-1, there are nearly confluent antigen-positive necrotic foci. These results suggest that endothelial and/or Kupfer cells may be infected by all three viruses but that spread into the parenchymal hepatocytes is greatly reduced with S_4R21. We have previously observed that both MHV-A59 (19) and a very weakly hepatotropic strain (C12) (unpublished results) replicate to very similar low titers, suggesting that the hepatocytes are not a major site of replication for MHV-A59. By comparing the highly hepatotropic MHV-3 with MHV-4, it has been also demonstrated that replication in hepatic endothelial cells but not hepatocytes correlated with hepatotropism (22, 34, 37, 38). Our data are consistent with the notion that all three strains may infect the vessel cells but that replication

and/or spread from these cells into hepatocytes might be much less efficient with MHV-4.

Belyarsky et al. (2) found that MHV-3, a strain that results in lethal fulminant hepatic necrosis in susceptible mice (9), is capable of inducing apoptosis in primary macrophage cultures. Schwartz et al. (40) have demonstrated that acute infection with MHV-A59, MHV-2, and the recombinant Penn98-1 induces apoptosis in hepatocytes and inflammatory cells. It is also known that the murine coronavirus spike contains both B-cell and T-cell epitopes (3, 4); thus, differences in spike sequences might have a major effect on the immune response. Interestingly, recent data obtained in various mouse hepatitis models for fulminant hepatitis indicate that Fas-mediated apoptosis plays an important role in the cytotoxicity induced by
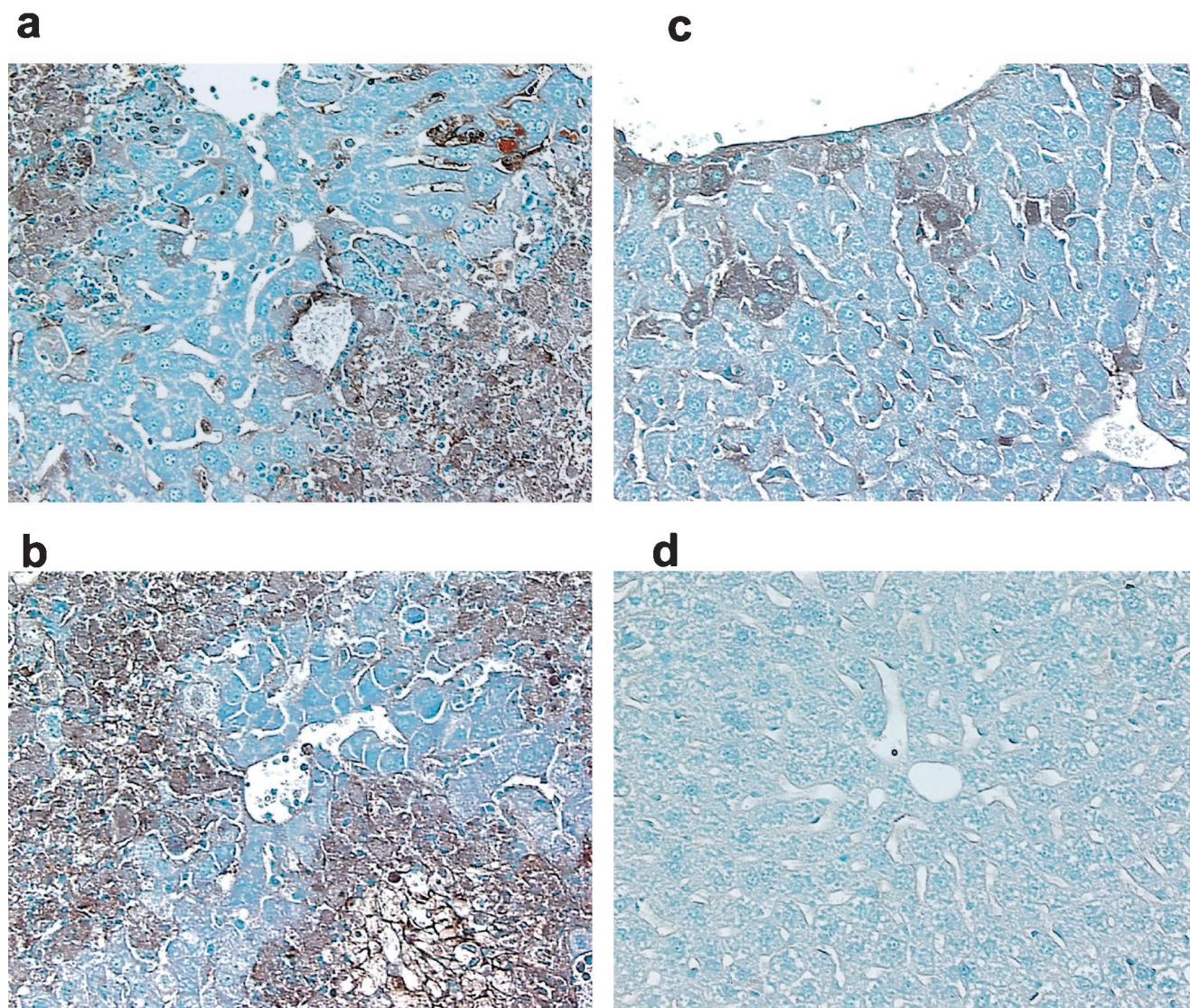
FIG. 5. Immunohistochemistry of liver sections of C57BL/6 mice infected with the recombinant viruses $S_{A59}R13$ (a), Penn98-1 (b), and S_4R21 (c) and a mock-infected mouse (d) at day 5 p.i. Immunolabeling was performed as described for Fig. 4. Viral antigen staining was found in sinusoidal lining cells (consistent with endothelial and/or Kupfer cells) of livers infected with $S_{A59}R13$ (a), Penn98-1 (b), and S_4R21 (c). Viral antigen colocalized with focal necrosis areas in $S_{A59}R13$ (a), confluent necrosis areas in Penn98-1 (b), and small clusters of hepatocytes in S_4R21 (c). No signs of pathology or viral antigen were found in a mock-infected control (d). Magnification, ×200.

hepatitis virus-specific cytotoxic T lymphocytes against virus-infected hepatocytes (1, 24, 27). Taking all these data together, it may be argued that both apoptosis and the immune response play a role in MHV-induced hepatitis.

Finally, it should be noted that there is a noticeable difference in the pathogenesis of MHV in the liver and the CNS in that the extent of replication of the recombinant viruses in the CNS did not predict the amount of inflammation and disease or the virulence of the recombinant viruses. In the CNS, while S_4R21 (and other MHV-4 spike-containing recombinant viruses) are approximately 1,000-fold more virulent than $S_{A59}R13$ and other A59 spike-containing wild-type recombinant viruses and induce more inflammation and significantly more viral antigen, both viruses replicate to similar levels (39). In the liver, the amount of replication correlates with the extent of hepatitis. Thus, S_4R21 and its parent MHV-4 replicate minimally in the liver and induce minimal hepatitis, while $S_{A59}R13$ and its parent A59 replicate to a significant degree, causing an intermediate level of viral antigen expression and necrosis in the liver. Finally, Penn98-1 and its parent MHV-2 replicate to the highest levels in the liver and induce extensive hepatocellular necrosis.

Future studies will be directed at elucidating the mechanisms which control the ability of the spike to induce various levels of hepatitis. We are starting by mapping the regions of the spike protein that control hepatotropism.

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