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Murine Coronavirus-Induced Hepatitis: JHM Genetic Background Eliminates A59 Spike-Determined Hepatotropism

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Recombinant murine coronaviruses, differing only in the spike gene and containing the strain A59 (moderately hepatotropic) and JHM (neurotropic) spike genes in the background of the JHM genome, were compared for the ability to replicate in the liver and induce hepatitis in weanling C57BL/6 mice. Interestingly, expression of the A59 spike glycoprotein within the background of the neurotropic JHM strain does not reproduce the A59 hepatotropic phenotype. Thus, the JHM genetic background plays a dominant role over the spike in the determination of hepatotropism.

The murine coronavirus mouse hepatitis virus (MHV) has a single-stranded, positive-sense RNA genome of approximately 31 kb (35). MHV infection of the central nervous system (CNS) has been used as a model for the study of chronic demyelinating diseases (3, 6, 9, 12, 19, 23, 26, 32). Some MHV strains cause a wide range of liver injuries that range from minimal changes to fulminant hepatitis (5, 11, 13, 20). The development of targeted RNA recombination (14, 24, 25, 38) has allowed the generation of chimeric recombinant viruses differing only in the spike gene (22). Targeted RNA recombination was developed by using the MHV-A59 strain (24, 25), and thus, until recently, only the A59 genetic background has been used for generation of recombinant viruses. Using chimeric A59 recombinant viruses, we have previously demonstrated that the spike protein of MHV is a major determinant of neurovirulence (33, 34), demyelination (4), and hepatotropism (27). We further demonstrated that the ability to induce hepatitis is largely determined by the spike gene when the rest of the viral genes are derived from A59 (27). We compared three isogenic recombinant viruses differing only in the spike gene and expressing the spike protein of A59 (RA59; formerly named S_{A59}R13), MHV-2 (Penn 98-1), or MHV-JHM (SJHM-RA59; formerly named S_4R21), all in the background of the A59 genome. We found that after intrahepatic inoculation with 500 PFU per mouse, the spike gene determined the viral load in the liver and that the amounts of antigen staining and necrosis in the liver correlated with the viral load (27). Our results were not surprising, since the spike glycoprotein is responsible for viral receptor attachment, entry, and cell-to-cell fusion (7, 8, 39). Thus, the spike would be expected to play a crucial role in initiation of infection, as well as in virus spreading (8). Our goal in this study was to explore the role of the viral genetic background in the development of murine coronavirus-induced hepatitis. Our data demonstrate that the presence of background genes from the neurotropic JHM strain of MHV eliminates A59 spike hepatotropism even after inoculation with 10^6 PFU directly into the liver.

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We have previously described the targeted recombination technology used to select recombinant viruses (in the A59 background) differing only in the spike gene, including the wild-type A59 recombinant (RA59) and the A59 recombinant virus expressing the JHM spike (SJHM-RA59) (27, 33). In this study, we have constructed two new recombinant viruses. Briefly, recombinant viruses were selected by using fMHV-JHM (clone B3b) (29), an interspecies chimeric JHM recombinant virus encoding the ectodomain of the spike gene of feline infectious peritonitis virus (15), and synthetic capped RNA transcribed from pJHM, a plasmid containing the HE gene through the 3′ end of the JHM genome in which spike genes can be switched. Here, we have changed our previous virus terminology in order to make it easier to identify the source of the spike and the source of the other viral genes. Thus, recombinant parental strains are named RA59 and RJHM and chimeric recombinant viruses are named after the source of the spike, followed by the type of viral genetic background. Thus, RJHM is a wild-type recombinant virus expressing the JHM spike protein whereas SA59-RJHM expresses the A59 spike in the JHM background. For each genotype, at least two independent recombinant viruses with the same spike gene sequence were evaluated in order to minimize the possible interference of spurious mutations outside of the spike gene. Note that independent recombinant viruses of the same genotype are not distinguished by name.

We initially compared the in vitro replication characteristics of the recombinant viruses. We and others have previously reported that MHV-A59 replicates to a high titer in a number of cell lines in vitro, whereas MHV-JHM replicates to a lower titer and displays higher levels of fusion and cytotoxicity (7, 8). Here, to determine whether non-spike genes affect replication properties, we analyzed the kinetics of both released and cell-associated virus production for the recombinant viruses SJHM-RA59 and SA59-RJHM, compared to those of RA59 and RJHM, in L2 cells (Fig. 1). In quantifying released virus, we found that recombinant viruses containing the JHM spike gene, SJHM-RA59, RJHM, and parental JHM (data not shown), all replicated with slower kinetics and to a lower final titer than RA59, confirming our previous results that in cell culture, the spike gene of JHM conferred an alteration in in vitro replication (33). Interestingly, JHM recombinants ex-

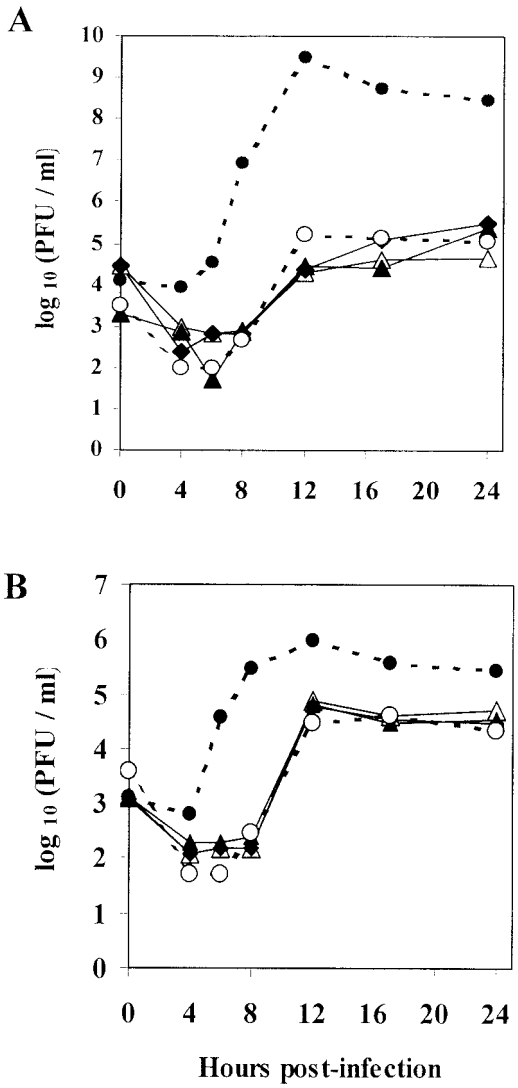

FIG. 1. Time course of released (A) and cell-associated (B) virus production in L2 cell cultures. L2 cells were infected in duplicate with RA59 (●), SJHM-RA59 (○), RJHM (♦), or SA59-RJHM (▲, △) at a multiplicity of infection of 1 PFU/cell. The data shown represent the mean titers of duplicate samples. In the case of SA59-RJHM, two independent recombinant viruses were analyzed. At the indicated times, virus titers were determined in cells and culture supernatants by plaque assay in L2 cells.

pressing the A59 spike gene (SA59-RJHM) displayed the same pattern of low virus production as RJHM and SJHM-RA59. Thus, introduction of the A59 spike gene into the JHM background did not alter the slower kinetics or the final extent of replication of JHM. Comparison of recombinant viruses with the same spike gene and differing only in non-spike genes (SJHM-RA59 versus RJHM and SA59-RJHM versus RA59) confirms that JHM genes other than the spike gene play a role in determining the extent of in vitro virus replication. When cell-associated virus production was quantified, however, the difference between RA59 and the other strains was much less dramatic. This suggests that the presence of high titers of RA59 in the supernatant may be due to factors such as virus stability rather than replication itself. (RA59 is, in fact, more stable than SJHM-RA59 [37].) Strikingly, SA59-RJHM exhibited a small-plaque phenotype (<1 mm), like JHM (data not shown), demonstrating that the A59 spike gene in the JHM background does not confer the large-plaque phenotype (>2 mm) characteristic of A59. Thus, in cell culture, JHM background genes contribute to plaque morphology, as well as replication kinetics.

TABLE 1. Virulence of recombinant viruses after intracranial and intrahepatic inoculations

Virus	Log_{10} LD_{50}	
	Intracranial	Intrahepatic
RA59	3.8	3.8
RJHM	0.7	>6.0
SJHM-RA59	1.0	>5.0
SA59-RJHM	2.0	>6.0

In order to determine whether replacement of the JHM spike gene with the A59 spike gene alters the in vivo JHM phenotype (SA59-RJHM), we evaluated virulence by both intracranial and intrahepatic inoculations of 4-week-old male C57BL/6 mice (NCI). We also evaluated the virulence of RA59, RJHM, and SJHM-RA59 (Table 1). Infected mice were observed for mortality, and the 50% lethal dose (LD_{50}) was calculated as previously described (27). We confirmed our previous results demonstrating that the spike gene of the highly neurovirulent JHM strain is sufficient to confer high neurovirulence in the A59 background. In contrast, substitution of the spike gene of neuroattenuated A59 for the JHM spike gene (SA59-RJHM) had lesser effects on JHM neurovirulence, suggesting that genes other than the spike gene contribute significantly to JHM neurovirulence. When virulence was assessed by direct inoculation into the livers of mice (which eliminates the CNS disease [10]), the LD_{50}s of the SJHM-RA59, RJHM, and SA59-RJHM viruses were dramatically higher while the virulence of RA59 was the same as that obtained by intracranial inoculation (Table 1). This attenuation following intrahepatic inoculation suggests that RJHM, SJHM-RA59, and SA59-RJHM are very poorly hepatotropic. These data confirm that the JHM spike gene does confer a dramatic increase in neurovirulence within the A59 background (33) and further demonstrate that genes other than the spike gene may contribute to JHM virulence.

Hepatotropism and pathogenesis in the liver of recombinant viruses RA59, RJHM, SJHM-RA59, and SA59-RJHM were evaluated by direct inoculation of the livers of 4-week-old male C57BL/6 mice that were sacrificed at day 5 postinfection (p.i.) (the peak of replication in the liver [27]). After perfusion with phosphate-buffered saline, livers were homogenized and the virus titer was determined on L2 cells as previously described (27). We first inoculated mice with 500 PFU, the same dose used in our previous study (27). RA59 replicated efficiently, whereas the A59 recombinant virus expressing the spike gene of JHM (SJHM-RA59) replicated to a minimal extent above the level of detection (RA59 versus SJHM-RA59, $P < 0.01$ [Wilcoxon rank sum test]), confirming our previous data (27) (Fig. 2A). Remarkably, after inoculation with 500 PFU, no infectious virus was recovered from livers infected with the

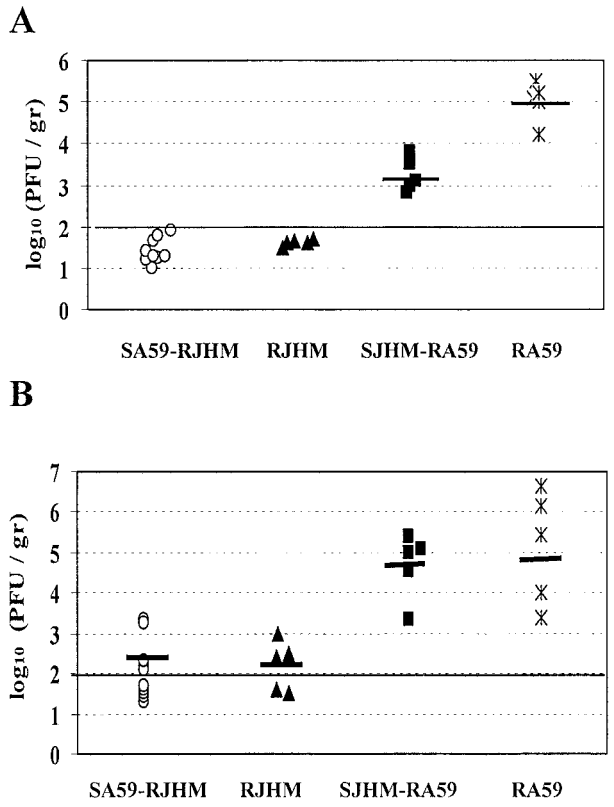

FIG. 2. Viral loads in livers of C57BL/6 mice after intrahepatic inoculation with 500 (A) or 10^5 to 10^6 (B) PFU of recombinant virus RA59, SJHM-RA59, RJHM, or SA59-RJHM, at 5 day p.i. Viral titers were determined by plaque assay and are presented as the $\log_{10}$ PFU per gram of liver. Each point represents viral titer of individual mice, and bars represent the $\log_{10}$ of the mean in each group. The limit of detection was 200 PFU/g of liver. The numbers of mice examined per viral dose were as follows: RA59, SJHM-RA59, and RJHM, 5; SA59-RJHM, 10. RJHM and SA59-RJHM did not induce a productive infection in the liver, irrespective of the viral dose ($P < 0.01$ [Wilcoxon rank sum test]). RA59 replicated efficiently irrespective of the viral dose, whereas SJHM-RA59 replicated to a minimal extent after inoculation with 500 PFU (RA59 versus SJHM-RA59, $P < 0.01$ [Wilcoxon rank sum test]). SJHM-RA59 was able to replicate to an extent similar to that of RA59 only after inoculation of large viral doses.

JHM recombinant virus expressing the A59 spike gene (SA59-RJHM) or RJHM (Fig. 2A). While a negative or very low burden of RJHM virus can be expected, the absence of SA59-RJHM virus production in the liver was surprising with respect to our previous report, in which we demonstrated that the spike gene is a major determinant of hepatotropism (27). This finding prompted us to further analyze liver tropism following inoculation with larger viral doses (10^5 and 10^6 PFU) (Fig. 2B). Strikingly, SA59-RJHM, like RJHM itself, was still unable to induce a productive infection in the liver (only a few mice showed minimal replication, just above the limit of detection), even after inoculation with 10^6 PFU (Fig. 2B). These data demonstrate that substitution of the A59 spike gene for the JHM spike gene does not alter the low-hepatotropism phenotype of JHM, suggesting that background genes do play a significant role in liver tropism. Supporting these data, we also found that after inoculation with 10^5 PFU, the A59 recombinant virus expressing the JHM spike gene (SJHM-RA59) was able to replicate to an extent similar to that of RA59 (Fig. 2B). This fact further supports a contribution of background genes to hepatotropism. These data may seem to contradict our previous report demonstrating that the spike is a major determinant of hepatotropism (27). However, in the present study, we confirm our previous finding that, at a low virus dose (500 PFU), the extent of hepatitis is determined by the spike gene within the A59 background; at this dose, the A59 recombinant virus expressing the JHM spike gene (SJHM-RA59) replicates to a minimal extent while RA59 replicates efficiently.

To further evaluate hepatitis induced by these viruses, histological assessment of liver sections was performed. Formalin-fixed, paraffin-embedded liver tissue was sectioned and stained with hematoxylin and eosin for histopathological diagnosis. Hepatitis was graded blindly and scored on a scale of 0 to 4 as previously described (1). Immunohistochemical analysis was performed on replicate sections as previously reported (27), by using a monoclonal antibody (MAb) against the nucleocapsid protein of MHV-JHM (MAb clone 1-16-1, kindly provided by J. L. Leibowitz, Texas A & M University). Because SA59-RJHM and RJHM exhibited a very low level of replication in the liver, an antigen retrieval technique (Antigen Unmasking Solution; Vector) was performed on all sections in order to enhance virus localization. Appropriate controls were performed (27), and slides were read in a blinded manner. The resulting data are shown in Fig. 3, 4, and 5.

After inoculation with 500 PFU, hepatitis induced by RA59 was mainly characterized by moderate hepatocellular damage although some mice developed mild or even severe hepatitis. This range of liver lesions has been previously observed for RA59 (21, 27). The A59 recombinant virus expressing the JHM spike gene (SJHM-RA59) induced minimal changes to mild hepatitis (RA59 versus SJHM-RA59, $P < 0.01$ [Wilcoxon rank sum test]), confirming our previous report (27). Interestingly, the JHM recombinant virus expressing the A59 spike gene (SA59-RJHM), like RJHM itself, induced minimal changes in the liver (RJHM or SA59RJHM versus A59, $P < 0.001$ [Wilcoxon rank sum test]). After inoculation with a larger viral dose (10^5 PFU), RA59 induced the same extent of moderate hepatitis as did inoculation with 500 PFU and, remarkably, the A59 recombinant virus expressing the JHM spike gene (SJHM-RA59) caused an exacerbation in the degree of hepatitis, inducing liver lesions to the same extent as RA59 does (Fig. 3B and 4C). Strikingly, after inoculation of 10^6 PFU, both the RJHM and JHM recombinant virus expressing the A59 spike gene (SA59-RJHM) caused mild hepatitis (Fig. 3B). Thus, JHM recombinant viruses, irrespective of the spike gene, caused very minimal (after 500 PFU) to mild (after 10^6 PFU) hepatitis whereas the A59 recombinant virus expressing the JHM spike gene, like RA59 itself, induced moderate hepatitis but only after inoculation with a large viral dose (10^5 PFU) (JHM background versus A59 background, $P < 0.01$ [Wilcoxon rank sum test]). We found a correlation between the viral replication titers and the degree of hepatitis induced by each virus (Fig. 3C). The extent of hepatocellular injury was also monitored by determining the number of nonconfluent necrotic foci per random field (three liver sections per sample, eight random fields per section)

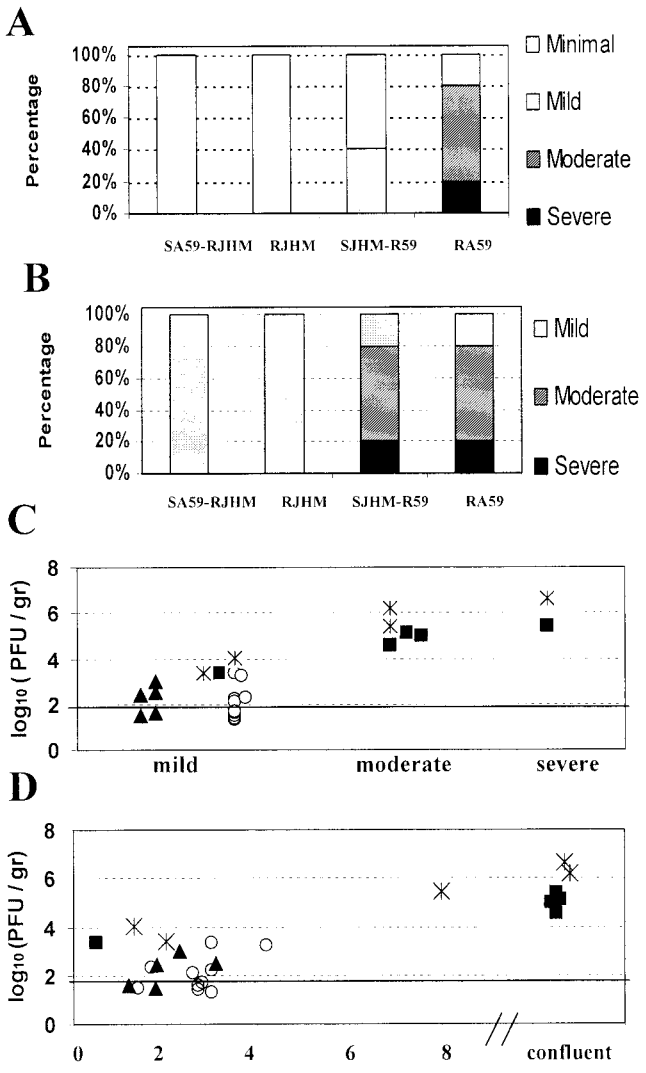

FIG. 3. Extent of hepatitis (scored as minimal [1], mild [2], moderate [3], or severe [4], as described in the text) and titers ($\log_{10}$ PFU per gram) of recombinant viruses in livers of C57BL/6 mice after intrahepatic inoculation with 500 (A) or 10^5 to 10^6 (B, C, and D) PFU of RA59, SJHM-RA59, SA59-RJHM, or RJHM virus. The results are shown as percentages of mice with minimal, mild, moderate, and severe hepatitis. The numbers of mice examined per viral dose were as follows: RA59, SJHM-RA59, and RJHM, 5; SA59-RJHM, 10. (A) After inoculation with 500 PFU, RA59 induced moderate hepatitis whereas SJHM-RA59, like RJHM and SA59RJHM, caused minimal hepatocellular damage (RA59 versus SJHM-RA59, $P < 0.01$; RA59 versus RJHM or SA59-RJHM, $P < 0.001$ [Wilcoxon rank sum test]). (B) After inoculation with 10^5 to 10^6 PFU, RJHM and SA59-RJHM induced mild hepatitis whereas SJHM-RA59 caused the same extent of hepatitis as RA59 (JHM background versus A59 background, $P < 0.001$ [Wilcoxon rank sum test]). (C and D) Correlation of the viral load with the degree of hepatitis induced by each virus and the number of necrotic foci.

(Fig. 3D), and this analysis showed that recombinant viruses with the JHM background induced one to four necrotic or inflammatory foci per field, whereas recombinant viruses with the A59 background showed more than eight foci per field or even confluent necrosis.

Figure 4 shows liver histopathology at day 5 after intrahepatic inoculation with 10^5 (RA59 and SJHM-RA59), 10^6 (RJHM and SA59-RJHM), or 500 (SJHM-RA59) PFU. The MHV-A59 and MHV-JHM strains are very different in liver histopathology. We found that our recombinant wild-type viruses (RA59 and RJHM) exhibited the same phenotype in the liver as the corresponding parental viruses (data not shown). Thus, RA59 caused moderate necrosis, with inflammation located in the portal areas, as well as in the lobular parenchyma (Fig. 4A), whereas RJHM induced scattered inflammatory foci with occasional spotty necrosis, even after inoculation with 10^6 PFU (Fig. 4B). The A59 recombinant virus expressing the JHM spike gene (SJHM-RA59) caused noticeable hepatocellular damage with increased inflammation after inoculation with 10^5 PFU (Fig. 4C); this is in contrast to the occasional necrosis found after inoculation of 500 PFU (Fig. 4E). Strikingly, the JHM recombinant expressing the A59 spike gene (SA59-RJHM), like RJHM itself, caused minimal changes to mild hepatitis (even after 10^6 PFU), with histopathological changes characterized by small, scattered inflammatory foci with some focal necrosis (Fig. 4B and D).

Figure 5 shows immunohistochemical staining of liver sections from mice inoculated directly in the liver with 10^5 (RA59 and SJHM-RA59), 10^6 (RJHM and SA59-RJHM), or 500 (SJHM-RA59) PFU by day 5 p.i. In general, viral antigen immunolabeling always colocalized with areas of hepatocellular degeneration and necrosis, as well as with individual or small clusters of hepatocytes. It is noticeable that the RA59 and SJHM-RA59 viruses exhibited differences in viral spreading in the liver; thus, SJHM-RA59 is not able to spread as efficiently as RA59 does (Fig. 5A versus C). Viral spreading was very limited for both RJHM and SA59-RJHM, showing isolated viral staining (Fig. 5B versus D).

Overall, our data suggest that although the spike gene is critical in the development of MHV liver pathogenesis, other genes do contribute to hepatitis development. These findings have led us to speculate that genes other the spike gene greatly influence postentry events that determine the outcome of infection, that is, the ability of the virus to replicate and spread within the liver. Thus, the JHM spike gene is likely inefficient at mediating entry into one or more liver cell types, but once entry is achieved, the A59 background genes allow efficient replication. Thus, at large virus doses, enough virus can enter the cells to allow efficient replication in the context of the A59 background genes. Conversely, A59 spike gene-mediated entry into liver cells may be efficient but the JHM background genes eliminate replication and hepatitis development.

There are many potential mechanisms, involving both nonstructural and structural proteins, by which genes other than the spike gene may influence pathogenesis. Thus, less efficient JHM replication in one or more cell types in the liver may account for the phenotype of SA59-RJHM. It has been previously proposed that the hepatotropism of a given MHV strain may be determined by its ability to replicate in nonparenchymal cells in the liver (30, 31, 36). Differences in replication would be due to the ability of the replicase or other nonstructural proteins to interact with cell type-specific factors needed for replication. Structural genes other than the spike gene may also play a role in the development

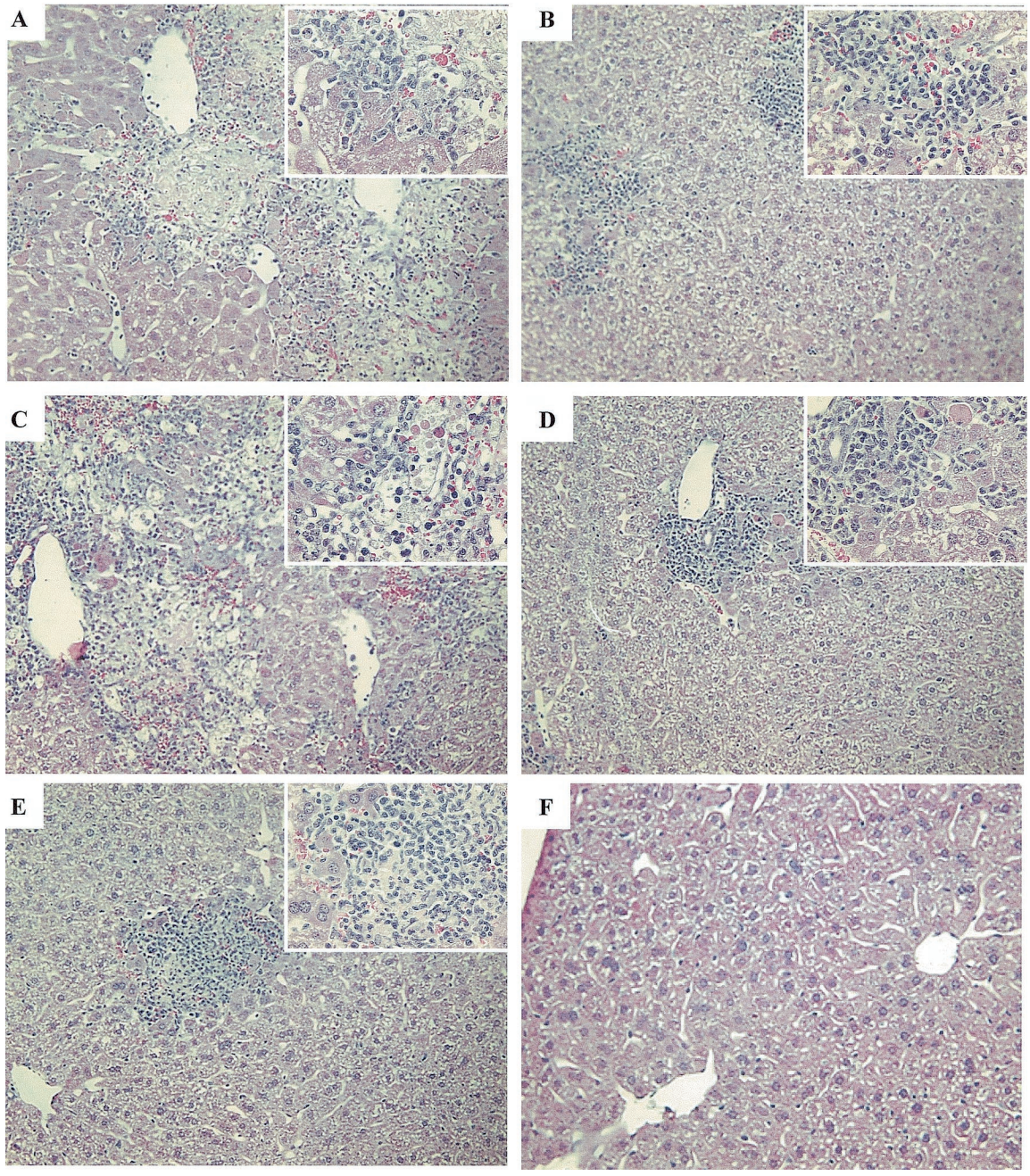

FIG. 4. Histopathologic changes in the livers of mice infected intrahepatically with 10^5 PFU of RA59 (A) or SJHM-RA59 (C), 10^6 PFU of RJHM (B) or SA59-RJHM (D), or 500 PFU of SJHM-RA59 (E), compared with a mock-infected control (F), at day 5 p.i. (hematoxylin-and-eosin staining). (A) RA59 showed noticeable necrosis and inflammation, whereas RJHM (B) and SA59-RJHM (D) induced occasional foci of necrosis. SJHM-RA59 caused noticeable hepatocellular damage and inflammation after inoculation with 10^5 PFU (C). In contrast, after inoculation with 500 PFU (E), SJHM-RA59 caused minimal changes in the liver. Insets (A to E) show inflammatory infiltrates, necrotic hepatocytes, and some apoptotic bodies. No signs of pathology were found in a mock-infected control (F). Magnifications: A to F, ×100; insets, ×400.

of hepatitis. For example, the nucleocapsid protein of MHV-3, a strain that causes fulminant hepatic failure, induces transcription of the *fgl2* prothrombinase gene in Kupffer and endothelial cells of the liver, causing microthrombosis and severe fibrin deposition, leading to acute hepatic failure (5, 28). Furthermore, transient expression of hemagglutinin-esterase by an MHV defective-interfering RNA alters A59 pathogenesis in the liver, causing less necrosis and viral antigen expression (40). Interestingly, the transmembrane glycoprotein (M) of coronaviruses induces alpha interferon (2). Thus, it is possible that the M protein may play a role in liver pathogenesis by its ability to induce interferon. The definitive role of these structural genes in hepatitis will be addressed in the future by the generation of

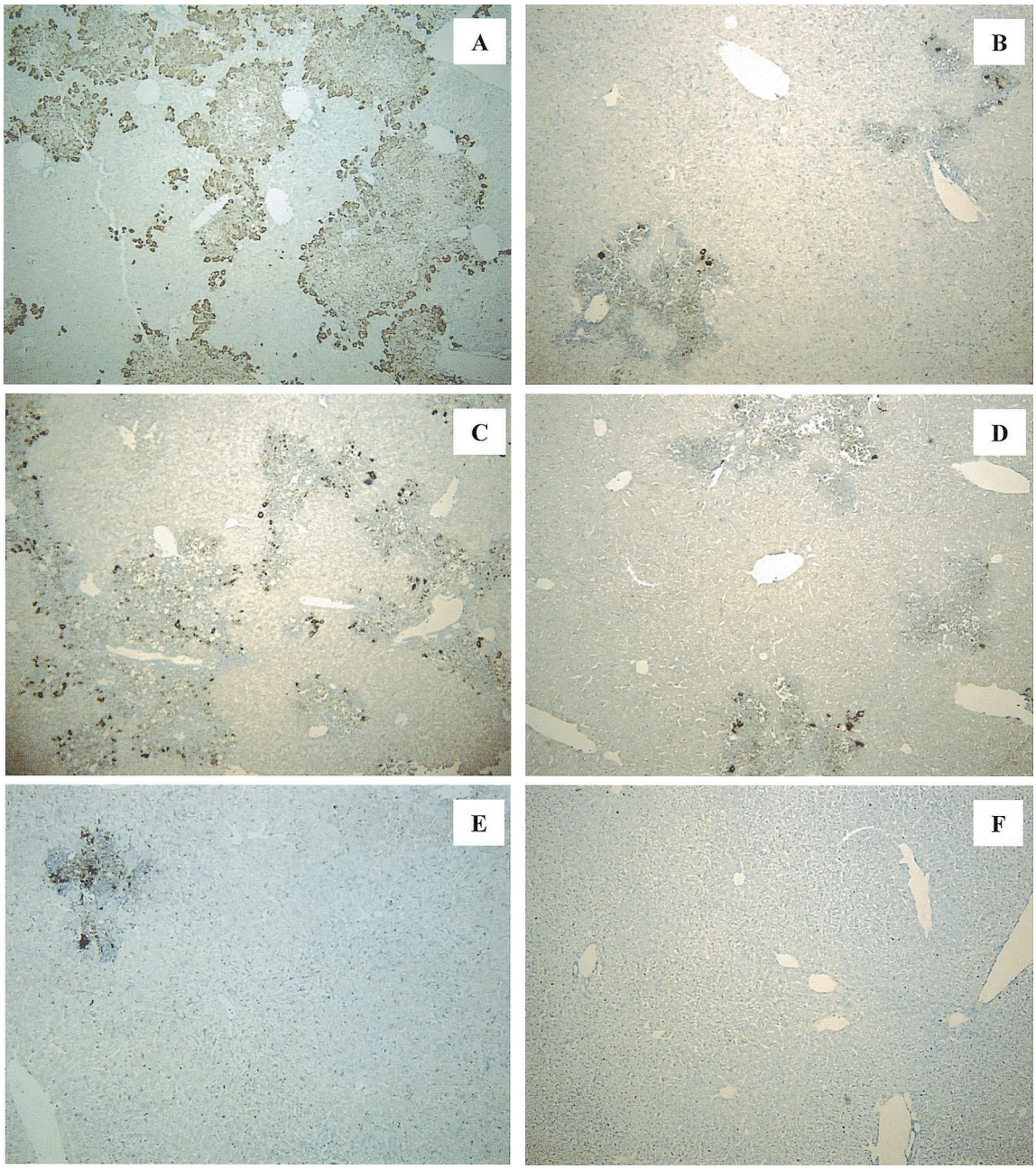

FIG. 5. Immunohistochemical analysis of liver sections of C57BL/6 mice infected with recombinant virus RA59 at 10^5 PFU (A), RJHM at 10^6 PFU (B), SJHM-RA59 at 10^5 PFU (C), SA59-RJHM at 10^6 PFU (D), or SJHM-RA59 at 500 PFU (E), compared with a mock-infected control (F), at day 5 p.i. MHV was detected by immunolabeling with a MAb against the nucleocapsid protein of MHV as described in the text. Viral antigen always colocalized with necrotic areas. In contrast to RA59 (A), SJHM-RA59 (C and E) exhibited limited viral spreading. RJHM (B) and SA59-RJHM (D) showed isolated viral staining. No signs of viral antigen were found in the mock-infected control (F). Magnification, ×40.

chimeric viruses with these structural genes in different MHV contexts.

Finally, the immune response may play a critical role in determining hepatitis outcome. Although the viral load of hepatotropic MHV in the liver peaks at day 5 p.i. (independently of the viral strain), infectious virus can be detected by day 1 p.i.; however, in mice that survive the acute phase, the virus is cleared by 7 to 10 days p.i. JHM background viruses do not induce a productive infection in the liver, even at day 1 p.i. (data not shown). Interestingly, in gamma interferon-deficient B6 mice, the MHV-JHM DL variant induces subacute fatal peritonitis and the virus persists for 30 days after infection (16, 17); furthermore, acute hepatic failure develops following infection of gamma interferon-deficient BALB/c mice (18). Thus, it is likely that a robust and protective innate immune response against JHM background proteins may account for the lack of productive infection in the liver. This hypothesis will be further investigated.

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