ALTERATION OF CLINICAL OUTCOME AND HISTOPATHOLOGY OF YELLOW FEVER VIRUS INFECTION IN A HAMSTER MODEL BY PREVIOUS INFECTION WITH HETEROLOGOUS FLAVIVIRUSES

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Abstract. Using a recently described hamster model of severe yellow fever (YF), we examined the hypothesis that prior infection with heterologous flaviviruses protects against severe or fatal YF. Hamsters were singly or sequentially infected with Japanese encephalitis, St. Louis encephalitis, West Nile, and/or dengue-1 viruses, and then challenged with a virulent strain of yellow fever virus (YFV). In contrast to control (naive) hamsters, many of which appeared clinically ill or died after YFV infection, the flavivirus-immune animals remained asymptomatic. The flavivirus-immune hamsters also had a reduced viremia and lower serum levels of alanine aminotransferase and total bilirubin, compared with naive hamsters, following YFV infection. Histologically, livers of animals in the flavivirus-immune and control groups showed comparable levels of multifocal necroapoptosis. However, steatosis was not observed in the flavivirus-immune animals, whereas naive hamsters developed extensive microvesicular steatosis in the liver following YFV infection. These findings suggest that hepatocytic steatosis is an adverse microscopic feature associated with severe disease in YFV infection. Our experimental results support earlier anecdotal reports that prior exposure of humans to heterologous flaviviruses reduces subsequent risk of fatal YFV infection.

INTRODUCTION

Monath1,2 and others3,4 have suggested that immunologic cross-protection by heterologous flaviviruses may provide partial protection against yellow fever (YF). In the 19th century, it was noted that indentured laborers from India and British troops who had served in India were less likely to contract severe YF during epidemics of the disease in Africa.5 Presumably, many of these people had been previously infected with dengue (DEN) viruses, and possibly West Nile (WN) or Japanese encephalitis (JE) viruses, during their residence in India. A retrospective investigation of a YF epidemic in Gambia in 1978 revealed that the inapparent-to-apparent infection ratio for YF was significantly higher in individuals with prior exposure to heterologous flaviviruses.5 In addition, there is limited experimental evidence from studies in monkeys supporting the concept that immunity to DEN and other flaviviruses may diminish the viremia and severity of yellow fever virus (YFV) infection.4,6

To test the hypothesis that prior infection with heterologous flaviviruses protects against severe or fatal YF, a series of laboratory experiments were done using a recently described hamster model of the disease.7,8 The present paper reports our results that support the earlier observations and hypothesis that heterologous flavivirus antibodies are protective against severe YF. In addition to reduced levels of viremia and less severe alternation in liver function, the flavivirus-immune hamsters did not develop microvesicular steatosis, one of the classic pathologic features of severe YFV infection. The significance of this lack of steatosis in the course of YFV infection is discussed.

MATERIALS AND METHODS

Viruses and animals. Five flaviviruses were used in this study; the Jimenez hamster-virulent (10th passage) strain of YFV;7 the Mochizuki high mouse passage (178x) strain of dengue-1 virus (DENV);9 the live attenuated SA14-2-8 vaccine strain of Japanese encephalitis virus (JEV);10 the New York 385-99 strain of West Nile virus (WNV);11 and the BeAr 23379 strain of St. Louis encephalitis virus (SLEV).12

The hamsters used were juvenile and adult female Syrian golden hamsters (Mesocricetus auratus) obtained from Harlan Sprague Dawley (Indianapolis, IN). Animals were cared for in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council) under an animal use protocol approved by the University of Texas Medical Branch. All work with infected animals was carried out in biosafety level-3 facilities.

Virus assay. Virus levels in blood of hamsters infected with YFV were titrated in cultures of the C6/36 clone of Aedes albopictus cells, as described previously.7 The presence or absence of viral antigen by immunofluorescence was used as the end-point. The YFV titers were calculated as the tissue culture infectious dose50 (TCID50) per milliliter of specimen by the method of Reed and Muench.13

Antibody determinations. Serum antibodies to YFV and the other four flaviviruses were measured by a hemagglutination inhibition (HI) test. Antigens for the HI test were prepared from brains of newborn mice inoculated intracerebrally with each of the viruses; the infected brains were processed by the sucrose-acetone extraction method.14 Hamster sera were tested by HI at serial two-fold dilutions from 1:20 to 1:5,120 at pH 6.6 (WNV, JEV, DENV, and SLEV) or 6.4 (YFV) with four units of antigen and a 1:200 dilution of goose erythrocytes, using commercial kits (Total Bilirubin® and ALT®; Sigma Diagnostics, St. Louis, MO), according to the manufacturer’s instructions.

Experimental infection of animals. Four separate experiments were done. The viruses used and the sequence in which they were administered to the hamster were as follows: Ex-
RESULTS

Establishment of heterologous flavivirus infection(s) in hamsters, their antibody response, and subsequent challenge with YFV. Experiment 1. Infection of naive hamsters with YFV (Group YF control). Sixteen adult hamsters (11–12 weeks old) were inoculated intraperitoneally with $10^{4.0}$ TCID$_{50}$ of YFV. The animals in this group showed clinical signs of severe YF (lethargy, anorexia, tremors) on the fifth and sixth days after infection, as described previously. Eight of the animals were bled daily for six consecutive days to monitor the level of viremia. On the sixth day after infection, these eight animals were exsanguinated to obtain serum for liver function studies, and tissue samples were taken at necropsy for histopathologic examination. The remaining eight hamsters were observed for 14 days; two of the animals died on sixth and seventh days, giving a mortality rate (25%) similar to that previously described.

Experiment 2. Infection with JEV, followed by YFV (Group JE/YF). Ten subadult hamsters (5–6 weeks old) were inoculated subcutaneously with JEV strain SA14-2-8. Twenty-eight days later, the animals were bled to determine the HI antibody titers to JE and YFV antigens, which are shown in Table 1. The hamsters in this experiment all developed a strong HI antibody response to JEV antigen (1:640–1:2,560), but they also had lower levels of cross-reacting antibodies to YFV antigen (1:80–1:160). Thirty-one days after JEV infection, the 10 hamsters were inoculated intraperitoneally with $10^{6.0}$ TCID$_{50}$ of YFV and were then bled for six consecutive days to determine the level of the resulting YFV viremia. Six days after infection with YFV, the hamsters were killed and necropsied to obtain tissue samples for histopathologic analysis. No sera were obtained for liver function tests from this group of animals. The animals in this group did not appear sick when killed on the sixth day of YFV infection.

Experiment 3. Sequential infection with DENV, WNV, and YFV (Group DEN/WN/YF). Twenty-six juvenile hamsters (3–4 weeks old) were inoculated intracerebrally with the Mo-chizuki strain of DENV. This virus strain produces an asymptomatic infection in juvenile and adult hamsters. Twenty-seven days after DENV infection, 10 of the animals were bled to determine their immune status. At this time, all of the hamsters had HI antibodies to DENV, with titers ranging from 1:160 to 1:640. The 26 animals were then inoculated intraperitoneally with $10^{4.0}$ TCID$_{50}$ of WNV strain New York 385-99. Thirty-one days after WNV infection, 18 of the hamsters were inoculated intraperitoneally with $10^{6.0}$ TCID$_{50}$ of YFV and 10 were bled for six consecutive days to determine the level of viremia. Ten of the 18 YFV-infected animals were killed on the sixth day for histopathologic analysis; the remaining eight survived and did not appear ill. The other eight DEN/WNV-infected animals did not receive YFV and were used as YFV-negative controls.

Experiment 4. Sequential infection with SLEV, WNV, and YFV (Group SLE/WN/YF). An additional 26 juvenile hamsters were inoculated subcutaneously with $10^{6.0}$ TCID$_{50}$ of SLEV strain BeAr 23379. Thirty days later, the animals were inoculated intraperitoneally with $10^{4.0}$ TCID$_{50}$ of WNV strain New York 385-99. Forty-five days after WNV infection, nine of the hamsters in this group were bled to determine their flavivirus HI antibody titers. Eighteen of the original 26 hamsters were then given $10^{6.0}$ TCID$_{50}$ of YFV. These animals

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The text continues with further details on the experiments and results, including histologic evaluation, in situ TUNEL assay, and proliferation analysis.
Hemagglutination inhibition (HI) antibody responses of hamsters to Japanese encephalitis virus (JEV) and yellow fever virus (YFV) antigens 28 days after infection with JEV and WNV antigens.

**Table 1**

<table>
<thead>
<tr>
<th>Hamster no.</th>
<th>JEV antigen</th>
<th>YFV antigen</th>
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<tbody>
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<td>160</td>
</tr>
<tr>
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</tr>
<tr>
<td>8100</td>
<td>640</td>
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</tbody>
</table>

* Reciprocal of highest positive serum dilution.

Table 2

Hemagglutination-inhibition (HI) antibody titers* in St. Louis encephalitis virus (JEV) and yellow fever virus (YFV) challenge

<table>
<thead>
<tr>
<th>Hamster no.</th>
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<tbody>
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<td></td>
<td>JEV Ag</td>
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<td>H-8109</td>
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</tbody>
</table>

* Antibody titers against corresponding antigens: JEV Ag = yellow fever viral antigen; WNV Ag = West Nile viral antigen; SLEV Ag = St. Louis encephalitis viral antigen. HI antibody titers are expressed as the reciprocal of highest positive serum dilution.
follicles. At the same time, there was splenic macrophage hyperplasia (Figure 5D). Among hamsters in the YF control group (Experiment 1), white pulp depletion was more prominent (Figure 5C). As summarized in the semi-quantitative analysis in Figure 4, the flavivirus immune groups (DEN/WN/YF and SLE/WN/YF) exhibited more lymphoid hyperplasia, less severe lymphoid depletion, and similar splenic macrophage activation compared with the YF control group in Experiment 1. The YFV-infected hamsters in Experiment 3 showed mild-to-moderate lymphoid hyperplasia in the spleen, but no significant lymphoid depletion or splenic macrophage hyperplasia.

Pancreatic tissue was also examined in some of the hamsters. As previously reported,8 moderate pancreatic necrosis was found in naive hamsters challenged with YFV (Experiment 1), but no necrosis was observed in the pancreas of the flavivirus-immune animals (Experiments 2-4) after YFV challenge.

In hamsters previously infected with JEV and then challenged with YFV, the liver showed mild-to-severe inflammatory cell infiltration of the portal tracts, as well as in the lobular parenchyma, predominantly by activated lymphocytes and plasma cells. Occasional eosinophils were also noted. The portal inflammation exhibited a perivascular pattern centered mainly around the portal veins. The bile ducts showed no evidence of injury. There was no interface hepatitis (piecemeal necrosis) in these lesions. The lobular inflammation was characterized by scattered to frequent hepatocytic necroptosis, accompanied by the accumulation of lymphocytes and plasma cells in the nearby sinusoidal spaces (Figure 6A). However, no bridging necrosis or large areas of confluent necrosis were noted as observed in the YF control group. Occasionally, prominent peri-central venule inflammation was seen. Again, no significant steatosis was observed in these hamsters. The spleen exhibited marked macrophage proliferation (Figure 6B), as well as lymphoid hyperplasia.

**In situ TUNEL analysis.** An in situ TUNEL stain was performed on the liver sections from each group of hamsters, and the apoptotic indices were scored individually, as previously described.8 The mean indices of the groups are shown in Figure 7. No statistically significant difference was found among any of the groups challenged with YFV (Experiments 1–4). No apoptosis was observed in livers of YFV-negative control hamsters from Experiments 3 and 4 (Figure 7).
FIGURE 3.  

A. Normal histology of liver from a hamster previously infected with Saint Louis encephalitis (SLE) and West Nile (WN) viruses.  
B. Normal histology of liver from a hamster previously infected with Dengue (DEN) and WN viruses.  
C and E. Liver from a hamster previously exposed to SLE and WN viruses six days after challenge with yellow fever (YF) virus. Moderate inflammatory cell infiltration and scattered Councilman bodies (necrapoptotic bodies) can be seen.  
D and F. Liver from a hamster previously infected with DEN and WN viruses six days after challenge with YF virus. Moderate inflammatory cell infiltration and scattered Councilman bodies (necrapoptotic bodies) can be seen.  
G and H. Liver from a YF virus-infected control (flavivirus naive) hamster. There is diffuse microvesicular steatosis, and scattered Councilman bodies can be seen. (Magnifications: ×50 in A, B, C, D, and G; ×100 in E, F, and H.)
FIGURE 4. Histologic scoring system used to quantitate liver and spleen pathology. SLE = St. Louis encephalitis; WN = West Nile; YF = yellow fever; D1 = dengue-1; Infl = inflammation; Necrapop = necrapoptosis; Steat = steatosis; Lymph Hyp = lymphoid hyperplasia; WP Depl = white pulp depletion; Mac Prolif = macrophage proliferation. The values for the SLE/WN groups were zero and thus are not visible on the graph.

FIGURE 5. A, Normal histology of spleen from a hamster previously infected with St. Louis encephalitis (SLE) and West Nile (WN) viruses. Note the distinct border of the lymphoid follicle surrounding a small arteriole (white pulp). B, Spleen from a hamster previously infected with SLE and WN viruses six days after challenge with yellow fever (YF) virus. Note the lymphoid hyperplasia characterized by the proliferation of immunoblastic cells, and macrophages (arrowheads). C, White pulp depletion of spleen from a flavivirus-naive (control) hamster six days after infection with YF virus. D, Splenic macrophage hyperplasia, from a hamster previously infected with dengue-1 and WN viruses six days after challenge with YF virus. Arrowheads show tangible-body macrophages. (Magnifications: ×25 in A; ×50 in B, C, and D.)
DISCUSSION

The results of this study support previous observations and speculation that immunity to heterologous flavivirus reduces the severity of YFV infection. In the present series of experiments, prior infection of hamsters with JEV, SLEV, WNV and DENV protected the animals from fatal disease after challenge with YFV. The protective effect of prior heterologous flavivirus infection was demonstrated by lower levels of viremia, less severe alteration of liver function (as measured by levels of TB and ALT), and an absence of hepatic steatosis in the flavivirus immune groups (Experiments 2–4) compared with the flavivirus-naive (YF control) group. A similar protective effect by prior heterologous flavivirus infection has been reported in a hamster model of WN encephalitis.

Our studies showed that hamsters previously exposed to other flaviviruses developed inflammation and hepatic necrapoptosis similar in extent to that observed in naive hamsters after YFV challenge. However, in the flavivirus-immune animals, no steatosis was found, in contrast to the extensive microvesicular steatosis observed in the flavivirus-naive hamsters following infection with YFV. The lack of steatosis was correlated with a clinically milder form of YFV infection as shown by lower viremia, less alteration of liver function, and generally asymptomatic infection. Histologic changes in the spleen were also different in the flavivirus-immune hamsters compared with the flavivirus-naive group in that there was more prominent lymphoid hyperplasia and much milder white pulp depletion in the former animals. Another difference noted between the immune hamsters and the naive group was the lack of pancreatic necrosis in the former. In summary, the flavivirus-immune hamsters demonstrated splenic lymphoid hyperplasia, a lack of microvesicular steatosis in the liver, and an absence of pancreatic necrosis compared with the pathology observed in naive animals infected by YFV.

In human cases of YF, although abnormalities are noted in multiple organs, the most consistent findings are seen in the liver. Microvesicular steatosis is a prominent microscopic feature of YFV infection in liver of susceptible vertebrate hosts, including humans, monkeys, or hamsters. In the hamster YF model, small numbers of minute fatty vesicles start to appear in some of the hepatocytes by the third or fourth day after YFV infection; by the fifth or sixth day, the fatty vesicles have spread to involve more cells in larger areas, and they usually occupy the entire cytoplasm (Figure 3G and H).

We have observed the association of liver steatosis with an adverse outcome in other experiments with YFV in the hamster model. For example, in studies comparing the virulence (mortality rate) of different YFV strains in hamsters, splenic lymphoid hyperplasia and a lack of liver steatosis were consistent findings in hamsters infected by less virulent strains of YFV (Fisher AF and others, unpublished data). These findings suggest that extensive steatosis is related to more severe clinical disease in YFV infection. However, steatosis may not be the cause for the adverse outcome of YFV infection in these hamsters; instead it may be simply a marker for severe liver dysfunction.

Massive hepatic steatosis (particularly the microvesicular type) occurs in other acute liver diseases, such as Reye’s syndrome. Its presence and rapid onset may indicate severe functional “shut-down” of the liver, and may explain the high mortality seen in diseases such as YF and Reye’s syndrome. This acutely developing phenomenon suggests dysfunction of mitochondria, since at the early stages, overt cellular degeneration is rarely observed. Mitochondrial changes also probably play an important role in the activation of apoptosis or necrapoptosis. The latter is a major component in YF liver pathology.

The hepatic necrapoptosis that occurs in YF ranges from scattered, individual Councilman bodies to foci of multiple hepatocytes forming coagula, and diffuse mid-zonal necrosis. In advanced or severe YF cases, the mid-zonal pattern can be obscured by the involvement of zones 1 and 3 as well. The pathologic features of necrapoptosis observed in the hamster model of YF suggest that a spectrum of changes occurs during the process. This hypothesis is supported by an in situ TUNEL staining, which revealed nuclear DNA fragmentation in the liver before discernable cell lysis occurred. In addition, typical apoptotic bodies of different stages were seen in livers of YFV-infected hamsters, using electron microscopy (Xiao SY, Popov VL, Tesh RB, unpublished data). We suspect that liver cell necrosis in human cases of YF also begins
as apoptosis, directly induced by virus infection, and not by necroinflammatory injuries. This view is based on the lack of consistent inflammatory cell infiltration in the disease, and the fact that the Councilman body, the morphologic evidence of apoptosis, was first described in YF livers. When viewed in the later stages of the disease, such as at autopsy, the necrosis is so extensive and advanced, and may be complicated by other secondary changes, that the process can no longer be simply attributed to apoptosis.

We suspect that YFV causes morphologic and functional compromise to the mitochondria of the hepatocytes, which contributes to cell apoptosis, and at the same time causes abnormal lipid peroxidation and microvesicular fatty changes. The pathogenesis of YFV-induced hepatic necrosis is largely unknown; but our future research will focus on how YFV triggers the cellular apoptosis pathways, as well as steatosis. Although no studies have been reported in examining the relationship between mitochondrial dysfunction and YFV infection, studies with another flavivirus, hepatitis C virus, indicate that it induces steatosis in human patients and transgenic mice by the binding of hepatitis C virus core protein with host cell mitochondrial protein. More recently, using immunogold electron microscopy, we have found YF viral antigen to be associated with hepatocyte mitochondria, in addition to rough endoplasmic reticulum (Xiao SY, Popov VL, Tesh RB, unpublished data). The functional compromise of the hepatocytes may also affect the synthetic functions of the liver, thus contributing to the bleeding diathesis observed in patients with YF.

Much has been written during the past three decades on the phenomenon of immune enhancement with sequential flavivirus infection. Most of epidemiologic and laboratory studies of this phenomenon have focused on DENV infection and an attempt to explain why some individuals develop dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). One dominant theory is that individuals with pre-existing antibody to one DENV serotype are predisposed to the more severe DHF/DSS when subsequently infected by a different DENV serotype. However, our experimental studies with hamster models of YF and WN encephalitis suggest that pre-existing flavivirus antibodies are protective and that they do not increase the severity of these two diseases. Thus, immune enhancement does not appear to be a general phenomenon with all flavivirus infections.