Experimental Studies on Subclinical Hepatitis E Virus Infection in Cynomolgus Macaques

Rakesh Aggarwal,1,2 Saleem Kamili,1 John Spelbring,1 and Krzysztof Krawczynski1

Serial subclinical transmission among susceptible humans may serve as a reservoir of hepatitis E virus (HEV) in areas in which HEV is endemic. This hypothesis was investigated in an experimental primate model. Four groups of 4 cynomolgus macaques each were inoculated intravenously with $10^4$–$10^5$ (group 1), $10^6$–$10^7$ (group 2), and $10^8$ (group 3) cynomolgus macaque HEV infectious doses. All 4 animals in group 1 had clinical disease marked by alanine aminotransferase (ALT) elevation, fecal virus excretion, viremia, and seroconversion. Of the animals in groups 2 and 3, only 1 had evidence of biochemical hepatitis, although most had virus excretion and viremia (3 animals each in groups 2 and 3), and evidence of seroconversion (1 animal in group 2 and 3 animals in group 3). Viral genomic titers in stool specimens of animals with or without ALT elevation were similar. Infectivity studies confirmed the viability and transmission potential of the virus excreted by animals without ALT elevation. These data suggest that subclinical HEV infection may represent an HEV reservoir.

Hepatitis E virus (HEV) infection is responsible for a large proportion of patients with acute viral hepatitis in several developing countries [1, 2]. It is endemic in the Indian subcontinent and in southeast and central Asia, where HEV causes large epidemics that affect hundreds to thousands of persons and recur at intervals of several years [3–5]. Smaller outbreaks have been observed in the Middle East, northern and western Africa, and North America (Mexico). In addition, in disease-endemic regions, HEV infection may cause sporadic acute viral hepatitis; for instance, this infection accounts for 50%–70% of all patients with sporadic viral hepatitis in India [6, 7]. Sporadic cases of hepatitis E that are observed during interepidemic periods usually lack a history of contact with a person with clinically overt HEV infection.

The reservoir for HEV during the interepidemic periods is not known. Several mechanisms have been proposed for the maintenance of the virus and, hence, its endemicity in a population. First, HEV may be maintained in environmental sources such as water and soil. Second, the virus may derive from patients with acute hepatitis E through fecal excretion for extended periods after clinical recovery [8]. Third, transmission of an HEV-like virus (swine HEV) from infected pigs to humans (swine handlers) may occur [9–12]. Finally, it has been suggested that symptom-free HEV carriers may serve as potential human reservoirs of the disease [13], and the virus may be repeatedly passaged as a subclinical infection in humans, somewhat akin to the continuous presence of poliovirus in areas where this infection once was endemic [14].

Cynomolgus macaques (cynos) reproducibly develop virologic, biochemical, immunologic, and histopathologic changes that model those observed in acute HEV infection in humans [15]. In infected cynos, a short incubation period is followed by viremia and fecal excretion of HEV, the presence of liver antigen in hepatocytes, anti-HEV (IgM and IgG), and elevation of serum transaminase levels with histopathologic evidence of acute hepatitis [15, 16]. This model of HEV infection has been used extensively for studies on pathogenesis, immune response, and the role of active and passive immunoprophylaxis in HEV infection [17, 18]. In this study, we investigated the hypothesis that serially passaged subclinical HEV infection is a potential reservoir of infectious virus by using the experimental model of HEV infection in cynos.
Materials and Methods

HEV inoculum and experimental design. The HEV inoculum used in this study was a 10% (wt/vol) suspension of a stool specimen obtained from a third-passage cyno (Cy73) infected with HEV Burma strain [15, 19]. Infectivity titer of the inoculum was determined (data not shown) by intravenous inoculation of serial 10-fold dilutions of the stool suspension into 2 cynos each. It was assumed that the highest dilution of the inoculum that caused either the presence of HEV RNA in stool or serum or the development of anti-HEV contained 1–10 cynos infectious doses (CyID) of HEV. The infectivity titer of the inoculum was $10^7$–$10^8$ CyID/mL; HEV RNA titer in this inoculum, expressed as the end-point logarithmic dilution, was $10^7$ viral genome equivalents (vge)/mL.

In the first part of the study, 12 male cynos (3–8 kg) were divided into 3 groups and were inoculated intravenously with 1 mL each of PBS-diluted stool suspension. Animals in group 1 (Cy9506, Cy9511, Cy9735, and Cy9736) received 10,000–100,000 CyID of HEV; animals in group 2 (Cy9726, Cy9730, Cy9739, and Cy9740) received 10–100 CyID of HEV; and animals in group 3 (Cy9731, Cy9732, Cy9508, and Cy9512) received 1–10 CyID of HEV. In the second part of the study, the infectivity of stools from a cyno with subclinical infection was tested in 4 HEV-naive female animals (Cy9802, Cy9803, Cy9804, and Cy9806; 3 kg each). The intravenously administered inoculum (1 mL of a 10% wt/vol stool suspension) was obtained from a subclinically infected cyno from group 2 (Cy9740) on postinoculation (pi) day 18; this animal had no alanine aminotransferase (ALT) elevation, but HEV RNA was present in stool. This suspension contained $10^7$ vge/mL HEV RNA.

Specimen collection. Stool specimens were collected on the day of inoculation and on alternate days thereafter for 9–12 weeks and were tested for HEV RNA. Serum specimens were collected on the day of inoculation and on alternate days thereafter for 9–12 weeks and were stored at $\text{-80}^\circ\text{C}$; these were tested for HEV RNA and for anti-HEV antibodies. Serum specimens also were tested for ALT activity, expressed in international units per liter; individual cutoff values (mean plus 3 SD) of the preinoculation values.

Histopathologic studies. Snap-frozen biopsy specimens were collected during the period of ALT elevation and were fixed in neutral buffered formalin.

Detection of HEV RNA in stool and serum specimens. We determined the presence of HEV RNA in stool and serum specimens, as described elsewhere [20]. In brief, RNA was extracted from 100 mL of a 10% stool suspension in 0.1% PBS (pH 6.8) or from serum by using commercial silica-gel microspin columns (QIAgen viral RNA isolation kit; Qiagen), which were subjected to reverse-transcription polymerase chain reaction (RT-PCR) with primers corresponding to the RNA-dependent RNA polymerase gene of the Burma strain of HEV, followed by Southern hybridization with an internal digoxigenin-labeled probe. Specimens showing a band of expected size on hybridization were considered to be positive. HEV RNA levels in selected stool specimens were estimated semiquantitatively by end-point dilution RT-PCR on 100 mL each of serial 10-fold dilutions of the 10% stool suspension. We then calculated the HEV RNA concentration and expressed the findings as the minimum number of vge/gram of stool, assuming 1 vge of HEV as the minimum amount of HEV RNA that leads to a positive RT-PCR test result.

Anti-HEV antibody detection. IgG anti-HEV was detected in serum by an HEV mosaic protein EIA, as described elsewhere [21]. In brief, the wells of a polystyrene microtitration plate (Immunolon II; Dynatech Laboratories) were coated with 100 mL of affinity-purified glutathione-S-transferase-HEV mosaic fusion protein at a concentration of 5 mg/mL in 0.1 M PBS (pH 7.5) at room temperature overnight. Cyno serum specimens were diluted 1:20 in 0.1 M PBS (pH 7.5) containing 0.1% Tween 20 and 10% normal goat serum. Binding of antibodies to the recombinant protein adsorbed to the surface of the wells of microtitration plates was identified with affinity-purified anti-human IgG conjugated to horseradish peroxidase (Pierce). For each animal, we tested 5–8 preinoculation serum specimens and fortnightly pi specimens in the same assay. Seroconversion was defined as an increase in optical density to above the mean plus 3 SD of the preinoculation values.

Histopathologic studies. Snap-frozen biopsy specimens were processed for detection of HEV antigen by immunofluorescence, as described elsewhere [22]; appropriate positive and negative controls were run in each experiment. Sections from liver biopsy specimens fixed in formalin were stained with hematoxylin-eosin.

Table 1. Biochemical, virologic, serologic, and histologic events in cynomolgus macaques (cynos) inoculated intravenously with different doses of Burma strain hepatitis E virus (HEV).

<table>
<thead>
<tr>
<th>Event</th>
<th>Inoculum dose (CyID), by animal</th>
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<tr>
<td></td>
<td>10,000–100,000</td>
</tr>
<tr>
<td>ALT elevation</td>
<td>Yes</td>
</tr>
<tr>
<td>No. of days</td>
<td>22–32</td>
</tr>
<tr>
<td>Peak (x cutoff)</td>
<td>4.6</td>
</tr>
<tr>
<td>Stool HEV</td>
<td>Yes</td>
</tr>
<tr>
<td>No. of days</td>
<td>6–42</td>
</tr>
<tr>
<td>Serum HEV</td>
<td>Yes</td>
</tr>
<tr>
<td>No. of days</td>
<td>7–36</td>
</tr>
<tr>
<td>Anti-HEV seroconversion</td>
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<tr>
<td>HEV antigen in liver biopsy</td>
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</tbody>
</table>

NOTE: ALT, alanine aminotransferase; CyID, cynos infectious doses; NT, not tested.
Results

**Group 1 infected with 10⁻⁴–10⁻⁵ CyID HEV.** All 4 animals in group 1 had HEV RNA excretion in their stools, beginning 4–6 days after intravenous inoculation, that persisted until days 38–50 (table 1 and figure 1A). All 4 cynos also had HEV viremia that was first detected on pi day 7 and lasted until pi days 28–36. Native HEV antigen was detected in liver biopsy specimens from 3 of the 4 macaques (on pi days 22 in Cy9506, on pi days 22 and 29 in Cy9511, and on pi days 14 and 21 in Cy9735). All animals seroconverted to anti-HEV between pi days 28 and 56.

HEV titers in stool specimens were measured in 2 cynos in group 1; those in Cy9506 were 10⁵, 10⁶, 10⁷, and 10⁴ vge/g on days 10, 18, 26, and 40, respectively, and those in Cy9736 were 10⁵, 10⁷, 10⁶, and 10³ vge/g on days 8, 24, 34, and 46, respectively. All animals in this group developed significant elevation of ALT levels above the cutoff level. The elevation of ALT level was first noticed on pi days 17–28 and lasted 2 weeks in 3 cynos and for nearly 4 weeks in Cy9511. The peak ALT levels in these cynos were 3–10 times higher than the cutoff level. Liver biopsy specimens also showed histologic evidence of hepatitis.

**Group 2 infected with 10⁻¹–10⁻² CyID HEV.** Of the 4 cynos in group 2, 1 (Cy9730) had no virologic or serologic evidence of HEV infection, but 3 excreted HEV RNA in feces (table 1 and figure 1B). In 2 animals (Cy9726 and Cy9740), excretion began on pi day 8 and lasted until pi days 30 or 32; both animals also had viremia. HEV titers in stool specimens were 10⁵, 10⁴, and 10⁵ vge/g on days 10, 16, and 24, respectively, in Cy9726, and 10⁵, 10⁴, and 10³ vge/g on days 10, 18, and 28, respectively, in Cy9740. In Cy9739, HEV excretion in stool was transient (pi days 40–44) and was not accompanied by detectable viremia. Of the 3 animals with evidence of fecal HEV excretion, none had demonstrable HEV antigen in serial liver biopsy specimens obtained weekly, and only 1 seroconverted to anti-HEV. None of the 4 animals in this group developed elevated ALT above their respective cutoff levels.

**Group 3 infected with 1–10 CyID HEV.** Three animals in this group excreted HEV RNA in their stools (table 1 and figure 1C), beginning on pi days 8–14. The HEV titers in stool specimens were 10⁴, 10⁴, 10⁶, and 10⁴ vge/g on days 16, 34, 40, and 48, respectively, in Cy9731. Cy9512 had no virologic or serologic evidence of HEV infection. All animals with stool excretion of HEV RNA also had viremia. Two of the 3 animals (Cy9731 and Cy9732) with evidence of HEV shedding in stool and viremia had demonstrable HEV antigen in hepatocytes in liver biopsy specimens on days 7–14 and 14–36 and days 36–50, respectively. Two of 3 animals with HEV RNA fecal excretion and HEV viremia (Cy9731 and Cy9732) had no elevated ALT activity above their individual cutoff points; the remaining HEV RNA–positive cynos (Cy9508) had a transient and mild ALT elevation, minimal focal intralobular hepatocytic necrosis, and minimal portal tract infiltration. All 3 cynos with evidence of HEV infection seroconverted to anti-HEV.

**Infectivity studies with HEV RNA–positive stool specimens from an animal with subclinical HEV infection and normal ALT activity.** All 4 cynos (Cy9802, Cy9803, Cy9804, and Cy9806) inoculated with 1 mL of 10% stool suspension containing 10⁵ vge/mL of HEV RNA from subclinically infected Cy9740 had detectable HEV RNA in feces, beginning on pi days 12–16 and lasting until pi days 20–50 (table 2 and figure 2). HEV RNA titers in stool specimens were 10⁴–10⁶ vge/g of stool, and all tested positive for HEV RNA in serum. None of these animals had HEV antigen detectable in liver biopsy specimens obtained weekly. Three cynos did not develop elevated serum ALT levels; animal Cy9804 had elevated ALT once (day 56) after cessation of HEV viremia and excretion. The ALT elevation may not be related to HEV infection in this animal.

Figure 1. Hepatitis E virus (HEV) infection in cynomolgus macaques inoculated intravenously with 10% suspension of stool from animal Cy73. Animals and HEV infectious dose (CyID), by panel: A, Cy9730, 1 mL of 1:10 dilution (10⁴–10⁵ CyID); B, Cy9740, 1:10⁶ dilution (10⁴–10⁶ CyID); and C, Cy9731, 1:10⁶ dilution (1–10⁴ CyID). In each panel, the X-axis shows days after inoculation, and the Y-axis shows alanine aminotransferase (ALT) levels in IU/L (solid line). Dotted lines, ALT cutoff levels based on >10 values obtained before inoculation. Diamonds, serum HEV RNA test results; squares, fecal HEV RNA test results; circles, presence of HEV antigen in frozen liver biopsy specimens by immunofluorescence assay. All filled symbols indicate positive test results; all open symbols indicate negative test results.
Table 2. Biochemical, virologic, serologic, and histologic events in 4 cynomolgus macaques (cynos) inoculated intravenously with stool suspension from a cynomolgus macaque with hepatitis E virus (HEV) RNA in stool but no alanine aminotransferase (ALT) elevation.

<table>
<thead>
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<th>Event</th>
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<th>9803</th>
<th>9804</th>
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<tbody>
<tr>
<td>HEV RNA found in stool sample</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>No. of days</td>
<td>12–20</td>
<td>12–28</td>
<td>16–50</td>
<td>12–36</td>
</tr>
<tr>
<td>HEV RNA found in serum sample</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>No. of days</td>
<td>12</td>
<td>19–26</td>
<td>29–46</td>
<td>22–34</td>
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<tr>
<td>Anti-HEV seroconversion</td>
<td>Yes, day 70</td>
<td>No</td>
<td>Yes, day 70</td>
<td>No</td>
</tr>
</tbody>
</table>

NOTE. All 4 cynos were inoculated with ~10^4–10^5 cynos infectious doses. None had elevated ALT or HEV antigen in liver biopsy specimen.

Discussion

Our experiments on HEV infection show that experimentally infected cynos excrete HEV in stools in the absence of biochemical evidence of liver injury. The excretion of virus by HEV-infected animals that had no ALT elevation was similar in magnitude to that observed in animals with ALT elevation. The excreted virus was viable and capable of transmitting HEV infection to HEV-naive cynos. Furthermore, HEV infection without ALT elevation occurred more frequently among animals that received a smaller virus inoculum dose than among those given a larger virus inoculum.

Only a few experimental observations are available regarding HEV infection in the absence of biochemical evidence of liver injury. In a previous study [23], cynos that received a smaller HEV dose had seroconversion but either mild or no ALT elevation; virus excretion and viremia were not determined. In another study, fecal excretion of HEV RNA in the absence of ALT elevation in a cyno model was reported; this occurred more often in animals that received a smaller inoculum [24]. The quantity or infectious potential of the excreted HEV RNA was not studied. For symptom-free healthy contacts of patients with acute hepatitis E, HEV RNA was detected in serum and stool specimens [13]. However, infectivity of the virus shed by these asymptomatic contacts has not been demonstrated. We believe that our results are the first to show that cynos with HEV infection but no biochemical (and histologic) evidence of liver injury excrete large amounts of viable HEV in their stools that are similar to those excreted by animals with ALT elevation or clinical infection and are capable of transmitting infection to HEV-naive animals.

Several aspects of subclinical HEV infection in cynos deserve further comment. First, subclinical HEV infection in some animals was associated with the failure of an immune response to develop or with a relatively delayed appearance of anti-HEV antibodies, compared with animals with clinical HEV infection. Further studies will be needed to determine whether this phenomenon occurs routinely during human subclinical HEV infection. Second, we did not study whether the animals with subclinical HEV infection were protected against rechallenge; this may be an important question for future research. Third, all 4 animals inoculated with stool suspension from an animal with subclinical infection developed subclinical infection. This result may be related to a lower virus dose in the inoculum used. Alternatively, it may be related to molecular changes in the virus that lead to attenuation of the virus, an aspect that we did not study, or to host factors that are difficult to study.

Because the cynos model of HEV infection faithfully reproduces the biochemical, virologic, and serologic events of clinical human HEV infection, in view of the data obtained, it is tempting to speculate that shedding of a viable virus in large quantities during subclinical infection (as observed in our study) may reflect a similar scenario in subclinical human HEV infection. Evidence of viremia and fecal shedding in symptom-free persons without antibodies to HEV recently was reported elsewhere [13]. In areas in which hepatitis E is endemic, a seroprevalence rate of 30%–50% was found among healthy subjects [25]. This seroreactivity is thought to reflect previous subclinical HEV infection.

In a prospective study in Nepal (where HEV is endemic) of a cohort of healthy persons, two-thirds of those who seroconverted to anti-HEV during an 18-month follow-up period had no history of jaundice [26], which suggests that subclinical HEV infection is common. Thus, HEV infection may persist in disease-endemic populations by serial transmission among susceptible persons in the form of subclinical and asymptomatic infection. These subclinical cases, which go unnoticed, may act as one of the reservoirs for persistence of this infection in the population and as a source of sporadic acute hepatitis E. This reservoir also may lead to contamination of drinking water supplies during periods of flooding of river systems, leading to large outbreaks during rainy seasons. Until recently, a similar continual transmission of subclinical infection was the main reservoir of poliovirus in areas in which the disease was endemic.

Many reservoirs of HEV have been postulated for maintaining the disease in a population. HEV RNA was detected in raw and...
treated waste water and in slaughterhouse sewage from pigs [27, 28]. Protracted viremia and prolonged fecal shedding of HEV in a small group of patients suggested that these persons may serve as temporary virus carriers who cause continuous contamination of sewage [8]; however, a recent study of a larger number of patients showed that HEV excretion ceased before serum ALT levels returned to normal [29]. Demonstration of a novel swine HEV strain [9] and of the presence of anti-HEV antibodies in the serum of pigs, cattle, sheep, and rodents in several areas where hepatitis E is and is not endemic [10, 12, 30–34] has led to the suggestion that a zoonotic reservoir of HEV may exist. However, the lack of molecular evidence that the same isolate causes infection in animals and humans indicates a need for further studies to substantiate this hypothesis of zoonotic spread of the disease. Our experimental data suggest that the hypothesis of serial transmission of the virus among susceptible persons as subclinical infection leading to a reservoir for HEV during inter-epidemic periods in disease-endemic areas is plausible.

Acknowledgments

We thank Dorrie Carson and Stephanie Goss (Division of Viral Hepatitis) for technical help, Gale Galland (Scientific Resources Program) for excellent animal care and management, and John O’Connor (Division of Viral and Rickettsial Diseases) for manuscript editing.

References


