Transmission of Hepatitis C Virus Infection to Tree Shrews

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Although hepatitis C virus (HCV) infection can be reproduced in chimpanzees, these animals are rare and expensive. Tree shrews (tupaia) are small animals, closely related to primates, which adapt easily to a laboratory environment. In this work we have investigated the susceptibility of *Tupaia belangeri chinensis* to HCV infection. Tupaia caught in the wild in Yunnan (China) were inoculated in China with HCV genotype 1b (study A) and in Spain with a mixture of genotypes 1b, 1a, and 3 (study B). In study B tupaia were divided into three groups: group I was inoculated without previous manipulation, group II received 750 cGy of X-ray whole-body irradiation before inoculation, and group III was used as control. Transient or intermittent viremia occurred in 34.8% (8/23) and anti-HCV in 30.4% (7/23) of tupaia in study A. In study B a transient viremia was detected in 20% (2/10) in group I and in 50% (2/4) in group II. Anti-HCV was found in 1 tupaia from group I and in 3 from group II. Viremia lasted for longer and anti-HCV tended to reach higher titers in animals which received total body irradiation. ALT elevations and nonspecific pathological changes occurred in inoculated tupaia; however, the wild nature of the animals precludes the interpretation of these changes as solely due to HCV infection. In summary our results show that *T. b. chinensis* are susceptible to HCV and that whole-body irradiation may possibly increase the efficiency of the infection. These animals may serve as an *in vivo* system for culturing HCV and addressing pathophysiological and therapeutic issues of HCV infection.

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Key Words: Tupaia; irradiation; PCR; ALT; antibodies.

INTRODUCTION

Hepatitis C virus (HCV)² infection is a major cause of chronic hepatitis, liver cirrhosis, and liver cell cancer worldwide (Schreier *et al.*, 1996; Zhang *et al.*, 1990; Alter *et al.*, 1990). Since the cloning of HCV in 1989 (Choo *et al.*, 1989), progress in the knowledge of the basic biology, pathogenic mechanisms, and therapy of hepatitis C has been hampered by the lack of an appropriate animal model (Abe *et al.*, 1993). Although chimpanzees are easily infected with HCV and develop acute or chronic hepatitis (Alter *et al.*, 1978; Bradley *et al.*, 1979), these animals are rare, expensive, and difficult to handle and thus inappropriate for use in research programs requiring an abundant number of animals. Therefore, the finding of a small animal, easy to breed and of low cost, which would be susceptible to HCV infection, would be of great importance in facilitating progress in HCV research.

Tree shrews (*Tupaia*) are small squirrel-like mammals which live in Southeast Asia (Wang *et al.*, 1991). The family Tupaiidae has been included either in the order Insectivora or in the order Primates, but more recently tree shrews are being considered in a single order called Scandentia (Martin, 1990). They have a relatively restricted geographic distribution, including southern China, Vietnam, Laos, Kampuchea, India, Thailand, Malaysia, Indonesia, Philippines, Burma, Bangladesh, and Bhutan (Martin, 1990). *Tupaia* includes eleven species; among these, *T. belangeri* has the widest distribution. Eight *T. belangeri* subtypes are found in China: *T. b. yaoshanensis*, *T. b. gaoligongensis*, *T. b. lepcha*, *T. b. tonquinia*, *T. b. chinensis*, *T. b. yunalis*, *T. b. modesta*, and *T. b. assamensis* (Wang *et al.*, 1991). Tree shrews have shown susceptibility to infection by human rotavirus (Wan *et al.*, 1982), herpes simplex virus type 1 and 2 (Darai *et al.*, 1978), and human hepatitis viruses A, B, and D (Li *et al.*, 1995; Walter *et al.*, 1996; Yan *et al.*, 1996a,b; Zan *et al.*, 1981). Susceptibility of *Tupaia* to HCV infection has not been reported. We tested this possibility in two transmission experiments using Chinese tree shrews. This paper shows that tupaia can be infected *in vivo* with HCV and that whole-body irradiation might facilitate HCV infection of these animals.

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² Abbreviations used: HCV, hepatitis C virus; PCR, polymerase chain reaction; HGV, hepatitis G virus; ALT, alanine aminotransferase.

TABLE 1

Results of Serum HCV RNA and Anti-HCV in Tupaia from Study A

Animal	Weeks postinoculation											
	0	2	5	7	9	14	18	23	27	31	36	47
1 ^a	—	—	—	—	—	—	—	—	—	nd	—	—
2 ^a	—	—	—	—	—	—	nd	—	—	—	—	—
4	—	nd	—	—	—	—	—	—	+	—	—	+
5	—	—	+	+	—	—	—	—	nd	—	—	—
6	—	—	—	—	—	—	—	—	—	—	—	—
7	—	—	—	—	—	—	nd	—	—	—	—	—
8	—	—	nd	—	+	—	—	—	—	—	—	—
9	—	+	+	+	—	+	—	—	+	—	—	—
10	—	—	—	—	—	—	—	—	—	—	—	—
11	—	—	—	+	—	nd	—	+	—	—	—	—
12	—	—	—	—	—	—	—	—	—	—	—	—
13	—	—	+	—	—	—	—	+	nd	—	—	—
14	—	—	—	—	—	—	—	—	—	—	—	—
15	—	—	—	—	—	—	—	—	—	—	—	—
16	—	—	—	+	—	—	—	—	+	—	—	—
17	—	—	—	—	—	—	—	—	—	—	—	—
18	—	—	—	—	—	—	—	—	—	—	—	—
19	—	nd	—	—	—	—	—	—	nd	—	—	—
20	—	—	—	—	—	—	—	—	—	—	—	—
21	—	—	—	—	—	—	—	—	—	—	—	—
22	—	nd	—	nd	—	—	nd	—	—	—	—	—
23	—	+	—	—	—	—	—	—	—	—	—	—
24	—	—	—	—	—	—	—	—	—	—	—	—
25	—	nd	—	—	—	nd	—	—	—	—	—	—
26	—	nd	—	—	—	—	—	—	—	—	—	—

Note. nd, not done. (+) Indicates positive HCV RNA and (–) negative HCV RNA. The shaded area indicates positive anti-HCV.

^aControl animals.

RESULTS

Study A

HCV RNA and anti-HCV were negative in all preinoculation blood samples obtained from experimental animals and in the two control tupaia. Eight inoculated animals (34.8%) developed HCV viremia, as determined by PCR at different times during the 47 weeks of follow-up (Table 1). HCV RNA was first detected 2 weeks postinoculation in two animals (Nos. 9 and 23). The viremia was transient in two tupaia and intermittent in six. In tupaia No. 9 HCV RNA was detected in serum from weeks 2 to 7 postinoculation, it became negative on week 9, and reappeared transiently on weeks 14 and 27. Anti-HCV was found transiently in serum in seven (30.4%) tupaia (Table 1). In three animals (Nos. 19, 22, and 23) the antibody persisted for 1 to 2 months. The fact that in 6 animals anti-HCV appeared after several weeks of seronegativity indicates that a carrying over of antibodies of the inoculum was not responsible for seropositivity. As seen in Table 1 three tupaia tested positively for both anti-HCV and HCV RNA at different time points during evolution but only two serum samples (one from animal No.9 and the other from No.13) were positive

simultaneously for the two determinations. Anti-HCV was also found in four animals (Nos. 7, 19, 22, and 26) that never exhibited detectable viremia. Peaks of transaminases (in most cases between weeks 7 and 18) were observed in five inoculated tupaia (Fig. 1A) and in one control animal. In all tupaia with increased serum transaminases HGV RNA determinations were performed, the results being negative in all animals tested. On week 50 postinoculation, liver biopsy specimens were obtained from four tupaia with previous elevation of ALT and/or detectable anti-HCV antibody (Nos. 13, 19, 22, and 24). The pathological examination showed ballooning degeneration of hepatocytes in all these animals, together with multinucleated liver cells and macrovesicular or focal necrosis with inflammatory infiltrate in some of them. One granulomatous lesion probably associated with parasite infection was also observed in one animal. The wild nature of the tupaia precludes the interpretation of biochemical and pathological changes as the solely due to HCV infection.

Study B

HCV RNA and anti-HCV antibodies were negative in all serum samples from the control group and in all prein-

FIG. 1. Evolution of ALT levels and serological HCV markers in two representative tupaia animals from study A (tupaia No. 16, A) and study B (tupaia No. 19, B). Solid and open squares represent, respectively, positive and negative assays for HCV RNA. Solid and open triangles represent positive and negative anti-HCV, respectively.

oculation samples from groups I and II (Table 2). In contrast, HCV RNA became positive in two tupaia animals from group I (20%) and in half of the animals (tupaia No. 7 and 19) from group II (Table 2). It was interesting to observe that while HCV RNA could be detected only for a brief period in group I, the viremia lasted longer in animals from group II. Positive viremia obtained with primers from the 5' untranslated region was confirmed with primers from the core region (Table 2). HCV genotyping and quantification of HCV RNA were performed in three of the animals with positive viremia (Nos. 14, 7, and 19) (Table 3). We found genotype 1b in all infected animals and coinfection 1b/3 in one of them. The viral titer was low in all the infected tupaia animals, ranging from less than 10^4 to 3.5×10^5 copies per milliliter of serum (c/ml).

Liver specimens from seven animals (three from group I, three from group II, and one from group III) were

processed for detection of HCV RNA. As shown in Table 2, HCV RNA could be detected in liver tissue in two tupaia animals from group II. The positive result obtained with primers from the 5' noncoding region was confirmed with primers from the core region of HCV. Viral loads in the liver of tupaia No. 7 and 19 were 10^5 and less than 10^3 copies/ μ g of RNA, respectively (Table 3).

Anti-HCV antibody appeared transiently in three animals and in a recurring manner in one. In three cases we found coincidence of positivity for anti-HCV and HCV RNA. In one case viremia was not accompanied by seropositivity and, conversely, anti-HCV developed in an animal that never presented positive viremia. Interestingly, while anti-HCV was detected in only one tupaia from group I, it was found in three of four animals from group II. In one of these tupaia animals (No. 19, week 21) the optical density in the ELISA test reached considerably high values (Table 2). Analysis of antigenic specificities using RIBA test showed that anti-C33c was the earliest and most frequent antibody (Table 4).

A transient peak of ALT elevation occurred in five animals from group I and in one from group II. The peak occurred between weeks 8 and 12 in most cases (Fig 1B). No significant ALT changes were observed in control animals. Testing for HGV RNA was performed in serum samples from animals showing elevated ALT at any time during the follow-up. In all cases HGV RNA was negative. Liver biopsy was obtained in all animals from the three groups on week 27 postinoculation. Histological examination of liver tissue in infected animals showed the presence of ballooning degeneration of hepatocytes, multinucleated liver cells, and fatty degeneration in most of these tree shrews. None of these changes could be interpreted as the sole result of HCV infection since multinucleated hepatocytes, fatty degeneration, and/or focal necrosis with inflammatory infiltrates were found in three animals from the control group. Granulomas possibly related to parasite infection were found in two tupaia animals from group I and in one from group III.

Liver biopsy was repeated on week 33 in two animals from group II with positive serum HCV RNA (tupaia No. 7 and 19) and in two control tupaia animals (No. 11 and 20) and the samples were processed for electron microscopy. Ultrastructural examination of the liver showed fat droplets, hyperplasia of smooth endoplasmic reticulum, and multiple membranous arrays resembling myelin figures. These changes were not found in tupaia animals from the control group. We did not find the typical cytoplasmic tubular structures described in chimpanzees with experimental HCV (Bradley *et al.*, 1981; Shimizu *et al.*, 1979).

DISCUSSION

In this paper we report the results of two studies which are concordant in showing that *T. b. chinensis* can be

TABLE 2

Results of Serum and Liver HCV RNA and Serum Anti-HCV Antibodies in Tupaia from Study B

Animal	Serum										Liver	
	Weeks postinoculation										27	33
	0	3	6	8	11	12	15	20	21	27		
	5' ^a C	5' C	5' C	5' C	5' C	5' C	5' C	5' C	5' C	5' C	5' C	5' C
1	—	—	—	—	—	—	—	—	nd	—	—	nd
2	—	—	—	—	—	—	—	—	nd	nd	nd	nd
3	—	—	—	—	—	—	—	—	—	—	nd	nd
4	—	—	—	—	—	—	—	—	—	—	nd	nd
5	—	—	—	—	—	—	—	—	nd	—	nd	nd
12	—	—	—	—	++	—	—	—	—	—	—	nd
13	—	—	—	—	—	—	—	—	—	—	nd	nd
14	—	—	—	++	—	—	—	—	—	—	—	nd
16	—	—	—	—	—	—	—	—	nd	—	nd	nd
17	—	—	—	—	—	—	—	—	nd	—	nd	nd
7 ^b	—	—	++	—	—	—	—	++	+-	++	++	—
8 ^b	—	—	—	—	—	—	—	—	nd	—	nd	nd
15 ^b	—	—	—	—	—	—	—	—	—	—	—	nd
19 ^b	—	—	—	—	—	—	—	++	++	+-	—	++
10 ^c	—	—	—	—	—	nd	—	—	nd	—	—	nd
11 ^c	—	—	—	—	—	nd	—	—	nd	—	nd	nd
20 ^c	—	—	—	—	—	nd	—	—	nd	—	nd	nd
21 ^c	—	—	—	—	—	nd	—	—	nd	—	nd	nd

Note. nd, not done. (+) Indicates positive HCV RNA and (–) negative HCV RNA. The shading area indicates positive anti-HCV; optical density of ELISA: tupaia No. 15 (week 3) = 0.291; tupaia No. 19 (week 3) = 0.357; tupaia No. 7 (week 6) = 0.303; tupaia No. 14 (week 8) = 0.230; and tupaia No. 19 (week 21) = 1.418.

^a HCV region of primers; 5', 5' untranslated region; C, core region.

^b Irradiated animals.

^c Control animals.

infected *in vivo* with HCV. Whether other subtypes of *T. belangeri* are also susceptible to this infection is not known. In fact attempts in our laboratory to transmit HCV to tupaia with their origin in Thailand and bred in a European primate center failed to produce positive results (unpublished observations).

In this work rigorous criteria have been adopted to classify serum samples as HCV RNA positive, namely,

TABLE 3				
Viral Load and HCV Genotypes in Infected Tupaia from Study B				
Tupaia No.	Week	Genotype	Viral load in serum ^a	Viral load in liver ^b
14	8	1b	3.5 × 10 ⁵	nd
7	6	1b	3.5 × 10 ⁵	nd
	20	3-1b	<10 ⁴	nd
	27	nd	nd	10 ⁵
19	20	1b	nd	nd
	21	1b	3 × 10 ⁵	nd
	33	nd	nd	<10 ³

Note. nd, not done.

^a Copies/ml of serum.

^b Copies/μg RNA.

the inclusion of multiple negative controls in each PCR run and the need for repeated positivity in two valid assays. In addition, PCR positivity was ascertained in several specimens using primers from the core region of HCV genome. Thus, there seems to be little risk of false positivities in our PCR findings.

Our data show that viremia occurred in 34.8% of tupaia from study A, inoculated in China with genotype 1b. In study B conducted in Spain, the proportion of nonmanipulated tupaia which developed viremia after inoculation with a mixture of genotypes was only 20%. The reason for this difference in the percentage of infected

TABLE 4						
Analysis of Antigenic Specificities in Anti-HCV-Positive Sera from Tupaia (Study B) Using RIBA HCV 3.0						
Tupaia No.	Week	Antigens used in RIBA HCV 3.0				
		C100	C33	C22	NS5	SOD
14	8	±	+	—	—	—
15	3	—	±	—	—	—
19	3	—	+	—	—	—
	21	±	+	—	2+	—

tupaia is not clear since the animals used in the two studies come from the same geographical area (Yunnan, China). It may be possible that HCV quasispecies of the Chinese inocula adapt better to tupaia biology than the quasispecies contained in the European inocula or that the amount of virus in the Chinese inocula was higher (unfortunately viral load in the latter could not be determined). Alternatively, the mixture of genotypes in the inocula used in study B might unfavourably affect the replication of the eventually dominant genotype. In fact, although in this study the inoculum contained genotypes 1b, 1a, and 3, genotype 1b was found to thrive better in tree shrews.

In study B we attempted to increase the efficiency of HCV transmission by subjecting animals to total body irradiation. We found that half of the surviving irradiated tupaia developed viremia and that HCV RNA tended to persist for longer in these tree shrews than in their nonirradiated mates. Moreover, while HCV RNA could not be detected in liver tissue from nonmanipulated tupaia, it was present in the liver of two irradiated animals. These findings indicate that irradiation, probably through its immunosuppressive effect, may enhance HCV replication in tree shrews. Our results open the way to explore other maneuvers which might facilitate HCV replication in tupaia, thus increasing the value of these animals as an experimental *in vivo* system of HCV infection.

In addition to developing viremia, tupaia also produce anti-HCV antibodies. In keeping with HCV RNA results, the prevalence of anti-HCV was highest in tree shrews from study A and in group II animals from study B. One of the irradiated tupaia developed very high titers of anti-HCV at week 21 postinoculation in coincidence with a rebound of HCV replication (Tables 2 and 4). In this case antibodies were directed against NS5 (2+) and C33c (1+). This was an exception since, in agreement with the chimpanzee model (Shindo *et al.*, 1992), the latter antigen was the main target of anti-HCV antibodies in most of the analyzed animals. It should also be noted that tupaia, similarly to chimpanzees, do not produce anti-C22 in response to HCV infection (Shindo *et al.*, 1992).

As mentioned before (see Results) the time pattern of presentation of anti-HCV in inoculated tupaia indicates that seropositivity is not a consequence of carrying over of antibodies from the inoculum. The long delay in the appearance of anti-HCV in some cases suggests that these antibodies are the result of ongoing HCV replication. It is intriguing, however, that some seropositive animals never exhibited detectable HCV RNA. Hence, it seems possible that HCV replication may occur in some tupaia at an undetectable level which could nevertheless be sufficient to induce a humoral response. In fact, in samples where HCV RNA could be quantitated, the level of viremia was found to be very low, equal to or

below 3.5×10^5 copies per ml, which is considerably inferior to the level (10^7 – 10^9) found in patients with chronic hepatitis C (Larrea *et al.* 1996; Gavier *et al.*, 1997).

Histopathological changes, mainly hepatocellular ballooning, fat degeneration, and the presence of multinuclear hepatocytes, occurred in many of the tree shrews which received HCV-positive inocula, but some of these morphological alterations were also observed in the control group. Occasional granulomas suggesting parasitic infection were found in some control and HCV-inoculated tupaia. It should be noted that the tree shrews used in the present study were captured in the jungle and wild tupaia are known to be frequently infected by viruses, such as tree shrew herpes virus, which may cause spontaneous hepatitis (Wu *et al.*, 1983). For similar reasons variations in transaminase levels should be interpreted with caution. We observed significant ALT elevations in 11 tupaia which received HCV-positive inocula and only in one control animal. The ALT peak took place in most of the animals between the 8th and 12th weeks, which is the approximate time of occurrence of the transaminase peak in the acute phase of experimental HCV infection in chimpanzees (Bradley *et al.*, 1990; Farci *et al.*, 1991).

On electron microscopy, tupaia with positive viremia exhibited microvesicular fat, abnormal cytoplasmic vesiculation with hypertrophy of smooth endoplasmic reticulum, but not the classical multilaminated tubular structures observed in the chimpanzee model (Bradley *et al.*, 1981; Shimizu *et al.*, 1979). These alterations were not found in control tupaia livers. However, whether the changes observed in the infected tupaia are related to HCV infection could not be established in the present study.

To summarize, this report shows that *T. b. chinensis* is susceptible to HCV infection and from our preliminary data it seems that susceptibility to this infection could possibly be increased by whole-body irradiation. The small size of tupaia, their relatively low cost, their easy adaptation to a laboratory environment, and their high reproductive activity in captivity make this animal a possible *in vivo* system for culturing HCV which may be valuable in testing antiviral agents and addressing relevant pathophysiological issues of HCV infection.

MATERIAL AND METHODS

Animals

Adult tree shrews, subtype *T. b. chinensis*, captured in the wild in Yunnan Province, China, were used in two different study protocols (A and B). All animals were negative for hepatitis B virus markers.

In study A, 25 tree shrews (10 male and 15 female; body weight 105.7–159.4 g, mean, 121.4 g) were kept in the Institute for Research on Zoology, Guangxi Medical University (Yunnan, China). They were housed in steel cages at a temperature of $23 \pm 2^\circ\text{C}$, relative humidity of

70–80% and a 12-h light/12-h dark photo cycle. Animals were fed basal diet supplemented with milk, eggs, and fruits. Water was supplied *ad libitum*.

In study B, 20 tree shrews (10 male and 10 female) were imported from China and kept at the animal facilities of the University of Navarra (Pamplona, Spain). Animals shrews (body weight 124.9–169.6 g, mean, 143.0 g) were housed individually in steel cages at $23 \pm 2^\circ\text{C}$, relative humidity 50–70%, and a 12/12-h light/dark cycle; they were fed commercial tree shrew chow *ad libitum* (Altromin, Germany) and fruits. They had free access to water.

Inoculation and study protocol

Study A. A pool of serum from four Chinese patients with chronic hepatitis C (one case of posttransfusion hepatitis, one case of chronic hemodialysis, and two cases of community-acquired hepatitis) was used for inoculation. The inoculum was HCV RNA and anti-HCV positive and negative for hepatitis B virus markers (HBsAg, Anti-HBs, HBeAg, anti-HBe, and anti-HBc). The HCV genotype of the inoculum was 1b. Virus concentration in the inoculum was not determined. Animals were inoculated intravenously with 0.15 ml of serum and the same volume of saline was administered to two animals which served as controls (one male and one female). Blood was drawn before and 2, 5, 7, 9, 14, 18, 23, 27, 31, 36, and 47 weeks after inoculation. Each time, 1 ml of blood was collected from the thigh vein and serum samples were distributed in three aliquots for each animal. One was immediately used for alanine aminotransferase (ALT) determination and the other two were stored at -40°C until used (for anti-HCV and HCV RNA determinations). In the 50th week postinoculation, surgical liver tissue samples were obtained from four animals with a previous elevation of ALT and/or detectable anti-HCV antibody. Liver biopsies were fixed in 10% Formalin and embedded in paraffin.

Study B. The inoculum consisted of a pool of the same amount of serum from five Spanish patients with chronic hepatitis C. The HCV genotype and virus concentration of these cases were as follows: patient 1, genotype 1b, concentration 5×10^7 c/ml; patient 2, genotype 1b, concentration 8×10^6 c/ml; patient 3, genotype 1b, concentration 1.2×10^7 c/ml; patient 4, genotype 1a, concentration 1×10^9 c/ml; and patient 5, genotype 3, concentration 1.1×10^9 c/ml. All sera were negative for hepatitis B virus markers (HBsAg, anti-HBs, HBeAg, anti-HBe, and anti-HBc). In this study protocol animals were divided into three groups: group I (10 animals: 5 male and 5 female) was inoculated without previous irradiation, group II (6 animals: 3 male and 3 female) received 750 cGy of X-ray whole-body irradiation 2 days before inoculation, and group III (4 animals: 2 male and 2 female) which were used as control. Two animals from group II died after irradiation, and thus this group was reduced to

only 4 tupaia. Animals from groups I and II were inoculated three times during the first week (2 days interval between inoculations) with 0.4, 0.8, and 0.8 ml of the inoculum. Group III received normal human serum instead of the HCV-positive inoculum with the same volume and time schedule as the other two groups.

Blood samples were collected before and at 3, 6, 8, 11, 12, 15, 20, 21, and 27 weeks after inoculation. Each time, 2 to 3 ml of blood was collected from the thigh vein and serum was aliquoted and stored at -80°C .

In the 27th week of follow-up, surgical liver biopsy was performed in all animals and in the 33rd week in selected cases. Liver samples were divided into three parts; one was fixed in 10% Formalin and then embedded in paraffin, and the other two were embedded in OCT (Sakura Finetek Europe, Zoeterwoude, Netherlands) and kept at -80°C for RNA extraction. Surgical liver biopsy was also performed in eight additional, not inoculated, tree shrews. For electron microscopy liver biopsies were fixed for 6 h at 4°C in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2 and postfixed in 1% phosphate-buffered osmium tetroxide. After dehydration in ethanol and washing in propylene oxide, the tissue fragments were embedded in Epon 812. Semithin sections, 1 μm in thickness, were cut, mounted on glass slides, stained with methylene blue, and studied by light microscopy. For electron microscopy, thin sections were double stained with uranyl acetate and lead hydroxide and observed with a Zeiss 10CR electron microscope.

Biochemical and serological determinations

ALT activity was determined using the Reitman–Frankel method in study A and by the Wroblewski and LaDue procedure using a commercially available kit (Sigma Diagnostic, St. Louis, MO) in study B. ALT activity was considered significantly elevated if higher than 2.5 times the mean preinoculation ALT values.

To determine the presence of anti-HCV antibodies, a passive hemagglutination assay (Abbott HCV PHA second generation; Dainabot Co., Tokyo, Japan) was employed in study A. Both structural and nonstructural antigens of HCV (C100-3, pHCV31, and pHCV34) were included in this assay. The test was performed according to manufacturer's instructions. Samples were considered positive when hemagglutination occurred at 2^{-5} dilution or higher. In study B, anti-HCV antibodies were assayed using a third generation ELISA (Ortho, Raritan, NJ) according to manufacturer's instructions.

HCV RNA was determined in serum or liver tissue using a reverse transcription-polymerase chain reaction (PCR) as described elsewhere (Gil *et al.*, 1993). Briefly, an Ultraspect RNA isolation system (Biotecx Lab, Houston, TX) was used to extract RNA from 100 μl of serum. RNA was subsequently reverse-transcribed, followed by 30 cycles of PCR amplification using a set of primers from

the 5' noncoding region of the HCV genome (outer primers: sense, 5'-CCT GTG AGG AAC TAC TGT CT-3'; anti-sense, 5'-CTA TCA GGC AGT ACC ACA AG-3'; 95°C 15 s for denaturation, 52°C 12 s for annealing, and 72°C 20 s for extension). One microliter of the PCR product was then subject to 30 cycles of amplification as described for the first PCR with a set of inner primers (sense, 5'-ACT GTC TTC ACG CAG AAA GC-3'; anti-sense, 5'-GAC CCA ACA CTA CTC GGC TA-3'). In each run of PCR, eight serum samples and three negative controls were tested. Multiple negative controls were included to detect contamination. The assay was considered valid only if all of the negative controls were negative. To further confirm our results, samples were taken as positive only if the positive result was repeated in two valid assays. In addition, in study B samples which were PCR positive using primers from the 5' noncoding region of the HCV genome were retested with two sets of primers derived from the core region (Viazov *et al.*, 1994). The two steps of amplification reactions (35 cycles each) were performed according to the following profile: denaturation, 95°C 15 s; annealing 50°C 12 s; extension, 72°C 20 s.

The quantification of HCV RNA was carried out using a competitive PCR procedure as previously reported (Larrea *et al.*, 1996). HCV genotyping was performed with a modified ELISA-hybridization method (Viazov *et al.*, 1994) using the amplified nested PCR product of the HCV core region with digoxigenin incorporation and specific probes for HCV genotypes 1a, 1b, 2a, 2b, and 3 as described elsewhere (Larrea *et al.*, 1996; Sarobe *et al.*, 1996).

Hepatitis G virus (HGV) RNA was analyzed in serum by a PCR-based procedure using a commercial kit (Boehringer Mannheim, Mannheim, Germany). This method consists of a PCR amplification after RNA extraction of two regions (5' noncoding region and NS5a) of HGV followed by capture of digoxigenin-labeled PCR products on plated specific probes and detection using anti-digoxigenin peroxidase-labeled antibody. Sera were considered negative when the two PCR products were negative.

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