Effect of Immune Globulin on the Prevention of Experimental Hepatitis C Virus Infection

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The efficacy of postexposure prophylaxis for the prevention of hepatitis C virus (HCV) infection was studied in experimentally infected chimpanzees. Three chimpanzees were inoculated with HCV: Two were treated 1 h later with anti-HCV–negative intravenous immune globulin (IGIV) or hepatitis C immune globulin (HCIG), and a third animal was not treated. HCV infection was detected in all 3 animals within a few days of inoculation. Once passively transferred anti-HCV declined in the HCIG-treated animal, there was an increase of HCV antigen (Ag)–positive hepatocytes followed by reappearance of anti-HCV; HCV Ag disappeared concordant with the development of acute hepatitis. Acute hepatitis C developed in both the IGIV-treated and untreated chimpanzees, with peak liver enzyme activity on day 59, but was delayed in the HCIG-treated animal until day 146. Postexposure HCIG treatment markedly prolonged the incubation period of acute hepatitis C but did not prevent or delay HCV infection. IGIV had no effect on the course of HCV infection.

Hepatitis C virus (HCV) is transmitted primarily through overt percutaneous exposures to infectious blood, although transmission may also occur through mucous membrane and inapparent parenteral exposures [1]. Specific measures to prevent HCV infection have been introduced in the transfusion and transplant settings and include the exclusion of donors judged to be at increased risk by history or by positive results on serologic screening for antibody to HCV (anti-HCV) or for surrogate markers [2]. However, most hepatitis C occurs outside these settings [1], and, when a defined exposure can be identified, the potential exists to prevent HCV infection with postexposure prophylaxis.

Several studies have attempted to assess the value of prophylaxis with immune globulin preparations against posttransfusion non-A, non-B hepatitis [3–6]. However, the results are difficult to compare and interpret because of a lack of uniformity in diagnostic criteria, mixed sources of donors (volunteer and commercial), different study designs (some lacked blinding and placebo controls), and different immune globulin preparations. In some of these studies, the immune globulin preparations seemed to reduce the rate of clinical disease but not overall infection rates; in one, treated patients were less likely to develop chronic hepatitis. These data have not been reanalyzed since anti-HCV testing became available. Moreover, in only one study was the first dose of the immune globulin preparation given after, rather than before, the exposure, making it difficult to assess the value of immune globulin for postexposure prophylaxis.

Although in experimental settings, the neutralization function of anti-HCV has been ineffective [7], isolate-specific neutralization antibodies seem to exist for a limited time after infection [8]. Experimental vaccine trials have also indicated that virus-specific protective antibodies can be induced in experimental conditions [9].

In 1992, the Food and Drug Administration recommended that all plasma be screened for anti-HCV. If protective antibody exists, such screening may affect its presence in immune globulin preparations manufactured from screened plasma pools. Using commercially prepared intravenous immune globulin (IGIV) manufactured from plasma screened for anti-HCV and a preparation of hepatitis C immune globulin (HCIG) made from plasma containing high titers of anti-HCV, we conducted a study in chimpanzees to assess the efficacy of postexposure prophylaxis for the prevention of HCV infection.

Materials and Methods

Immune globulin preparations. IGIV (Miles Biological, Naperville, IL) was prepared from 5 plasma pools, 4 of which had
been screened with a multiantigen (i.e., second-generation) anti-HCV EIA and 1 with a single-antigen EIA. The preparation was processed using standard cold ethanol fractionation with the pH of the solution adjusted to 4.0–4.5. The product was negative for anti-HCV and HCV RNA.

HCIG was prepared from 23 plasma donations: Each was anti-HIV–positive at a dilution of ≥1:1000. All donations contained antibodies to core, NS3, and NS4 proteins as determined by a second-generation supplemental anti-HCV immunoblot assay (MATRIX HCV; Abbott Laboratories, Abbott Park, IL). Standard, cold-ethanol fractionation [10] of a 200-mL plasma pool was carried out. The final preparation was acidified (to bring the pH to 4.3) and kept at room temperature for 3 weeks to inactivate any residual HCV [11]. The final preparation was positive for anti-HCV at a titer of 2330 (HCV EIA 2.0 [Abbott Laboratories]; HCV EIA 2.0 [Ortho Diagnostic Systems, Raritan, NJ]) and was HCV RNA–negative. The protein content was 54.1 mg/mL, and purity was 97.9%.

Inoculation and passive immunization of chimpanzees. Three male chimpanzees (CH.1493, CH.1487, and CH.1489; weight, 14–16 kg) not previously exposed to HCV were inoculated intravenously with 30 chimpanzee infectious doses (CID) of HCV (strain HCV-1; CDC/Chiron) in 10% autologous serum in PBS. One hour after inoculation, CH.1487 received 35 mL of IGIV, and CH.1489 received 35 mL of HCIG by intravenous infusion; CH.1493 served as the untreated control. Serum samples were obtained 24 h after inoculation and at 3- to 7-day intervals for the duration of the study (240 days) and tested for alanine aminotransferase (ALT) activity and markers of HCV infection under code. Liver tissue specimens were obtained weekly and prepared for routine histopathology examination and for immunohistochemistry by snap freezing at −80°C.

Serologic analysis. Serial serum samples obtained before and after inoculation from the 3 chimpanzees were tested for anti-HCV with a commercial second-generation EIA (HCV EIA 2.0) and with the MATRIX HCV (both Abbott). The sera were also tested by an EIA for antibodies to the putative envelope glycoprotein E2 (anti-E2) using the C′ terminally truncated form of E2 protein (aa 384–661) purified from a Chinese hamster ovary cell by affinity and ion exchange column chromatography [12].

HCV RNA detection and sequencing. Reverse transcriptase polymerase chain reaction (RT-PCR) detection of HCV RNA was done as described [13, 14]. Briefly, RNA extracted from 200 μL of serum was reverse transcribed and then amplified using nested primers within the conserved 5′ untranslated region of HCV at a final MgCl2 concentration of 1.5 mM. The sensitivity of the assay was ≥0.5–1 CID [13].

Selected serum samples from CH.1487 (days 40 and 130 after inoculation) and from CH.1493 and CH.1489 (day 40 after inoculation) were used for sequencing of the NS5 region. After serum was reverse transcribed with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Indianapolis) in the presence of RNAsin and random primers (1000 μg; Promega, Madison, WI) to generate a stock solution of cDNA; one-tenth of this stock was used for subsequent PCR amplification.

PCR amplification was done with HCV-1 external and internal primers corresponding to K1/K2 and 122/123, respectively [15, 16]. Amplifications were hot-started at 95°C for 1 min in the presence of 5% dimethyl sulfoxide followed by 30 cycles of denaturation at 95°C for 60 s, annealing at 40°C for 60 s, and extension at 72°C for 60 s for both the external and internal amplifications (25 cycles each). The products were purified by PAGE, eluted, and phenol-chloroform was extracted and alcohol was precipitated. Cycle sequencing reactions were done with the internal positive or negative sense primers using dye terminators (Applied Biosystem Division, Perkin-Elmer, Foster City, CA) in the presence of 5 ng of single-strand DNA binding protein (SSB; United States Biochemicals, Cleveland). Before electrophoresis, the SSB was digested with 0.1 μg of proteinase K at 65°C for 20 min. Sequence comparisons to other strains of HCV were done with the computer program Pileup (version 8; University of Wisconsin Genetics Computer Group, Madison, WI).

HCV antigen (Ag) detection in the liver. HCVAg was determined by direct immunofluorescence with the use of a fluorescein isothiocyanate (FITC)–labeled IgG preparation obtained from a patient with chronic HCV infection as described previously [17]. This immunohistochemical reagent had been documented to be virus-specific by absorption studies with recombinant HCV proteins. Cryostat sections from liver biopsy specimens were fixed in chloroform, stained with the FITC-labeled anti-HCVAg reagent, and evaluated under code. HCVAg was graded semiquantitatively on the basis of the percentage of hepatocytes positive for the antigens: 1+, <5%; 2+, 5%–20%; and 3+, >20%. The intensity of fluorescence usually matched the number of cells that stained positive.

Results

Untreated (control) and IGIV-treated chimpanzees. The pattern of HCV infection and disease progression was similar for the control (CH.1493) and IGIV-treated (CH.1487) animals (figure 1). HCV RNA was detected in the serum within 72 h after inoculation, and HCVAg was identified in hepatocytes on day 7 after infection. Viremia lasted 121 days in the control animal and 139 days in the chimpanzee treated with IGIV. The number of focally distributed hepatocytes positive for HCVAg reached 70% in the control chimpanzee and 50% in the IGIV-treated chimpanzee by week 7 after inoculation. Detectable antigen disappeared just before or at the time of anti-HCV seroconversion. Both the control and the IGIV-treated chimpanzees developed histopathologic features of acute hepatitis, with peak ALT values of 592 and 878 U/L, respectively, by day 59 after inoculation. ALT values returned to the normal range 12 weeks after inoculation. Antibodies to HCV were detected 63 days after inoculation in both animals. Antibodies to NS3, NS4, and envelope (E2) proteins were detected in both animals, whereas the antibody to the core protein was detected only in a single serum sample from the control animal and in none of the samples from the IGIV-treated animal (figure 2).
Figure 1. Temporal relationship between markers of HCV infection: HCV RNA in serum, HCV antigen (HCVAg) in liver cells, and anti-HCV measured by EIA and alanine aminotransferase values in control, anti-HIV–negative immune globulin (IGIV)–treated, and hepatitis C immune globulin (HCIG)–treated chimpanzees (CH). IGIV (CH.1487) and HCIG (CH.1489) were infused 1 h after challenge with HCV-1 inoculum on day 0. S/CO, signal-to-cutoff ratio.
In the IGIV-treated animal, anti-HCV became undetectable 146 days after seroconversion. The control animal was anti-HCV positive throughout follow-up.

**HCIG-treated chimpanzee.** Passively transferred anti-HCV, when measured by the second-generation EIA, was detectable 24 h after the HCIG infusion and persisted for the next 70 days. The antibodies became undetectable in samples obtained during the next 20 days but were again present in serum from day 97 until the end of observation (figure 1). Between days 76 and 90, when anti-HCV was undetectable by EIA, MATRIX testing detected antibody to the core protein and NS3. Passively transferred anti-E2 measured in selected serum samples became undetectable by day 97 and again tested positive from day 125 (table 1).

HCV RNA was detected in serum 1 day after the HCV inoculation and the infusion of HCIG and was present in subsequent serum samples for the next 20 weeks (until day 139). Hepatocytes positive for HCVAg were found in the first liver biopsy specimens obtained after inoculation (day 7). The proportion of hepatocytes positive for HCVAg were consistently <5% for the first 9 weeks after inoculation but then increased in concordance with a decline in the level of passively transferred anti-HCV. HCVAg was found in ~50% of liver cells between days 118 and 125 and in 70% of hepatocytes at day 133. At the time of increasing expression of HCVAg in the liver, signal-to-cutoff ratio values for anti-core increased sharply; anti-core was the only HCV antibody detected in samples taken between days 97 and 125 (figure 2). In 2 subsequent weekly liver biopsy specimens, a sharp decrease of HCVAg-positive cells was observed (to 30% by day 139), and HCVAg became undetectable on day 146. At the time HCVAg disappeared from the liver, anti-E2 was strongly reactive (table 1).

Acute hepatitis and peak ALT levels were significantly delayed compared with control and IGIV-treated animals (figures 1, 2). The peak ALT value (415 U/L; 10.6 times the negative cutoff) was observed on day 146, although slightly elevated ALT values (<2.7 times the negative cutoff) were observed on days 31–111 (figure 1). During the initial phase of ALT elevation, morphologic lesions in the liver consisted of nonconspicuous changes, such as sinus-lining cell activation, accumulation of lymphocytes in sinuses, rare single foci of cell necrosis, and minimal infiltrations in portal tracts. Morphologic features of acute hepatitis were observed in specimens obtained at and near peak ALT elevation.

**Sequencing of HCV RNA.** HCV nucleotide (nt) sequences in the amplified region within the NS5 between nt 8299 and nt 8584 (287 bases) in all samples from all 3 chimpanzees were identical to each other and to those of the HCV used as the infectious inoculum (HCV-1 strain, "Rodney pooled serum"/CDC).

**Discussion**

In this study, we investigated the effectiveness of postexposure prophylaxis for preventing HCV infection in experimen-

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**Figure 2.** Profiles of antibody responses against recombinant structural (core, envelope E2) and nonstructural (NS3, NS4) HCV antigens. Horizontal bars, elevated alanine aminotransferase (ALT) values. CH, chimpanzee; neg, negative.
Table 1. Dynamics of hepatitis C virus antigen (HCVAg) presentation in the liver and anti-HCV response in an HCV-infected chimpanzee (CH. 1489) treated with hepatitis C immune globulin (HCIG).

| Day of experiment* | HCVAg-positive liver cells (%) | Anti-HCV (S/CO) | Anti-E2
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<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.0 0.2 0.2</td>
<td>Negative (0.024)</td>
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<tr>
<td>1</td>
<td>18.9 17.9 11.6</td>
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<td>Positive (&gt;2.0)</td>
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<tr>
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<td>&lt;5</td>
<td>17.6 16.8 9.0</td>
<td>Positive (0.818)</td>
</tr>
<tr>
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<td>5</td>
<td>9.0 11.2 2.4</td>
<td>Positive (0.169)</td>
</tr>
<tr>
<td>63</td>
<td>5</td>
<td>2.9 4.5 0.8</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>5-15</td>
<td>2.8</td>
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</tr>
<tr>
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<td>5-20</td>
<td>1.8 2.9 0.5</td>
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<td>90</td>
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<td>10.9 1.8 0.6</td>
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</tr>
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<td>20</td>
<td>14.8 0.9 0.5</td>
<td>Negative (0.066)</td>
</tr>
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<td>Positive (0.776)</td>
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<tr>
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<td>16.7 1.7 0.6</td>
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<tr>
<td>146</td>
<td>0</td>
<td>13.0 2.8 2.8</td>
<td>Positive (1.134)</td>
</tr>
<tr>
<td>203</td>
<td>0</td>
<td>6.7 0.8 1.1</td>
<td>Positive (0.208)</td>
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* Day after HCV-l inoculation and HCIG treatment.

1 S/CO, signal-to-cutoff ratios. Values > 1.0 are positive.

2 E2 recombinant protein expressed in CHO cells. OD, optical density.

tally infected chimpanzees. The challenge dose of 30 CID was comparable to the amount of the virus in 3 μL of plasma of an infected human with a level of viremia equal to 10⁶ CID and roughly equivalent to the amount of the virus estimated to be transmitted during a needlestick exposure. The identification of HCV RNA in serum and HCVAg in liver cells showed that neither anti-HIV−negative IGIV nor HCIG prevented HCV infection in the postexposure setting. In both the IGIV-treated and control chimpanzees, the course of the infection and the disease were identical when abnormal ALT values, HCV RNA in serum, and HCVAg in liver cells were compared.

In our experiment, the 8-week incubation of acute hepatitis was similar to that observed for chimpanzees infected with the same inoculum in our laboratory and elsewhere [9, 18]. The only difference between the control and IGIV-treated chimpanzees was in the profiles of anti-HCV response. In the IGIV-treated animal, the antibodies against structural and nonstructural HCV proteins disappeared from serum as early as 120 days after the inoculation. It remains to be established whether infusion of normal globulins of human origin influences the dynamics of humoral anti-HCV response in experimental animals. The overall characteristics of the viral replication and liver pathology characterized by typical enzyme elevation and histopathologic changes of acute hepatitis were similar in the IGIV-treated and control chimpanzees. This observation clearly indicated the lack of a protective function of anti-HIV−negative immune globulin administered intravenously after HCV infection. The results of our experiments in primates contributed to the recently revised recommendations of the Advisory Committee on Immunization Practices (ACIP). In February 1994, the ACIP reviewed the available data and concluded there was no support for the use of immune globulin for postexposure prophylaxis of hepatitis C (unpublished data).

The infusion of high-titered HCIG as early as 1 h after HCV inoculation did not prevent infection as shown by the presence of HCV RNA in serum and HCVAg in liver cells. Observations on the dynamics of viral replication and liver pathology indicated, however, that the course of the infection and acute hepatitis in the HCIG-treated animal was different from that of the 2 other chimpanzees. Immunohistochemical findings suggested that the rate and extent of the spread of the infection in the liver was inversely correlated with the level of passively transferred anti-HCV. The number of infected HCVAg-positive hepatocytes remained low during the first 63 days of the experiment when passively transferred anti-HCV was detected in serum. It was assumed that the level of viremia also remained low, since we had previously found that the number of HCVAg-positive liver cells reflects the level of viremia as measured by a bDNA assay (unpublished data) [19, 20]. The containment of the spread of HCV infection in the liver may have been related to the presence of antibodies against envelope proteins (anti-E2) in the HCIG. The variability of the envelope region of HCV and the quasispecies nature of the virus observed in several studies [21, 22] would result in a wide range of antibody specificity within a pool of anti-HIV−positive serum samples used for preparation of HCIG.

Recent infectivity experiments in chimpanzees and neutralization studies in tissue culture using antibodies from different phases of HCV infection suggest that the neutralizing activity of antibodies changes during various phases of the infection
The potential presence of neutralizing anti-HCV in the pool of serum samples collected from multiple donations and used for the preparation of immune globulin may be responsible for the modification of posttransfusion hepatitis observed in the early clinical studies [3, 4, 6].

In the liver of the HCIG-treated animal, the number of HCVAg-positive cells began to increase when the optical density values of anti-E2 declined 10-fold from those of anti-E2 passively transferred from the HCIG on the day after inoculation. The increase of viral synthesis and HCVAg in liver cells was followed by a sharp increase in anti-core levels, whereas the signal-to-cutoff ratios for anti-NS3 and anti-NS4 fell below 3.0. At that time, the EIA showed an antibody-negative “window” after the elimination of passively transferred anti-HCV. The level of anti-core response significantly exceeded those observed in chimpanzees inoculated with the same HCV strain in other studies [13]. A similar increase or decrease of anti-core related to the rising or falling amount of HCV in serum was observed in HCV-infected patients treated with interferon and suggests that the humoral immune response against the HCV capsid protein reflects the rate of the viral synthesis [24, 25]. The sharp quantitative rise in HCVAg and subsequent elimination of the antigen from the liver was observed simultaneously to the increasing amount of anti-E2 in serum. This rapid elimination of the antigen from the liver cells and intermittent presence of HCV RNA in serum suggests that in experimental HCV infection the increase in the neutralizing fraction of envelope antibodies during the humoral immune response may play a significant role in elimination of the virus. The coexistence of anti-E2 and circulating HCV, as observed in the HCIG-treated chimpanzee, has been reported in studies of HCV-infected patients with acute and chronic hepatitis [26, 27].

It is important to note that enzymatic and histopathologic manifestations of acute hepatitis in the HCIG-treated animal were significantly delayed compared with those in the control and IGIV-treated animals. It was shown in previous studies that the HCV inoculum used in this study induces acute hepatitis in chimpanzees, with peak ALT activity between 40 and 60 days after inoculation regardless of the infectious HCV dose [18]. In HCV vaccine studies, 4 control chimpanzees inoculated with 10 CID of the same inoculum had the highest ALT elevations between 35 and 56 days after inoculation [9]. The significant delay in the occurrence of acute hepatitis and elimination of virus observed in our experiment is similar to the vireologic and pathologic events observed in a chimpanzee during passive immunoprophylaxis of hepatitis B virus infection with the use of monoclonal antibodies to the a determinant of hepatitis B surface antigen [28].

The prolonged viremia and the delayed onset of a marked rise in ALT levels within the HCIG-treated chimpanzee raised the question of whether the original HCIG contained virus that was controlled by the homologous antibodies and, after their decline, resulted in disease within the HCIG-treated chimpanzee. Several lines of evidence suggest that the chimpanzee treated with HCIG was infected by the original inoculum (HCV-1) and not by potential residual virus in the HCIG. The HCIG preparation, although it was prepared from a pool of plasma samples that may have contained HCV, was negative for HCV RNA when tested by nested RT-PCR. The Cohn-Oncley fractionation procedures result in an overall reduction in HCV RNA by a factor of 4.7 × 10^6 from the starting plasma [10], and additional room temperature incubation at pH 4.3 for 3 weeks further reduces HCV infectivity by a factor of 10^3 [11]. Although the reasons for the loss of the infectivity in immune globulin preparations are not clear, the removal of HCV during the fractionation process, virus inactivation during manufacture, and the existence of neutralizing antibodies were considered. In addition, the NS5 sequence of the inoculum and all the samples from the infected animals used in this study was identical to the NS5 sequence of HCV-1, indicating that the disease in all animals was caused by the original titered inoculum and not by residual virus in either the IGIV or the HCIG.

The overall results of this experiment indicate that neither HCIG nor IGIV prevented or delayed HCV replication. Passive transfer of HCIG markedly prolonged the incubation period of acute hepatitis C and suggested that the course of liver infection was controlled first by passive immunity and later by an active immune response. It seems, therefore, that the passively transferred antibodies from the HCIG may have a neutralizing activity that can limit the extent of viral replication within the liver in the initial phase of the infection. It is conceivable that a larger dose of antibodies (larger volume or higher titer of anti-HCV) could further modify infection. Further experiments are needed to investigate the effect on the course of HCV infection of repetitious infusions of HCIG to maintain adequate levels of neutralizing antibodies after challenge. Virologic, clinical, and pathologic observations from these experiments may be of significance in developing preventive modalities in HCV-infected liver transplant patients.

Acknowledgments

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References