Hepatitis C Virus Core and Envelope Proteins Do Not Suppress the Host’s Ability To Clear a Hepatic Viral Infection

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Several hepatitis C virus (HCV) proteins have been shown in vitro to interact with host cellular components that are involved in immune regulation. However, there is a paucity of data supporting the relevance of these observations to the in vivo situation. To test the hypothesis that such an interaction suppresses immune responses, we studied a line of transgenic C57BL/6 mice that express the HCV core and envelope proteins in the liver. The potential effects of these proteins on the hepatic immune response were evaluated by challenging these mice with a hepatotropic adenovirus. Both transgenic and nontransgenic mice developed similar courses of infection and cleared the virus from the liver by 28 days postinfection. Both groups of mice mounted similar immunoglobulin G (IgG), IgG2a, interleukin-2, and tumor necrosis factor alpha responses against the virus. Additionally, BALB/c mice were able to clear infection with recombinant adenovirus that does or does not express the HCV core and envelope 1 proteins in the same manner. These data suggest that HCV core and envelope proteins do not inhibit the hepatic antiviral mechanisms in these murine experimental systems and thus favor a model in which HCV circumvents host responses through a mechanism that does not involve general suppression of intrahepatic immune responses.

Hepatitis C virus (HCV), a major cause of chronic liver disease worldwide, currently infects about 1.8% of the U.S. population (1). Most HCV-infected patients become chronically infected, and a substantial proportion of such patients develop clinical manifestations of liver disease. Although the natural history of the chronic infection is not well understood, the high frequency with which infection progresses to chronicity suggests that HCV has evolved specific mechanisms to circumvent the host’s immune responses.

A number of distinct models have been proposed to explain the phenomenon of persistent HCV infection. Several studies have described the emergence of viral variants that could escape from the immune system. During the course of a chronic infection, antibodies (Abs) recognizing hypervariable region 1 of the viral E2 envelope protein undergo changes in their epitope specificities. In addition, cytotoxic T lymphocytes (CTL) from a chronically infected chimpanzee failed to recognize the HCV quasispecies present at 4 months postinfection (3, 25). Collectively, these studies suggest that, despite active immune responses, a viral population may emerge with the selection of variants that possess an enhanced ability to persist in the host.

On the other hand, the establishment of a persistent HCV infection may not always be due to genetic variation of the virus (15). It has been suggested that HCV, like adenovirus, herpes simplex virus, and many other viruses, may encode one or more proteins that act to inhibit viral clearance by the host. Indeed, two viral proteins, N55A and the envelope glycoprotein E2, have been shown to repress the host’s double-strand-RNA-inducible protein kinase R (PKR), resulting in a state of selective interferon (IFN) resistance (7, 21). When expressed in mammalian cells, N55A confers IFN resistance on vesicular stomatitis virus, which normally is sensitive to the antiviral actions of IFN (6). In addition, the HCV core protein has been shown to bind to the cytoplasmic domains of tumor necrosis factor (TNF) receptor 1, lymphotoxin β receptor, and gC1q receptor (4, 13, 17, 28), resulting in interference with Fas/TNF-α-induced apoptosis and T-cell proliferation in vitro (8, 16).

Given the roles of these cellular proteins in the normal development of peripheral lymphoid organs, transduction of apoptotic signals, and initiation and resolution of inflammation (18, 24), these data raise the possibility that the HCV structural proteins (core, E1, and E2) have significant immunomodulatory functions. However, there is a paucity of data either supporting or refuting the relevance of these in vitro observations to the in vivo situation. To distinguish between the relative contributions of evasive and suppressive factors in HCV persistence in vivo, we took advantage of a recently developed HCV transgenic (tg) mouse (14a) and examined the effects of the HCV structural proteins on intrahepatic immune responses during a viral infection. Our data suggest that the HCV core and envelope proteins are not inherently immunosuppressive in murine experimental systems and thus favor a model in which HCV circumvents immune responses through a mechanism that does not require profound immune suppression.

**MATERIALS AND METHODS**

**Mice.** Generation of HCV tg mice has been described in detail elsewhere (14a). Briefly, cDNA corresponding to the structural protein coding region (core, E1, and E2/p7) of the genotype 1b HCV-N strain of HCV was subcloned into plasmid pGEMAlbSVPA under the control of the liver-specific murine albumin promoter-enhancer. The insert was excised by digestion with XhoI and injected...
into F1 hybrid zygotes (C3H/HeJ × C57BL/6J). The offspring were screened for the presence of tg DNA by Southern blot analysis, and a tg F2, founder (S-N/66s) was mated with C57BL/6 mice (H-2d; Jackson Laboratories, Bar Harbor, Maine) to produce F2 and subsequent generations of animals. BALB/c mice (H-2d) were purchased from Jackson laboratories. All mice were housed in a specific-pathogen–free facility. The Institutional Animal Care and Use Committee of the University of Texas Medical Branch approved the study.

**Viral infection of the liver.** Tg and non-tg mice were infected with a replication–impaired (with E1 deleted) adenovirus (AdΔgal) that expresses bacterial β-galactosidase (β-Gal), as previously reported by Herz and Gerard (9). Ten tg mice, 2 to 3 months of age, were each inoculated intravenously with 3 × 10^9 PFU of AdΔgal. Equal numbers of age-matched non-tg littermates were infected as controls. In separate experiments, 20–22-month-old female BALB/c mice were similarly infected with 3 × 10^9 PFU of AdΔgal and a recombinant adenovirus expressing the core and E1 segments of the HCV polyprotein (AdΔCE1) (2). At 3 to 6, 14, and 28 days postinfection, two to four mice in each group were sacrificed by CO₂ asphyxiation. Liver tissues were snap frozen in liquid nitrogen for DNA isolation or placed in OCT cryostat-embedding compound (Tissue-Tek, Torrance, Calif.) for morphological analyses. Serum samples were stored at −70°C for subsequent enzyme-linked immunosorbent assays (ELISAs) and ala- nine aminotransferase (ALT) measurements.

**Immunochemistry and Western blotting.** After being fixed in acetone and blocked with normal goat serum and avidin-biotin (Avidin/Biotin blocking kit; Vector Laboratories, Burlingame, Calif.), cryosections were incubated at 4°C overnight with a rabbit anti-β-antibody specific for the E2 protein of HCV N (1:500). The sections were incubated with biotinylated goat anti-rabbit immunoglobulin G (IgG) followed by avidin-conjugated peroxidase (Vectorstain Elite ABC kit) and developed in 3,3′-diaminobenzidine tetrahydrochloride. Detection of lymphocytes in tissue samples was carried out as described previously (20). For immunoblotting, 2 μg of protein extracted from the livers of mice infected with AdΔCE1 and AdΔgal or from a stably transfected cell line, Huh7/191-20, that expresses HCV core, E1, and E2 proteins were electrophoresed in sodium dodecyl sulfate–12% polyacrylamide gel (unpublished data). The pro- teins were electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, Calif.) and probed with a monoclonal antibody specific for HCV core (Anogen, Mississauga, Canada), β-actin (Sigma, St. Louis, Mo.), and adenovirus hexon protein (Chemicon, Temecula, Calif.), followed by a horseradish peroxidase-conjugated anti-mouse IgG (Southern Biotechnology Associates, Birmingham, Ala.) and enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Little Chalfont, England).

**Detection of AdΔgal in liver cryosections.** Following a brief fixation with 0.5% glutaraldehyde, frozen liver sections were incubated with 0.2 μg of X-Gal (5-bromo-4-chloro-3-indoly-β-D-galactopyranoside)/ml at 37°C for 60 to 120 min. Infected cells expressing β-Gal activity were stained blue, whereas uninfected cells were counterstained red (neutral red). Five images from two liver sections were randomly selected and captured with a Zeiss Axioskop microscope equipped with a Sony DXC-970MD video camera (10× objectives). Infected and uninfected regions were quantified using the MetaView Imaging System 4.0 software (Universal Imaging System, West Chester, Pa.). Thresholds were se- lected with colors representative of the desired color range of the infected or uninfected cells. Intensity of the hepatocytes was expressed as the average ratio of infected to uninfected cells.

**Determination of hepatic viral load.** The AdΔgal copy numbers in the liver were assessed by real-time quantitative PCR. Total DNA was extracted from 50 mg of liver tissue from each mouse by using a QIAamp DNA Mini Kit (Qiagen Inc., Valencia, Calif.). Analysis was carried out on a Roche LightCycler with its core reagent kit (DNA Master SYBR Green 1). The PCR primers corresponding to the adenovirus hexon gene were 5′-GAGCCGACATTAATTGATGC A-3′ and 5′-AGATAGTCTTAAAGGACTTGCTTGC-3′. The extracted DNA samples were diluted 1:100 with Tris-EDTA buffer, and 5-μl aliquots were placed in glass capillary cuvettes (Roche) containing 15 μl of PCR master mix. Reaction cycles included denaturation at 94°C for 30 s, annealing at 60°C for 3 s, and extension at 72°C for 10 s. The reaction product was detected by a 126-bp product. The observed threshold cycle for a no-template control was typically around 35 cycles. The standard curve of the threshold cycles versus the template abundance was linear over 5 orders of magnitude, with a regression correlation typically greater than 0.98. All data were combined from two independent experiments.

**Measurements of serum IgG and cytokines.** Serum samples were analyzed for adenovirus-specific IgG by ELISA with a procedure modified from a previous report by Chirnule et al. (5). Briefly, 96-well Immunolon-IV microtiter plates (Dynatech, Chantilly, Va.) were coated with AdΔgal (10^5 particles/ml). Serum dilutions (1:50) were added to duplicate wells, and the plates were incubated at 4°C overnight. The plates were incubated with biotinylated rabbit anti-mouse IgG or IgG2a, followed by avidin-conjugated peroxidase (BD PharMingen). The enzyme activity was determined colorimetrically using O-phenylenediamine dihydrochloride reagent (Sigma) and read at 492 nm. Interleukin-2 (IL-2) and TNF-α in the serum samples were measured with commercial ELISA kits (BD PharMingen) according to the manufacturer’s recommendations. The paired MABS specific for IL-2 and TNF-α were JES6-1A2/JES6-5H4 and G281-2626/MP6-XT3, respectively. Serum samples (1:3) were incubated in the plates at 4°C overnight, followed by incubation with a biotinylated anti-IL-2 or anti-TNF-α MAb. Avidin-conjugated peroxidase was added to the plates, and the enzyme activity was determined colorimetrically as described above.

**TUNEL assay for detection of apoptosis in situ.** The terminal deoxynucleotidyltransferase–mediated dUTP–biotin nick end labeling (TUNEL) assay was carried out on deparaffinized, formalin-fixed liver sections treated with proteinase K (20 μg/ml). After endogenous peroxidase was blocked by immersing the sections in 3% H₂O₂ for 30 min, slides were incubated with a buffer solution containing terminal deoxynucleotidyltransferase and digoxigenin-labeled deoxynucleotide triphosphates (Apoptag Peroxidase; Intergen Company, Purchase, N.Y.) in a humidified chamber at 37°C for 1 h. An antidigoxigenin-peroxidase conjugate was added for 30 min, followed by 3,3′-diaminobenzidine tetrahydrochloride as a chromogen. The slides were scored blindly with respect to the tg status of the animal. One section from each mouse was examined in each of two independent assays. The total number of apoptotic cells in 10 randomly selected microscopic fields (40×) was scored in each section.

**Statistical analysis.** All statistical evaluations were conducted using analysis of variance for two factors, independently and interactivity. Tabulations were fa- cilitated by using Abstat 1.90 software (Anderson-Bell Corp., Arvada, Colo.).

**RESULTS**

Since the intrahepatic immune system has a number of unique properties, including lymphocyte populations that are distinct from those of the spleen and lymph nodes (19), it is important to examine the potential immunosuppressive effects of HCV proteins in the context of the hepatic microenvironment. To assess the role of HCV structural proteins expressed at levels similar to those encountered in infected humans, we used a tg mouse line with liver-specific expression of HCV core, E1, and E2/p7 proteins. Immunocompetent mice are capable of eliminating recombinant adenovirus primarily through a cellular immune response (27, 29). Thus, the prolonged presence of adenovirus in these tg mice would support the view that the HCV structural proteins suppress the immune system.

First, we evaluated the expression of the HCV transgene in the tg mice. The E2 protein was expressed in tg liver tissue with a distribution that was cytoplasmic and particularly prominent in the pericentral regions (Fig. 1A). No E2 antigen was present in non-tg mice. RNA transcripts of the transgene were detectable in the livers of tg mice by Northern blot analysis (14A). Next, we infected these mice and their non-tg littermates with a recombinant adenovirus (AdΔgal) that expresses a reporter β-Gal. When 3 × 10⁹ PFU of AdΔgal was injected through the tail vein, the hepatotropism of the virus was evidenced by β-Gal staining of the liver but not the spleen, kidneys, or lungs (data not shown). Three groups of mice in each of two experiments were sacrificed during the early (days 3 to 6), intermediate (day 14), and late (day 28) stages of the infection. Both tg and non-tg mice demonstrated a striking hepatic infection during the early stage (Fig. 1B). The β-Gal activity was easily detectable in infected hepatocytes, and the excellent contrast between β-Gal and neutral red (counterstain) allowed accurate enumeration of infected hepatocytes. The results from these experiments showed that tg and non-tg mice followed similar courses of infection, with the disappearance of most AdΔgal-infected hepatocytes by 28 days postinfection (Fig. 1B).
and 2A). To provide an independent measure of viral clearance, we also determined the viral load in the liver tissues by a highly sensitive real-time quantitative PCR. Consistent with functional loss of viral β-Gal activity, there was an average of 90 and 94% reduction of the viral genome in tg and non-tg mice, respectively, by 28 days postinfection (Fig. 2B). At about 30 viral particles per cell in both groups of animals, these viral loads may draw near the threshold of the lacZ assay (Fig. 2A) and thus prohibit further identification of infected cells beyond that point. Together, these results demonstrated no impairment in the ability of the HCV tg mice to respond to and successfully eliminate an intrahepatic infection with an unrelated virus.

Since both humoral and cellular immune responses have been demonstrated to play critical roles in adenovirus clearance and recovery from experimental adenovirus infections (10, 27, 29), we examined whether these responses to the viral infection were quantitatively affected in tg mice. Despite the expression of the HCV structural proteins in tg animals, tg and non-tg mice produced similar quantities of adenovirus-specific IgG, as well as IgG2a, which is generally accepted as an indicator of the Th1-mediated immune response (Fig. 3A and B). Immunohistological examinations showed that the two groups of mice had comparable abilities to recruit CD4 and CD8 T cells to inflammatory lesions in the liver (Fig. 1C). Also, the serum levels of TNF-α and IL-2 in tg mice were equivalent to those found in non-tg littermates (Fig. 3C and D), suggesting no apparent compromise in either the humoral or cellular effector mechanisms.

In immunocompetent mice, adenovirus infection of the liver is accompanied by a transient elevation of serum ALT activity, which is followed by a significant reduction in virus within 3 weeks (27). Thus, we determined whether the presence of the HCV structural proteins was associated with exacerbated liver injury and/or delayed resolution of the biochemical lesion. The peak of the chemical liver injury occurred at 14 days postinfection in both tg and non-tg mice, and ALT levels declined significantly in both groups of animals thereafter (Fig. 4A). The resolution of hepatic inflammation in both groups of mice was also confirmed by measurements of the total liver weight, as well as resolution of acute histopathological abnormalities (data not shown). Since the tg mice demonstrated robust TNF-α production in response to Adβgal infection (Fig. 3C), we also examined the possibility that expression of the HCV core protein in the liver could result in an exacerbated TNF-induced apoptosis in the tg mice (4, 8, 17, 28). Using TUNEL assays, we found that both tg and non-tg mice had elevated numbers of apoptotic cells in the liver during the early stages of infection. However, the number of apoptotic cells declined in both groups as the infection progressed to the later stages (Fig. 4B). There were no significant differences between tg and non-tg animals in these studies.

To determine whether the absence of immunosuppression due to HCV structural proteins that we observed in the HCV tg mice could be restricted to the H-2^d^ genetic background or the level of tg expression, we infected BALB/c (H-2^d^) mice with 3 x 10^7 PFU of Adβgal and a recombinant adenovirus (AdCE1) expressing the core and E1 segments of the HCV genome (2). Such infection resulted in a substantially higher abundance of the intracellular HCV core protein (Fig. 5A). Despite this, we observed a significant reduction of viral load in...
the BALB/c mice infected with AdCE1, which was comparable to that in the mice infected with Adβgal (Fig. 5B). Both groups of mice recovered from the infection, as reflected in a decrease in serum ALT activities from early peak values (data not shown). These results lend strong support to the conclusion that in mice the HCV core protein does not hinder the host’s ability to resolve an intrahepatic viral infection. This absence of immunosuppression is neither mouse strain specific nor dependent on the level of HCV core expression.

DISCUSSION

Using a series of vaccinia virus/HCV recombinants, Large et al. have examined the influence of specific HCV gene products on the immune responses to vaccinia virus in a murine model (14). In that study, vaccinia virus recombinants expressing various regions of the HCV polyprotein were inoculated intraperitoneally into naïve BALB/c mice. The HCV core protein contributed to a greater pathogenicity of vaccinia virus/HCV recombinants in BALB/c mice (H-2d), suggesting that it might contribute to viral persistence by directly suppressing host immune responses, in particular, the generation of virus-specific CTL. In the present study, however, we evaluated the effect of the HCV core protein in tg mice that express an abundance of the HCV structural proteins that is much closer to that found in infected human livers. Surprisingly, we found no evidence of suppression of intrahepatic viral clearance mechanisms in these mice (H-2b), suggesting that the HCV core protein does not hinder the host’s ability to resolve an intrahepatic viral infection. This absence of immunosuppression is neither mouse strain specific nor dependent on the level of HCV core expression.

FIG. 3. Production of virus-specific IgG and cytokines following an intrahepatic viral infection. Mice were injected with Adβgal as for Fig. 2A, and their serum samples were collected at the indicated time points. The error bars represent the standard deviations among individual animals. The relative concentrations of virus-specific IgG (A) and IgG2a (B) are expressed as absorbance, or optical density (O.D.). The levels of circulating TNF-α (C) and IL-2 (D) were determined by ELISA. Tg+, tg mice; Tg−, non-tg mice.

FIG. 4. Recovery from liver injury following Adβgal infection in HCV tg (Tg+) and non-tg (Tg−) mice. The experiments were carried out as described in the legend to Fig. 2A. (A) Serum ALT levels are shown in individual animals as a function of time following infection. There was no significant difference in ALT values between tg and non-tg mice (P > 0.05). (B) TUNEL assay for detection of apoptosis in the liver. Shown are the numbers of apoptotic cells present in 10 randomly selected fields (magnification, ×40) in individual sections. No difference in the number of apoptotic cells, either overall (P > 0.05) or at any given time point (P > 0.05), was found between two groups of mice. The number of apoptotic cells decreased significantly during the course of infection in both groups of mice (P < 0.01). The slides were scored blindly for apoptotic cells with respect to the date and tg status of the animal.
Apoptosis has been suggested as a common pathway to virus clearance by host CTL and NK cells. Many virus genomes encode proteins that suppress apoptosis in order to escape immune attack by the host (18). Conflicting results have suggested that the HCV core protein can either protect transfected cells from Fas- and TNF-α-induced apoptotic cell death or sensitize them to it (8, 16, 28). Our results suggest that HCV core neither exacerbates nor inhibits apoptosis in vivo in the face of a robust, endogenous TNF-α response. Furthermore, as our tg-mouse experiments have shown, the HCV E2 protein does not hamper the host’s ability to mount effective intrahepatic immune responses, even though it has been suggested to interfere with and suppress PKR responses (21).

These data clarify previous contradictory reports regarding the functions of core and E2 proteins, as they are derived from an in vivo system in which the abundances of these proteins are more reflective of the situation in human patients. While our data favor a model in which HCV circumvents the immune responses through a mechanism that does not involve general immunosuppression, they do not exclude the possibility that the virus modulates the complex interplay between the dynamics of viral diversity and the early breadth of CTL responses. For instance, viral escape mutants have been shown to give rise to T-cell receptor antagonists capable of inhibiting CTL clones that recognize prototypic viral epitopes (3, 11, 22). In addition, the additive or synergistic effects of other HCV proteins not expressed in these studies (e.g., NS5A) may conceivably contribute to viral resistance to the IFN-mediated antiviral mechanism, leading to persistent infection (7).

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