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Suppression of Host Immune Response by the Core Protein of Hepatitis C Virus: Possible Implications for Hepatitis C Virus Persistence

Mary Kathryn Large,* David J. Kittlesen,* and Young S. Hahn2*†

Hepatitis C virus (HCV)3 is a positive strand RNA virus belonging to the family Flaviviridae (1, 2). HCV was a major cause of transfusion-associated hepatitis before the development of serologic tests for HCV-contaminated blood products, and it remains a major agent of community-acquired hepatitis (3, 4). Characteristic features of HCV infection include a high incidence of persistent infection and progression to chronic hepatitis (3). Chronic HCV infection of the liver is a strong risk factor for development of hepatocellular carcinoma (5).

The high incidence of HCV persistence after infection suggests that this virus has evolved a mechanism to evade the host response, probably by inhibiting the immune response necessary for viral clearance during acute infection. One proposed mechanism of immune evasion is the generation of viral variants during infection that could escape from Ab or CTL recognition. This strategy is consistent with the high error rate of the viral polymerase during viral RNA replication and may account for the high frequency of HCV quasispecies detected in HCV-infected patients (6). Alternatively, as observed for many of the large DNA viruses, such as adenovirus and herpes simplex virus (7–9), HCV may encode one or more products that act to inhibit viral clearance by the host and can lead to progressive or persistent viral infection.

The induction of virus-specific CTLs during infection is a well-established mechanism for virus elimination during infection. In the case of HCV, CTLs have been recovered from both the liver (10) and the periphery (11) of chronically infected patients. However, the frequency of HCV-specific CTL precursor (CTLp) and effectors is much lower than that observed during infection with other persistent viruses, such as hepatitis B virus or HIV (12, 13). It is currently unknown whether this low CTLp in chronically infected patients reflects an immunosuppressive mechanism caused by the ability of the HCV to establish and maintain viral persistence after infection.

The HCV RNA genome is approximately 9.5 kb in length and encodes a long polyprotein. Ten discrete proteins are produced from this polyprotein by proteolytic processing. The structural proteins include the core (C) and the E1 and E2 glycoproteins and are found in the N-terminal portion of the polyprotein. The nonstructural (NS) proteins involved in RNA replication are found in the remainder of polyprotein. Among the HCV gene products, only the highly conserved HCV core protein has been suggested to have an immunomodulatory function. This is based on the in vitro demonstration that the core protein binds to the cytoplasmic domain of certain members of the TNF receptor superfamily (14) and can modulate the sensitivity of cells expressing HCV core to TNF-mediated lysis in culture (15).

Since HCV infects only humans and chimpanzees and replicates inefficiently in cell culture, studies aimed at assessing the abilities of individual HCV-encoded proteins to modulate host immune responses during HCV infection have been difficult to perform. To examine this issue in a model system, we have used infection of mice with recombinant vaccinia viruses (VV) expressing HCV gene products as a way to assess possible immunomodulatory functions.

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Abbreviations used in this paper: HCV, hepatitis C virus; CTLp, precursor of CTL; C, core; NS, nonstructural; VV, vaccinia virus; pfu, plaque-forming units.

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effects of specific HCV polypeptides on VV infection. The contributions of specific components of the immune response to VV infection in mice have been well defined, and recovery from VV infection has been demonstrated to be strongly correlated with the generation of a virus-specific CTL response (16, 17).

In the study described here, we examined the virulence of various VV/HCV recombinants in mice and studied the host response induced by recombinant viruses. Surprisingly, we found that a recombinant VV expressing the structural protein of HCV produced a lethal disseminated infection in mice and concomitantly suppressed the VV-specific CTL response and the production of proinflammatory cytokines as well. Using a series of VV recombinants expressing various C-terminally truncated polypeptides, this immunosuppressive effect was mapped to the core protein. These results suggest that expression of HCV core during HCV infection could account for the low frequency of CTLp observed in chronically infected patients and that HCV core may play a critical role in establishing and maintaining persistent HCV infection.

Materials and Methods

Cell lines

The P815 (H-2d) mastocytoma and BSC40 monkey kidney cell lines were maintained in DMEM supplemented with 10% (v/v) FBS and 1% (w/v) glutamine.

Plasmid constructions and generation of VV recombinants

VV/HCV recombinants are designated by the region of the HCV (H strain; genotype 1a) polyprotein sequence expressed. Two recombinants, vHCV-S and vHCV-NS, have been described previously (18, 19). For mapping studies, a nested set of C-terminal deletions was constructed using PCR to introduce a stop codon at or near the end of each individual protein. VV insertion vectors were either pTM3 (20) or pBRTM (19), and the HCV-specific portions amplified by PCR were verified by sequence analysis. The resulting plasmids were then used to rescue the corresponding VV/HCV recombinants by standard methods (21). The salient features of the six VV constructs used in this study are summarized in Fig. 1. The VV/HCV-S expresses HCV C, E1, E2, p7, NS2, and a portion of NS3. The VV/C-p7, vHCV-C/E2, vHCV-C/E1, and vHCV-C express the C to p7, C to E2, C to E1, and C proteins, respectively. The VV/NS expresses the major portion of NS2 (which begins at residue 900) through the end of polyprotein (NS5B). VV recombinants were rescued, plaque purified, and expanded in BSC40 cells. Their titers were determined by standard plaque assay using BSC40 monolayers in six-well plates for 1 h at 37°C. Inocula were removed and replaced with DMEM containing 1% FCS and 1 mM MgCl2. Virus was released from the pellets containing 1% FCS and 1 mM MgCl2. Virus was released from the pellets by centrifugation at 500 × g for 10 min, and pellets were resuspended in PBS containing 1% FCS and 1 mM MgCl2. Virus was released from the pellets by three freeze-thaw cycles followed by sonication.

To assay for plaque-forming virus, 200 μl of 10−1–10−3 dilutions were incubated with confluent BSC40 monolayers in six-well plates for 1 h at 37°C. Inocula were replaced with DMEM containing 10 μg/ml penicillin/streptomycin, 10% FCS, and 2 mM t-glutamine followed by incubation for 2 days at 37°C. Monolayers were fixed with 7% formaldehyde and stained with crystal violet. Plaques were counted to determine the viral titer of each liver sample, and the viral titer was calculated as log plaque-forming units (pfu) per gram of tissue.

Measurement of primary VV-specific CTL responses

To determine the primary VV-specific CTL response, mice were injected i.p. with 5 × 103 pfu of a vHCV-S recombinant or control recombinant vHCV-NS. On day 5 postinoculation, mice were sacrificed, and spleens were harvested. A single cell suspension of splenocytes was prepared and purified through Isopaque-Ficoll. Cells were harvested from the layer between the Isopaque-Ficoll and medium, and washed twice with medium to remove the residual Ficoll. To measure VV-specific CTL responses, these purified splenocytes were tested using 31Cr-labeled P815 target cells previously infected with wild-type VV. The percentage of specific 31Cr release was determined by standard procedures (23). Values for 31Cr release are the mean of quadruplicate samples; SDs were typically <5%.

Quantitation of VV-specific primary CTL

To determine CTLp frequency by limiting dilution (24), three BALB/c mice per group were infected with 5 × 103 pfu of vHCV-S or vHCV-NS (as a control). At 5 days postinfection, spleen cells from infected mice were pooled and diluted to 5 × 104 to 4 × 106 cells/well. Diluted splenocytes were cultured with VV-infected syngeneic splenocytes as stimulators (103 cells/well). Cells in individual wells were harvested after 5 days, and CTL activity was measured on VV-infected P815 target cells. P815 (H-2d) target cells were infected for 1 h at 37°C with the wild-type VV and were labeled with 150 μCi of 31Cr for 2 h at 37°C. After washing to remove free label, target cells were incubated with VV-specific CTL effectors for 6 h at 37°C, as described previously (23, 25). Calculations using the Taswell method generate a frequency estimate (1/f), a corresponding 95% confidence interval, and the χ2 estimate of probability (p) for the frequency estimate. In these studies, p > 0.05 indicates that the frequency estimate is statistically acceptable.

Cytokine analysis of bulk cultures

Primary mixed lymphocyte cultures were prepared using spleens harvested on day 5 from mice infected with vHCV or vSC11 VV recombinants as described above. Splenocytes were restimulated with irradiated VV-infected splenocytes. Responder cells were cultured at 4 × 106 cells/ml with stimulators at a 5:1 responder:stimulator ratio in Iscove’s DMEM containing 10 μg/ml penicillin/streptomycin, 10% heat-inactivated FCS, 2 mM t-glutamine, and 0.05 mM 2-ME. Cultures were incubated at 37°C, and a portion of each culture (culture supernatant and cell pellet) was harvested for 48 h. Supernatants were harvested and stored at −70°C until analysis. Supernatants were evaluated for IFN-γ by ELISA. The ELISA for IFN-γ was performed essentially as described with the following modifications. All reagents were purchased from Scieix (Caliper, Hopkinton, MA). Plates were coated overnight at 4°C with 1 μg/ml of mouse IFN-γ capture antibody. Plates were then washed and blocked with 3% (w/v) nonfat dry milk in PBS containing 0.05% Tween 20 (PBS-T) and then washed. Supernatants were then added to the wells and incubated for 1 h at 37°C. A goat antimouse IFN-γ (BD Biosciences) was then added for 1 h at 37°C. The plates were washed, and a 1:10,000 dilution of rabbit antimouse IFN-γ (BD Biosciences) was added for 1 h at 37°C. The plates were washed, and a 1:50,000 dilution of biotinylated sheep antimouse IgG (BD Biosciences) was added for 1 h at 37°C. The plates were washed, and a 1:10,000 dilution of streptavidin-alkaline phosphatase (BD Biosciences) was added for 1 h at 37°C. The plates were washed, and the substrate was added and the plates were incubated until the reactions were complete. The reactions were stopped by the addition of 1% (w/v) sodium carbonate. Absorbance of each well was determined using the Microwell Scan Reader (Scieix). Absorbance values were tabulated, and the data were analyzed as described previously (25). The data were analyzed using the Taswell method (23). A p > 0.05 indicates that the frequency estimate is statistically acceptable.
after 24, 48, 72, and 96 h of stimulation. Culture supernatants were assayed for cytokine levels by ELISA (26, 27). A standard ELISA protocol (PharMingen, San Diego, CA) was used to measure the quantities of IL-2 and IFN-γ in culture supernatants at each time point. For the standard curve to determine the amount of produced cytokines, IL-2 and IFN-γ purchased from PharMingen were serially diluted and used for ELISA assay.

**Assay for target cell recognition by VV-specific CTL**

VV-specific CTL were generated by a standard method. Two BALB/c mice (6–8 wk old) were infected with 10^5 pfu of recombinant virus (vSC11) encoding a β-galactosidase gene. Spleens were harvested from vSC11-primed mice and were stimulated in vitro with vSC11-infected and irradiated (2000 rad) naive splenocytes. The level of target cell lysis was determined by a standard 51Cr release assay using P815 (H-2d) as target cells. The lysis of 51Cr-labeled VV-infected target cell by VV-specific CTL was inhibited by unlabeled VV-infected target cells (data not shown), suggesting that the VV-specific CTL activity on P815 targets is mediated by class I MHC-restricted CD8+ T cells.

**Results**

**HCV structural gene expression enhances viral virulence**

To evaluate the possible effect of expression of HCV gene products on the virulence of and the host response to VV in the mouse, we infected BALB/c (H-2b) mice i.p. with 10^9 pfu of a recombinant VV (rVV) expressing either the 5′ half (vHCV-S) or the 3′ half of the HCV genome (vHCV-NS). The vHCV-S virus expresses the HCV structural proteins, i.e., C, E1, E2, and p7, as well as the nonstructural proteins, NS2, and a portion of the NS3 protein, while the vHCV-NS expresses the nonstructural proteins of HCV exclusively (Fig. 1).

Mice infected with vHCV-S became clinically moribund by days 1–2 postinfection, and 90% of these mice died by 5–7 days after infection (Fig. 2A). By contrast, mice infected with 10^8 pfu of the HCV-NS virus, which expresses the HCV nonstructural protein, survived, showed no clinical signs of infection or mortality, and recovered from infection as efficiently as mice infected with the control vSC11 virus (Fig. 2A). This latter virus is a rVV expressing the pSC11 recombination vector that is used for foreign gene insertion into the VV genome (see Materials and Methods). These findings suggested that expression of one or more HCV structural proteins dramatically increased the virulence of the normally avirulent VV.

To assess the relationship of viral inoculum size to disease severity and mortality, groups of eight mice were infected i.p. with varying doses of the structural protein expressing vHCV-S virus. Lethal infection was demonstrable in a virus dose-dependent fashion. All recipients of 10^9 pfu of vHCV-S virus died by day 5 postinfection, and recipients of 5 × 10^7 pfu of virus also succumbed to lethal infection with 100% mortality evident by day 8 of infection (Fig. 2B). In addition, mice receiving smaller viral inocula, i.e., 10^7 or 10^6 pfu, recovered from infection (Fig. 2B) and showed neither morbidity nor mortality up to 21 days postinfection (data not shown).

To determine the pathological changes associated with lethal infection of vHCV-S-infected mice, recipients of 10^9 pfu of vHCV-S virus were sacrificed on days 4–5 postinfection. Recipient livers were harvested daily over 5 days, and virus titers in vivo. Three mice per group were infected with 10^8 pfu of vHCV-S, vHCV-NS, or the control recombinant vSC11 virus. Recipient livers were harvested daily over 5 days, and virus titers in the infected livers were determined. As shown in Table I, after an initial burst of virus replication on day 1 postinfection, recipients of the control vSC11 virus and the vHCV-NS virus expressing the HCV nonstructural proteins rapidly cleared virus from their livers, and liver viral titers from these mice were below detectable levels by day 4 of infection. This finding is consistent with the uniform survival of mice infected with these viruses and suggests a vigorous host response to infection with these rVV.

By contrast, mice infected with the vHCV-S virus expressing the HCV structural proteins failed to clear virus and maintained high titers of infectious virus up to 5 days after inoculation, the time period when we observed the lethality during vHCV-S infection. These results are in agreement with our necropsy findings and
strongly suggest that these recipients of vHCV-S died from overwhelming infection with this rVV. It is also noteworthy that the effect of HCV structural protein expression on virus replication and presumably the host response to infection was evident early in the infectious process, as virus titers in the livers of vHCV-S recipients were 10-fold higher than titers of vHCV-NS virus or the control VV on day 1 postinfection (Table I).

**Suppression of the VV-specific CTL response by HCV protein(s)**

The finding that expression of the HCV structural proteins by rVV resulted in the lack of viral clearance suggested that expression of one or more HCV structural proteins drastically inhibited the host response to viral infection. Since virus-specific CTL have been demonstrated to play a critical role in virus clearance and recovery from experimental viral infections, including VV infection (16, 17), it was of interest to determine whether the CTL response to primary VV infection was suppressed in mice infected with vHCV-S virus.

When mice were infected with \(5 \times 10^7\) pfu of the vHCV-NS virus, their immune splenocytes, harvested on day 5 postinfection, exhibited significant cytolytic activity on VV-infected targets in a standard in vitro cytotoxicity assay (Fig. 3A). The magnitude of the in vivo primary VV-specific CTL response in vHCV-NS infected mice on day 5 postinfection was comparable to that in mice infected with the control VSC11 virus (data not shown). As expected, infectious virus was also not detectable in the livers of vHCV-NS-infected mice on day 5 postinfection (Fig. 3B).

By contrast, the in vivo primary CTL response to VV virus was markedly reduced (>5-fold) on day 5 postinfection in the spleens of mice infected with \(5 \times 10^7\) pfu of the vHCV-S virus (Fig. 3A). A comparable inhibition of in vivo primary CTL activity was observed on day 7 postinfection in vHCV-S-infected infected recipients that survived to day 7 (data not shown). Commensurate with the low levels of VV-specific CTL activity in the spleens of vHCV-S-infected recipients, the livers of vHCV-S-infected mice had high levels of infectious virus on day 5 postinfection (Fig. 3B). Liver virus titers were at least 1000-fold greater than those of the vHCV-NS-infected recipients on day 5 postinfection. These results suggest that the enhanced virulence of the vHCV-S virus may be due in part to the inhibition of a VV-specific CTL response in infected recipients through the action of one or more HCV structural proteins expressed in vHCV-S-infected cells.

To more precisely determine the degree of suppression of VV-specific CTL activity induced by the vHCV-S virus, we determined the frequency of CTLp in spleens of mice on day 5 postinfection with vHCV-S or vHCV-NS using limiting dilution analysis according to the methods of Taswell (4). We found that mice infected with the rVV expressing the HCV structural proteins had approximately a 10-fold lower frequency of CTLp on day 5 postinfection than recipients infected with virus expressing the nonstructural proteins (Fig. 4). The frequency of CTLp was \(1.6\) CTLp/10^6 immune splenocytes for the virulent vHCV-S virus and \(12.5\) CTLp/10^6 immune splenocytes for the avirulent vHCV-NS virus. This result is in keeping with the above findings on the magnitude of the in vivo primary CTL response to these two viruses.

Taken together, these results reinforce the view that the expression of one or more HCV structural proteins in infected cells in vivo leads to inhibition of the host response to viral infection, which is reflected in the suppression of the induction of virus-specific CTL. As discussed below, we also observed a marked inhibition of IFN-γ production by immune splenocytes from vHCV-S-infected mice in response to antigenic stimulation in vitro. Again, this finding suggests that one or more HCV gene products can profoundly alter the host response to VV infection.

**Identification of HCV core protein as the gene product involved in immune modulation**

Since the HCV-S virus expresses four putative HCV structural proteins, C, E1, E2, and p7, it was important to determine whether the immune suppression induced by infection with the vHCV-S virus was mediated by a specific HCV structural gene product. To approach this question, we constructed a panel of rVV expressing

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Table I. **VV titers on BSC40 cells (log pfu/g tissue)**

<table>
<thead>
<tr>
<th>Day</th>
<th>vSC11</th>
<th>vHCV-S</th>
<th>vHCV-NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>D 1</td>
<td>4.41 ± 0.44</td>
<td>5.80 ± 0.14</td>
<td>4.56 ± 0.28</td>
</tr>
<tr>
<td>D 2</td>
<td>3.80 ± 0.04</td>
<td>5.84 ± 0.49</td>
<td>4.32 ± 0.64</td>
</tr>
<tr>
<td>D 3</td>
<td>3.00 ± 0.54</td>
<td>5.98 ± 0.13*</td>
<td>2.83 ± 0.43*</td>
</tr>
<tr>
<td>D 4</td>
<td>&lt; 1.40</td>
<td>5.83 ± 0.50</td>
<td>&lt; 1.40</td>
</tr>
<tr>
<td>D 5</td>
<td>&lt; 1.40</td>
<td>6.10 ± 0.15</td>
<td>&lt; 1.40</td>
</tr>
</tbody>
</table>

* Three mice per group were inoculated with recombinant viruses expressing HCV gene products. Liver tissues were harvested from mice each day after virus inoculation and pooled from three mice per group. VV titer from liver tissues was determined on BSC 40 cells. The value (± SD) represents a SD calculated from two separate assays for VV titer. Experiments were reproducible from three independent experiments. * Statistically significant difference between indicated pairs (p < 0.01).

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FIGURE 3. The VV-specific CTL response is suppressed in vHCV-S-infected mice. A, Recognition of VV-infected target cells by primary VV-specific CTL. BALB/c mice were inoculated with \(5 \times 10^7\) pfu of vHCV-S or vHCV-NS. On day 5 after virus inoculation, spleens were harvested from infected mice, and splenocytes were purified on a Isoaque-Ficoll gradient. Purified splenocytes from vHCV-S-infected mice (n = 3) and vHCV-NS-infected mice (n = 2) were added to VV-infected P815 target cells prelabeled with 51Cr. The E:T cell ratio was 100:1. The percentage of spontaneous lysis on uninfected target cells was 4–5% (data not shown). The spontaneous release was <10%. B, Determination of VV titer. Liver tissues were harvested from rVV-infected mice on day 5 after virus inoculation. Tissues were homogenized, and VV from liver tissue was released by freezing and thawing of the cell pellet. VV titer was determined on BSC40 monkey kidney cells and is presented as log plaque-forming units per gram of tissue. Error bars indicate the SD of quadruplicate wells (A) or the SD of duplicate virus titers (B). Experiments were repeated with three times with similar results. * Statistically significant difference between indicated pairs (p < 0.01).
progressively larger deletions in the cDNA encoding the HCV structural proteins (see Fig. 1). To evaluate the impact of the incremental loss of HCV structural genes on the host response to infection, we evaluated in vivo primary VV-specific CTL responses to each of these deletion mutants, to vHCV-S virus, and to the vHCV-NS virus on day 5 after infection. As shown in Fig. 5A, the immunosuppressive activity exhibited by the vHCV-S virus expressing the full complement of HCV structural proteins was also demonstrable in mice infected with a rVV expressing HCV residues 1–192, which encodes the full length of the HCV core protein exclusively. This result strongly suggests that the HCV core protein is the viral gene product that suppresses the host response to infection and enhances the virulence of VV. In agreement with this cytotoxicity data, we also found that mice infected with the rVV expressing only the HCV core protein (vHCV-C) had elevated virus titers in their livers on day 5 of infection (Fig. 5B) and succumbed to lethal infection (data not shown). Virus titers in vHCV-C-infected mice were comparable to those in mice infected with the vHCV-S virus expressing the full complement of HCV structural proteins.

Alteration of cytokine synthesis by HCV gene product(s)

As noted above, along with the assessment of in vivo primary CTL responses to infection, we also compared the in vitro production of IFN-γ and IL-2 in response to wild-type vaccinia-infected APCs of immune splenocytes taken on day 5 after infection with the vHCV-NS virus or the vHCV-S and vHCV-C viruses. IFN-γ production by immune splenocytes from mice infected with core-expressing vHCV-C virus was profoundly suppressed (Fig. 6). The degree of inhibition of IFN-γ production in vitro was comparable to that observed in splenocytes of mice infected with the vHCV-S virus expressing the full complement of HCV structural proteins. Infection with these core-expressing rVV also resulted in a modest, but significant, inhibition of Ag-stimulated IL-2 production by immune splenocytes from vHCV-C- and vHCV-S-infected mice. Immune cells from mice infected with the vHCV-NS virus produced high levels of both IFN-γ and IL-2 (Fig. 6).

These results suggested that the HCV core protein was responsible for the diminished virus-specific CTL activity and IFN-γ production as well as the enhanced virulence observed in mice infected with the vHCV-S virus. Since activated CD8+ T lymphocytes effectors have been reported to produce high levels of IFN-γ in response to Ag stimulation (28–30), inhibition of the induction of virus-specific CTL by an HCV core-dependent suppressive mechanism would lead to decreased numbers of CTL effectors and diminished in vitro IFN-γ production in response to viral antigenic stimulation.

One potential mechanism by which HCV core could inhibit CTL induction is that HCV core may interfere with viral Ag presentation at a step along the MHC class I processing pathway in cells expressing core protein. To determine the impact of HCV core on viral Ag presentation by MHC class I molecules, we examined the capacity of VV-specific CD8+ CTL generated in response to infection with wild-type VV to lyse target cells displaying VV peptides after infection with the core-expressing vHCV-S, vHCV-C, or with the core-negative vHCV-NS virus. The VV-specific CTL do not lyse uninfected P815 target cells, and the effects of VV-specific CTL are MHC restricted. As shown in Fig. 7, core protein expression had no significant effect on the recognition of VV epitopes by VV-specific CTL. This result suggests that core protein does not disrupt Ag processing and/or presentation of viral Ag to CD8+ T lymphocytes as shown by adenovirus E3/19K protein (8). Therefore, the inhibitory effect of core on the

**FIGURE 4.** The CTLp frequency in vHCV-S-infected mice. On day 5 after virus inoculation, spleens were harvested from mice inoculated with vHCV-S or vHCV-NS (n = 3). Purified splenocytes were diluted several-fold and cultured with VV-infected splenocytes as stimulators. The CTL activity for stimulated responder CTL was determined on VV-infected target cells. The frequency of CTLp was calculated by the Taswell method. The p value was determined by χ 2 minimization, p > 0.10 for all estimates (p > 0.05 indicates significance).

**FIGURE 5.** Identification of the HCV gene product involved in suppression of the VV-specific CTL response. A, Primary VV-specific CTL response in mice inoculated with various vHCV recombinants. Two BALB/c mice per group were inoculated with 5 × 10 5 pfu of vHCV-S, vHCV-NS, and vHCV recombinants expressing the deleted polyproteins shown in Fig. 1A. Purified splenocytes were prepared from spleens harvested on day 5 after virus inoculation. Primary VV-specific CTL from pooled splenocytes was determined as described in Fig. 3. B, Determination of VV titer. The VV titer was determined on BSC40 monkey kidney cells from liver tissues harvested on day 5 after virus inoculation. Error bars represent the SD of quadruplicate wells (A) or the SD of duplicate virus titers (B). Experiments were reproducible from two independent experiments. *, Statistically significant difference between the indicated pairs (p < 0.01).
host response to viral infection is unlikely to be at the level of CD8\(^+\) T lymphocyte recognition of virally infected APCs.

**Discussion**

In this report we examined the effect of expression of HCV gene products in a rVV on the in vivo virulence of this rVV and on the regulation of the host response to VV in a murine model. We found that the selective expression of the HCV structural proteins in rVV(vHCV-S) dramatically enhanced the virulence of an otherwise avirulent VV strain. This enhanced virulence led to an increase in mortality of mice infected with this VV recombinant and persistently elevated VV titers in primary targets of vaccinia replication in vivo, i.e., liver, spleen, and ovaries. We also observed that this enhanced virulence and the sustained elevation of virus titers in recipients of the vHCV-S were associated with a suppressed in vivo primary cytotoxic T lymphocyte response to VV Ags in these recipients. In addition, immune cells from the spleens of mice undergoing primary infection with this rVV (vHCV-S) produced markedly lower levels of IFN-\(\gamma\) in vitro in response to VV Ags than immunocytes from animals infected with a rVV expressing vHCV-NS. Cells from these animals also exhibited a modest, but significant, decrease in in vitro production of IL-2. Using a series of rVV expressing various deletions in the gene complex encoding the HCV structural proteins, we identified the HCV core protein as the structural gene product that both accounted for the suppression of the host response to infection and enhanced VV virulence.

Viruses have evolved a variety of mechanisms to ensure their replication and survival and to circumvent the host immune response (31). One of the best characterized mechanisms is the inhibition of CD8\(^+\) CTL response to the virus by the ability of viral gene products to inhibit the processing of viral proteins and/or the presentation of viral peptide/MHC complexes on the surface of virus-infected target cells (32–34). Indeed, as noted above, expression of the HCV core protein does suppress CTL responses in this model system. However, we could not detect any effect of core protein on the presentation and recognition of processed viral Ag by activated virus-specific CD8\(^+\) CTL effectors. Rather, our data from the analysis of the cytolytic activity of primary CD8\(^+\) cytolytic effectors taken directly from infected animals as well as from limiting dilution analysis of the frequency of activated CD8\(^+\) T lymphocytes giving rise to cytolytic effectors argue for an effect of core protein primarily at the level of CD8\(^+\) CTL induction. Therefore, it should be emphasized that one or more HCV gene product(s) may modulate the host response during natural HCV infection for the inhibition of viral Ag processing and presentation by MHC class I molecules. In our murine model, however, we have no evidence that the core protein enhances virulence and suppresses the host response by this mechanism.

Available evidence suggests that core protein has several functions in HCV replication, including viral RNA encapsidation into viral nucleocapsid (35, 36). Because of the localization of the core gene at the 5’ end of the HCV polyprotein transcript, the core protein is likely to be the first viral gene product produced in the virus-infected cells. If, as our data suggest, core protein expression may profoundly suppress the host response to virus infection, the effect of core on the host immune response would be evident in an earlier phase of virus infection before primary virus-specific CD8\(^+\) T lymphocyte precursors give rise to CTL effectors. In this connection, it is noteworthy that mice infected with vHCV-C already demonstrate a 10-fold higher titer of virus in the liver as early as day 1 postinfection compared with mice comparably infected with control vaccinia (vSC11) or vHCV-NS (Table I). This finding reinforces the view that HCV core protein acts to suppress the host immune response at an early point in the process of viral infection.

Multiple mechanisms could account for the increase in viral titers observed within 24 h of infection. Both type 1 IFNs, \(\alpha\) and \(\beta\), have been shown to have important and nonredundant roles in the control of VV infection (37). NK cells are also significant to the early control of viral infections, and are greatly stimulated by IL-12 or IFN-\(\alpha\) and -\(\beta\). IL-12 produced by macrophages or NK cells synergize with TNF-\(\alpha\) to stimulate the production of IFN-\(\gamma\) by NK cells. IL-12 also appears to be critical for the development of Th1 responses, which can, in turn, augment CTL responses. Therefore, a disruption in the expression or function of any of these cytokines could explain not only the increased early viral titers observed, but also the deficiencies in IL-2, IFN-\(\gamma\), and CTL as shown here. Precedent for such a mechanism has been demonstrated with the inhibition of IL-12 synthesis by measles virus (38).
The induction of an effective CD8+ CTL response and the production of IFN-γ during infection have been implicated as important factors in resistance to as well as recovery from VV infection in the mouse (16, 17). Available evidence suggests that an effective CD8+ CTL response to virus infection can be mounted in the absence of either IFN-γ production or a functional IFN-γR (27). Therefore, in the murine model of HCV core-mediated immune suppression described here, it is unlikely that core protein acts by inhibiting IFN-γ production early in infection and thereby suppresses an IFN-γ-dependent step in CD8+ T lymphocyte differentiation into activated effectors. Since CD8+ CTL effectors produce high levels of IFN-γ in response to Ag (28–30), it is more likely that the diminished IFN-γ production by immune splenocytes after infection with core expressing rVV reflects a core protein-dependent inhibition of activation of virus-specific CD8+ T cells into IFN-γ-producing CTL effectors. One intriguing mechanism that could account for both the enhanced replication of core expressing VV early in infection and the suppression of the virus-specific CTL response reported here is suggested by the recent report of binding of HCV core protein to the cytoplasmic tail of the human lymphotxin-β receptor (14, 15). If HCV core can directly interact with the intracellular signaling domains of one or more members of the murine TNF receptor family in infected cells and inhibit the function of these receptors, then this inhibitory effect on TNF receptor signaling might account for the suppression of both the early innate response to virus infection and the induction of a specific CTL response observed here.

Although we observed a profound effect of HCV core expression on IFN-γ production by immune splenocytes, the effect of core on IL-2 production was less dramatic. One possible explanation for this result is suggested by the recent finding by Dr. Charles Rice’s laboratory (unpublished observation) that HCV core can up-regulate transcription of the IL-2 promoter. It is not yet clear whether this activity reflects another strategy employed by HCV to dysregulate the host response, but it does point out the potential for this viral gene product to alter multiple steps in the host response to infection with HCV.

In the studies reported here, enhanced virulence of VV was used as a functional readout of HCV core protein-dependent suppression of the host response. Although rVV have been used by other investigators to evaluate the effect of expression of foreign genes on the immune response to VV infection, it was of concern that the immunosuppressive effect of core observed by us in this model system may reflect a suppressive effect dependent upon an interaction between HCV core and one or more VV gene products unique to this class of DNA viruses. Although this possibility cannot as yet be formally excluded, it appears unlikely, since we have been able to demonstrate that HCV core expression enhances the replication and persistence of Sindbis virus (a member of the positive strand RNA alphavirus) in vivo in mice when the core gene is expressed in a recombinant Sindbis virus (Y. S. Hahn, unpublished observation). This result adds support to our view that core protein may likewise have an immunomodulatory effect during HCV infection in humans.

One of the unique features of human HCV infection is the high incidence of development of persistent HCV infection (3). This fact strongly suggests that HCV has evolved one or more strategies to suppress the host response during acute infection and thereby facilitate the development of viral persistence. The results reported here provide compelling evidence that the expression of the HCV core protein may play a critical role in the establishment and possibly the maintenance of persistence during HCV infection in humans.


