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Genetic Immunization Generates Cellular and Humoral Immune Responses Against the Nonstructural Proteins of the Hepatitis C Virus in a Murine Model

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Exposure to hepatitis C virus (HCV) is associated with a high prevalence of persistent viral infection and the development of chronic liver disease and hepatocellular carcinoma. Recovery from acute infection may depend upon the generation of broad-based cellular immune responses to viral structural and nonstructural proteins. We used the DNA-based immunization approach in BALB/c mice to determine whether the HCV nonstructural proteins NS3, NS4, and NS5 will induce Ab responses, CD4+ Th cell proliferation, and cytokine release in response to stimulation by recombinant proteins as well as generate CD8+ CTL activity both in vitro and in vivo. We found that the nonstructural proteins were particularly good immunogens and produced cellular immune responses when administered as a DNA construct. Indeed, a tumor model was established following inoculation of syngenic SP2/0 cells stably transfected with NS5. We observed protection against tumor formation and growth only in mice immunized with the NS5-encoding DNA construct, establishing the generation of significant CTL activity in vivo by this technique. The results indicate that genetic immunization may define the cellular immune response of the host to HCV nonstructural proteins and is a promising approach for vaccine development. The Journal of Immunology, 1998, 161: 4917–4923.

Hepatitis C virus (HCV) is the major cause of posttransfusion and sporadic non-A, non-B hepatitis (1) and is found throughout the world. There is a prevalence of 0.6 to 2% in western countries and ≤15% in other regions of the world (2). Approximately 60% of individuals exposed to HCV will develop chronic infection and hepatitis; 20 to 40% will eventually progress to cirrhosis and liver failure (3). More important, persistent HCV infection is associated with a high risk of primary hepatocellular carcinoma, particularly in the setting of hepatic fibrosis and cirrhosis (4). Effective therapy of chronic HCV infection has been limited, at best, and only IFN and ribavirin have been shown to exhibit beneficial antiviral activity (5). Indeed, ~10 to 15% of individuals treated with IFN alone will respond and eradicate HCV from the liver. However, recent studies have revealed that individuals who recover from acute HCV infection develop substantial CD4+ T cell proliferative responses against the nonstructural proteins as compared with those individuals who develop persistent HCV infection (6, 7). This type of cellular immune response suggests that the nonstructural proteins may be the more critical immunogens to eradicate persistent viral infection from the host. In this context, direct injection of DNA encoding for viral genes in combination with different facilitators into the muscle or skin has been shown to induce broad-based humoral and, more important, cell-mediated immune responses, and is especially effective in generating protective cytotoxic T cell responses against a variety of pathogens (8–11). However, the generation of such protective immune responses in humans remains to be established.

In the present investigation, we evaluated in vitro and in vivo humoral and cellular immune responses generated by DNA-based immunization against the three different nonstructural proteins of HCV in a murine model. It was found that the cDNAs encoding for the NS3 serine protease and helicase and NS5 RNA-dependent RNA polymerase were particularly effective in generating high-level CD4+ and CD8+ activities against epitopes that reside on these nonstructural proteins.

Materials and Methods

Plasmid construction

As a source of viral genes, a plasmid designated pBRTM/HCV1-3011 covering the full-length open reading frame (ORF) of HCV was used to clone into expression vectors (12). Constructs pAp031-NS3, pAp031-NS4, and pAp031-NS5 were PCR-cloned after inserting engineered start and stop codons as well as restriction enzyme sites using the following primers: for NS3, 5’-GG TCT AGA TTG ATG GCG CCC ATC ACG GC-3’ (XbaI), 5’-CAC ACG CGT TCA CCG GTT GGG GAG GAG GT-3’ (MluI); for NS4, 5’-GTC TAG ATG AGC ACC TGG GTG CTC-3’ (XbaI) and 5’-CCA GGA TCC TCA GCA TGG AGT GGT ACA-3’ (BamHI); and for NS5, 5’-T CAG TCT AGA ATG TCG GGC TCC TGG CTA AGG GA-3’ (XbaI) and 5’-A GCT ACG CGT TCA CCG GTT GGG GAG GAG GT-3’ (MluI). After PCR amplification using a high-fidelity PCR system (Boehringer Mannheim, Indianapolis, IN), the cDNA fragments were inserted into the plasmid expression vector pAp031 containing a Rous sarcoma virus enhancer element and a CMV promoter (Apollon, Malvern, PA). Constructs were transformed into DH5α cells, and plasmid DNA was subsequently purified by either 2X cesium chloride centrifugation or with a Qiagen Giga kit using the Endofree buffer system (Santa Clara, CA). Correct insertion of cDNAs coding for of the nonstructural proteins was verified by sequencing analysis using standard methods. To establish stable NS3-, NS4-, and NS5-expressing cell lines as target cells for the CTL assays, the nonstructural protein-encoding gene fragments were also cloned into the pCDNA3 and pCDNA3.1/Zeo (+) expression vectors (Invitrogen, San Diego, CA) with a neomycin selectable marker. An XbaI and MluI

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fragment of NS5 and NS5 was subcloned into the Nheol/Mul site of Litmus-38 vector (New England Biolabs, Beverly, MA), cut with EcoRI and SalI, and ligated into the EcoRI/XhoI multiple cloning site of pcDNA3 and pcDNA3.1/Zeo(−), respectively. An XbaI and BanHI fragment containing NS4 was ligated into Litmus-29 (New England Biolabs), recut with KpnI and EcoRI, and subsequently ligated into the pcDNA3 vector. Plasmids were designated pcDNA3-NS3, pcDNA3-NS4, and pcDNA3.1/Zeo(−)-NS5.

In vitro expression

The HuH-7 human hepatoma cell line was transiently transfected with the various constructs by the calcium phosphate method to assess expression levels of HCV nonstructural proteins. In brief, cell lysates were prepared in modified RIPA buffer (0.15 M NaCl, 1% Nonidet P-40, 50 mM Tris, 0.5% deoxycholate, and 1% SDS) after metabolic labeling with [35S]methionine and cysteine for 4 h. Cell lysates were precleared with horse serum and subsequently bound to Sepharose A by preincubation overnight with polyclonal antiserum to WU 110 (NS3), WU 148/151 (NS4), and WU 115 (NS5) (12). After separating the proteins by SDS-PAGE, the gels were dried and exposed. NS5 protein expression was also determined by Western blot and immunofluorescence analysis using a murine mAb (Biogenesis, Sandown, NH). To generate stably transfected cell lines expressing NS3, NS4, and NS5, the syngenic BALB/c mouse myeloma derived cell line SP2/0 was transfected by electroporation with pcDNA3-NS3, pcDNA3-NS4, or pcDNA3.1/Zeo(−)-NS5. Cells growing in selection medium were cloned by limiting dilution (0.3 cell/well) and screened by the methods described above. However, attempts to clone stable NS4-expressing cell lines were unsuccessful.

Immunization protocol

Female BALB/c (H-2b) mice were maintained under standard pathogen-free conditions in the animal facility of the Massachusetts General Hospital. Mice were obtained from Charles River Laboratories (Wilmington, MA) and used at the age of 6 to 20 wk for the in vivo studies. A total of 100 μg of plasmid DNA in 100 μl of 0.9% NaCl was injected two and three times over five different sites into the quadriceps muscle of the mice. Booster injections were given into the opposite leg every 14 days. As a positive control for all immunologic experiments, 5μg of recombinant proteins overnight at 4°C (0.5×10^6) were incubated with recombinant HCV core or HBsAg proteins at the same concentrations. Cells were stimulated with recombinant HCV core or HBsAg proteins were harvested and washed in serum-free medium; a 4 h [3H]thymidine release assay was performed in 96-well round-bottom plates (total volume of 140 μl) using [3H]-labeled SP2/NS3-3 or SP2/NS5-21. These cells (1 × 10^6) were incubated for 1 h with 100 μl of 51Cr (1 mCi/ml) and subsequently washed three times in DMEM containing 10% FCS (4°C). Parental SP2/0 or SP2/19 cells expressing the HCV core protein were used as controls for Ag specificity of lysis and background activity. Assays for CTL activity were performed at lymphocyte E:T ratios of 100:1, 30:1, 10:1, and 3:1, respectively, using 1 × 10^6 51Cr-labeled target cells/well. All depletion experiments were conducted by incubating effector cells with either an anti-CD4+ or CD8+ mAb containing hybridoma supernatant (GK1.5 anti-CD4, rat or 3.155 anti-CD8, rat) for 30 min at 4°C; next, the cells were washed and then incubated at 37°C with complement (1/5 dilution of low-toxicity rabbit complement; Cedarlane Laboratories, Hornby, Canada) before performing the CTL assay described above.

Assessment of CTL activity in vivo

Mice were immunized i.m. three times with either Mock DNA or pApNS5 vector. Some animals were also immunized i.p with recombinant NS5 protein or a combination of both. Recombinant proteins (5 μg i.p.) were administered as a mixture of NS5-4 (aa 2622–2806) and NS5-12 (aa 2007–2268). At 1 wk after the last immunization with the various plasmid constructs or recombinant protein, 2 × 10^6 syngenic SP2/0-derived cells stably expressing NS5 were washed, resuspended in 200 μl PBS, and inoculated s.c. into the right flank. SP2/0 cells that stably expressed HCV core protein (SP2/19) were used as a control in selected animals. Tumor formation was assessed at 15 days postinoculation, and the number of animals with tumors and tumor weight was determined.

Results

Expression of HCV nonstructural proteins in mammalian cells

HCV is a positive-strand RNA virus with a genome length of ~9.5 kb. One large ORF encodes for a polyprotein precursor of ~3000 aa that is processed by a combination of host and viral proteases into ≥10 different structural and nonstructural proteins (12–14). We cloned the genes encoding for the individual nonstructural proteins with engineered start and stop codons into an expression plasmid driven by a CMV promoter and a Rous sarcoma virus enhancer (pAp031). The expression vector pcDNA3 containing a neomycin selection marker was also used to generate stable SP2/0-derived cell lines (Fig. 1a). The plasmid constructs were sequenced across the cDNA inserts, and protein expression was analyzed in vitro in HuH-7 cells after transient transfection and in SP2/0 target cells after stable transfection, respectively. Signals corresponding to proteins with molecular masses of ~70 kDa for NS3, 30 kDa for NS4, and 125 kDa for NS5 were observed in cellular lysates but not in supernatant from transfected cells (Fig. 1b).

Humoral immune responses

Specific Ab responses directed against all three nonstructural proteins were found in all animals by ELISA following three immunizations. No Ag-specific immune responses were detected in mice immunized with mock DNA (Fig. 2a). As positive controls, mice were vaccinated three times i.p. with recombinant NS3, NS4, and NS5 proteins in combination with CFA; as expected, the mice demonstrated a strong humoral immune response (data not shown).

Cellular immune responses

To investigate cell-mediated immune responses to the nonstructural proteins, spleen cells were harvested and restimulated either...
with recombinant Ag or with Ag expressed by stably transfected cell lines in vitro. Substantial lymphocyte proliferation was induced by all nonstructural proteins at different Ag concentrations as measured by \[^{3}H\]thymidine incorporation (Fig. 2b). Immunization with recombinant Ag i.p. as a means of generating maximum stimulation produced a 5- to 10-fold higher lymphocyte proliferative rate for all three proteins (data not shown). The cytokine profile determined after DNA-based immunization demonstrated a classic Th1 response, with high levels of IFN-\(\gamma\) (Fig. 2c) and IL-2 (Fig. 2d) secreted into the cell culture supernatant. The cytokine release after incubation with recombinant NS3 could only be studied at a concentration of 0.1 \(\mu\)g/ml, since higher concentrations of NS3 (1 \(\mu\)g/ml) were toxic to the cells. In contrast, very little IL-4 production was observed after genetic immunization with genes encoding for the HCV nonstructural proteins (Fig. 2e).

Because CTL responses are essential to eliminate virus from infected cells, we studied the ability of splenocytes derived from immunized mice to lyse syngenic SP2/0 murine myeloma target cells stably expressing NS3 and NS5 proteins in a \(^{51}\)Cr release assay. The NS3- and NS5-immunized mice exhibited a specific

**FIGURE 1.** Expression of nonstructural proteins following transient transfection of HuH-7 and stable transfection of SP2/0 cells. a, a single large ORF of HCV encodes for a polyprotein precursor of \(\sim 3011–3030\) aa that is cleaved by host signal and virus proteases into the different structural and nonstructural proteins (arrows). Gene sequences of NS3, NS4, and NS5 were PCR-amplified, inserted into pcDNA3 or pcDNA3.1(−), and sequenced. b, lanes 1–6: After transient transfection of HuH-7 cells with these constructs and controlling for transfection efficiency with a \(\beta\)-galactosidase assay, cells were starved for 30 min in methionine and cysteine-free medium and labeled for 4 h with \([^{35}\text{S}]\)methionine and cysteine. Cell lysates were immunoprecipitated with polyclonal rabbit sera specific for the nonstructural proteins, captured by Sepharose A beads, and analyzed by SDS-PAGE followed by autoradiography. Lanes 1, 3, and 5 are mock DNA-transfected cells and serve as negative controls (Mock). Lanes 2, 4, and 6 show specific bands of \(\sim 70\) kDa for NS3, \(\sim 30\) kDa for NS4, and 125 kDa for NS5. Lanes 7–10: SP2/0 cells were transfected with pcDNA3-based constructs containing the genes for NS3, NS4, and NS5. After antibiotic selection, cells were cloned by limiting dilution (0.3 cells/well), and expanded and analyzed either by radioactive labeling and immunoprecipitation for NS3 or by Western blot for NS5 as described above. Lanes 7 and 9 represent cell lysates derived from cells stably expressing HCV core protein as a negative control (SP2/19); Lanes 8 and 10 indicate a specific expression of NS3 and NS5. These cells were used for in vitro stimulation and as target cells in the CTL assays.
cytotoxic T cell response after 5 days of in vitro stimulation, whereas low activity was observed against SP2/0 or SP2/19 (stably expressing HCV core protein) cells used as controls for target cell specificity (Fig. 3, a and b). To demonstrate the phenotype of cells producing the specific lysis, splenocytes were incubated with CD8<sup>+</sup>- or CD4<sup>+</sup>-specific mAbs in the presence of complement. These studies revealed that the cytotoxic activity was mediated by CD8<sup>+</sup> cells (Fig. 3c). We were unable to establish SP2/0 cell lines stably expressing NS4 protein; therefore, CTL activity was not measured against this HCV nonstructural protein.

In vivo CTL activity was assessed by a tumor model. Only 40% of mice immunized with a cDNA encoding for NS5 protein and challenged with a syngenic murine myeloma cell line (SP2/NS5-21) stably expressing NS5 developed tumors after 15 days. Moreover, tumor size was significantly less (p < 0.0001) as determined by the measurement of tumor weight when compared with mice immunized with mock DNA or recombinant NS5 protein or mice immunized with the same syngenic SP2/0 cell line expressing a different HCV structural protein (HCV core) as a control (Fig. 4, a and b). Indeed, 90 to 100% of mice immunized with mock DNA or challenged with SP2/0 cells demonstrated tumor formation, confirming the specificity of the CTL activity in this tumor model. It is important to emphasize that immunization with recombinant NS5 protein in CFA did not protect animals against tumor formation. To assess the effect of a combination of DNA-based immunization and recombinant protein vaccination, one group of animals was immunized with both. There was partial but significant (p < 0.03) protection against tumor formation, but combined immunization was not as effective as immunization three times with a DNA construct encoding for NS5 protein alone (Fig. 4a).

**Discussion**

During active HCV infection, humoral and cellular immune responses have been shown to be polyclonal and multispecific. It is likely that the host immune response produced during persistent
HCV infection is responsible, in part, for production of the liver cell injury. However, it may not be sufficiently broad-based or vigorous enough to promote viral clearance and generate protective immunity in individuals with chronic HCV infection (15). Individuals who have recovered from acute HCV infection have recently been shown to develop strong proliferative CD4 T cell responses directed against the nonstructural proteins (6, 7). More important, the generation of HCV-specific CTL activity appears to be associated with control of viral replication in individuals with chronic hepatitis (16, 17).

It is not known whether the nonstructural proteins NS3, NS4, and NS5 are sufficiently immunogenic to generate broad-based and vigorous CTL responses in vivo. The genetic immunization approach was employed to test this hypothesis, since this technique has been shown previously to induce cellular immune responses of different levels against a variety of pathogens in animal model systems (9–11, 18, 19). The advantage of this method compared with immunizations with soluble recombinant proteins or peptides is its ability to induce a more Th1-like immune response with the production of inflammatory CD4+ T cell as well as cytotoxic T cell activity, presumably due to the intracellular processing of viral proteins into peptides and subsequent loading onto MHC class I molecules in transfected muscle cells as well as to yet to be defined interactions of the complex with APCs. In contrast, immunization with soluble protein primarily leads to a humoral immune response due to processing through the MHC class II pathway. Immunization with synthetic peptides has several disadvantages, since only a limited number of epitopes are available for stimulation of the host immune response. In contrast, all naturally occurring B and T cell epitopes encoded for each protein by the DNA construct of interest are presumably preserved for recognition by TCRs and consequently will generate very broad-based humoral and cellular immune responses (20).

During active viral replication, HCV has a very high mutation rate, and several genotypes and subtypes have been described previously (13, 14). In this regard, the Ags are processed intracellularly in infected hepatocytes, and a large number of epitopes are presented to the immune system. However, neutralizing Abs generated against the envelope region of HCV have been found to be insufficient to provide protection and tend to promote immunoselection of quasispecies (21). In this study, we present evidence that DNA-based vaccination with plasmids encoding for three different nonstructural proteins of HCV is capable of eliciting Ag-specific immune responses in both effector pathways of the immune system. It was noteworthy that all animals developed detectable Ab responses after three immunizations. In this regard,
these nonstructural proteins are far better Ags to stimulate humoral immune responses compared with previous studies by us using the HCV core structural protein (22, 23). Similar to the findings of HCV core, the humoral immune response to the NS3 protein was weak; therefore, it may be necessary to activate APCs by the co-administration of cytokine-expressing plasmids such as IL-2 and granulocyte macrophage CSF to achieve optimal humoral and cellular immune responses, (23, 24). Nevertheless, the generation of inflammatory CD4\(^+\) T cell responses with a predominant Th1 phenotype was demonstrated for all three plasmids encoding for NS3, NS4, and NS5. Most important, a specific CD8\(^+\) CTL response was generated for NS3 and NS5 with lysis values that have been shown previously to induce protection against a variety of pathogens in animal model systems (18, 19). It was not possible to measure CTL responses to NS4, since we were unable to establish stable NS4-expressing SP2/0 myeloma cell lines. However, CD4 T cell responses and IL-2 and IFN-\(\gamma\) release were in the range observed for NS3 and NS5, and NS4 appears to be an attractive candidate protein for this immunization approach as well. Since no small animal model is currently available for HCV infection, we determined whether the CTL responses generated by DNA-based immunization would protect animals against tumor formation using syngenic SP2/0 tumor cells stably transfected with a cDNA encoding for NS5 protein. Approximately 60% of mice were protected against tumor formation, indicating the in vivo CTL activity produced by this immunization approach. Moreover, tumor weight in those animals that developed tumors was significantly reduced compared with mice immunized with mock DNA or recombinant NS5 protein. This study emphasizes the capability of assessing cellular immune responses against HCV nonstructural proteins in an animal model as measured by inhibition of tumor growth. It should now be possible to determine the fine specificity of CTL epitopes with overlapping peptides using these techniques.

In contrast to the data presented here, DNA immunization using a construct encoding for the HCV core structural protein produced less vigorous cellular and humoral immune responses (22, 23, 25). The envelope region has great sequence diversity among the various genotypes and may not be a good target region because of immunoselection of viral variants known to occur during natural viral infection (22, 25, 26). The NS3 gene encodes for a serine protease that cleaves the viral polyprotein precursor posttranslationally at several junctions and also serves as the viral helicase. The NS5 region encodes for the RNA-dependent RNA polymerase of the virus. Both genomic regions are believed to be highly important and critical for viral replication; therefore, these regions may serve as important molecular targets for antiviral approaches (27–29). Based on both previous clinical studies, which demonstrate the importance of the cellular immune response to the nonstructural proteins with respect to preventing persistent viral infection in humans (6, 7), and the experimental results presented here, which demonstrate that the nonstructural proteins are particularly potent candidates in generating cellular immune responses in mice, we are led to believe that DNA-based immunization with genes encoding for the HCV nonstructural proteins is an attractive approach for the construction of therapeutic and prophylactic vaccines against HCV. However, the clinical efficacy of DNA-based immunization in generating antiviral immune responses against
HCV in humans remains to be established. Finally, it will be important in the future to determine whether different genotypes or subtypes of HCV may circumvent the immune responses induced by one genotype following DNA-based immunization.

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