Immune Responses to Plasmid DNA Encoding the Hepatitis C Virus Core Protein

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Received 13 March 1995/Accepted 1 June 1995

Hepatitis C virus (HCV) is a major causative agent of parenterally transmitted non-A, non-B hepatitis. The genomic region encoding the virion-associated core protein is relatively conserved among HCV strains. To generate a DNA vaccine capable of expressing the HCV core protein, the genomic region encoding amino acid residues 1 to 191 of the HCV-1 strain was amplified and cloned into an eukaryotic expression vector. Intramuscular inoculation of recombinant plasmid DNA into BALB/c mice (*H-2^d*) generated HCV core-specific antibody responses, lymphoproliferative responses, and cytotoxic T-lymphocyte activity. Our results suggest that the HCV core polynucleotide warrants further investigation as a potential vaccine against HCV infection.

Hepatitis C virus (HCV) has been identified as the major causative agent of parenterally transmitted non-A, non-B hepatitis (3). It accounts for a large portion of community-acquired acute and chronic non-A, non-B viral hepatitis and is associated with liver cirrhosis and hepatocellular carcinoma (1, 3). Approximately 1% of the world's population (greater than 50 million people) is chronically infected. Chronic hepatitis is the ninth leading cause of death in the United States, and recent estimates suggest that HCV contributes to over 40% of these deaths (11).

HCV is classified as a member of the family *Flaviviridae*. The HCV genome is a linear positive-strand RNA molecule of ~9,500 bp and encodes a polyprotein precursor of ~3,000 amino acids (3). The genomic region encoding the HCV core protein (~21 kDa) is located at the N terminus of the polyprotein precursor and is believed to be cleaved by a signal peptidase located in the lumen of the endoplasmic reticulum (9). In contrast to the putative envelope glycoproteins, the virion-associated core protein shows greater sequence conservation among HCV strains (10). Cytotoxic activity against the HCV core protein has been observed in the liver-infiltrating lymphocytes of patients with HCV-associated chronic hepatitis (14). Immunization of BALB/c mice with recombinant vaccinia virus expressing HCV structural proteins has been shown to generate cytotoxic activity in spleen cells (21). A highly conserved antigenic site (amino acid residues 129 to 144) of the HCV core protein recognized by both murine and human cytotoxic T lymphocytes (CTL) was identified by those investigators. The murine epitope was further mapped to a decapeptide (amino acid residues 133 to 142) presented by class I major histocompatibility molecules (*H-2D^d*) to conventional CD4⁺ CD8⁺ CTL.

Polynucleotide vaccines have recently been shown to induce both humoral and cellular immune responses against a number of infectious agents, including influenza virus, rabies virus, human immunodeficiency virus, bovine herpesvirus 1, and malaria (4, 5, 8, 19, 20, 22–24, 26). Following intramuscular inoc-

ulation, DNA is taken up by muscle cells with subsequent endogenous expression of the gene products (25). On the basis of these previous studies, we investigated the role of DNA immunization in the generation of immune responses to HCV. Because the core protein is the most conserved HCV structural protein, we evaluated the immunogenic potential of recombinant plasmid DNA encoding the HCV core protein in BALB/c mice. Our results demonstrate that this strategy of DNA immunization generates HCV core-specific antibody responses, lymphoproliferative responses, and CTL activity.

Cloning and analysis of the HCV core gene product. A partial cDNA clone (Blue4/C5p-1) of strain HCV-1 containing the 5' untranslated region, C, E1, E2, and a portion of the NS2 region was used as a template for amplification of the core region (amino acids 1 to 191) by PCR. Two synthetic oligonucleotide primers, sense (GTGCTTGCGAATTCCCCGGGA) and antisense (CGTGGGAATTCGCACTTAGTAGG), containing *EcoRI* restriction enzyme sites were used for PCR amplification by a procedure similar to that previously described (12). The amplified DNA was inserted into the *EcoRI* site of the pcDNA3 mammalian expression vector (Invitrogen, San Diego, Calif.). HCV core protein expression by the recombinant pcDNA3 construct (pcDNA3-HCVcore) was verified by *in vitro* translation by using the TNT coupled reticulocyte lysate system (Promega Corp., Madison, Wis.). A major translation product of ~22 kDa was obtained from the recombinant core plasmid DNA and corresponded to the appropriate size of the HCV core protein (data not shown).

Immunization of mice. Female 5- to 8-week-old BALB/c (*H-2^d*) mice were procured from Jackson Laboratory (Bar Harbor, Maine). To test the HCV core polynucleotide vaccine, each mouse was immunized with 200 µg of recombinant (pcDNA3-HCVcore) or control plasmid DNA (pcDNA3) per injection intramuscularly in the thigh. Mice were immunized twice at 0 and 2 weeks or three times at 0, 2, and 4 weeks. Plasmid DNA was injected into the right thigh muscle at 0 and 4 weeks and into the left thigh muscle at 2 weeks. Six weeks after the first immunization, mice were sacrificed, and sera and spleen cells were collected to study the immune responses generated against the HCV core protein. Recombinant vaccinia virus (6) expressing the HCV structural proteins C, E1, and E2, and a portion of NS2 (vv/HCV₁₋₉₆₇) and recombinant

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FIG. 1. Detection of antibody responses to the HCV core protein in experimental mice by ELISA. Pooled sera (1:50 dilution) from each group of 12 mice immunized two or three times with DNA encoding the HCV core protein (pcDNA3-HCVcore) were incubated in wells coated with recombinant HCV core protein and detected by a goat anti-mouse secondary antibody (immunoglobulin) conjugated to alkaline phosphatase. The results are expressed as the optical densities (O.D.) at 405 nm for three independent experiments. Bars indicate standard errors. Antibody responses in a similar group of mice immunized three times with control DNA (pcDNA3) are also shown.

vaccinia virus expressing bacteriophage T7 RNA polymerase (vT7) (provided by Bernard Moss, National Institute of Allergy and Infectious Diseases, Bethesda, Md.) were used for comparison of immune responses. Mice were immunized intraperitoneally with 10^7 PFU of vaccinia virus per injection according to the immunization schedule described above.

Antibody response in immunized mice. Sera from immunized mice were pooled and tested for antibody responses to the HCV core protein by enzyme-linked immunosorbent assay (ELISA). BALB/3T3 cells stably transfected with pcDNA3-HCVcore and known to express the HCV core protein were freeze-thawed, homogenized, and sonicated. Cell extracts were used for coating ELISA plates, and antibody responses in the sera of immunized mice to the HCV core protein were analyzed by a procedure similar to that already described (17). Mice immunized with HCV core recombinant DNA developed increased antibody to the HCV core protein (Fig. 1). Additionally, 12 mouse sera were tested individually against purified recombinant HCV core protein (12) by immunoblot analysis. There was variable intensity of staining, but all sera obtained from recombinant DNA-immunized mice reacted with the recombinant HCV core protein (data not shown). Sera from mice immunized with control DNA did not react with the purified recombinant core protein in Western blot (immunoblot) analysis. However, antibody responses against HCV may not be important for protection. Antibodies to internal viral proteins such as the HCV core protein may not be able to bind to intact virions during infection. Moreover, the persistence of HCV despite antibodies reactive to the putative envelope glycoproteins (2, 18) has previously been observed in humans.

Isolate-specific neutralizing antibody responses (7) and serotypic variations among viral isolates have been suggested as important features of HCV (16). The high degree of genetic heterogeneity of HCV in vivo, manifested both in the generation of viral quasispecies and in the continuous emergence of neutralization escape mutants, poses an obstacle to the development of a broadly reactive HCV vaccine based on antibody reactivity to HCV envelope glycoproteins (7).

Lymphoproliferative responses in immunized mice. A synthetic decapeptide, LMGYIPLVGA (C7-A10), from the HCV core protein (amino acid residues 133 to 142), earlier shown to represent a murine T-cell epitope in BALB/c mice (21), was synthesized (Research Genetics, Huntsville, Ala.) and tested for in vitro stimulation of spleen cells from experimental mice. Spleen cells obtained 6 weeks after primary immunization of mice were added to 96-well flat-bottom plates (10^5 cells per well) and incubated in the presence of 1 or 10 μ M C7-A10 peptide at 37°C in 5% CO₂. After 4 days, cells were pulsed for 16 h with [³H]thymidine (Amersham Corp., Arlington Heights, Ill.) and harvested with a Skatron semiautomatic cell harvester (Skatron Instruments, Inc., Sterling, Va.). Lymphocyte stimulation indices (SI) were calculated by the following formula: SI = counts per minute measured after antigen stimulation/counts per minute after medium incubation alone. The results reported are the means of 12 values. The SI values for C7-A10 in spleen cells from HCV core DNA-immunized mice (7.5 to 10.5) were similar to those for cells harvested from vv/HCV₁₋₉₆₇-immunized mice (Fig. 2) and did not increase with a third booster immunization. SI values were found to be slightly higher when spleen cells were stimulated with 10 rather than 1

FIG. 2. Proliferative responses of splenic lymphocytes from immunized mice following in vitro stimulation with 10 μ M C7-A10 peptide for 5 days. Data are the mean SI $\pm$ standard errors for 12 experimental mice immunized with recombinant DNA encoding the HCV core protein (pcDNA3-HCVcore) and control DNA (pcDNA3). Proliferative responses in mice immunized with recombinant vaccinia virus expressing HCV structural proteins (vv/HCV₁₋₉₆₇) and recombinant vaccinia virus expressing bacteriophage T7 RNA polymerase (vT7) are also included for comparison.

μ M C7-A10 peptide (data not shown). Spleen cells from experimental and control animals showed similar background medium counts, as well as similar SI values after concanavalin A (Sigma Chemical Co., St. Louis, Mo.) stimulation. In general, proliferation assays predominantly measure CD4⁺ T-cell responses; therefore, our results may suggest that the C7-A10 peptide contains both CD4⁺ and CD8⁺ T-cell epitopes. Alternatively, because the peptide used in our assay binds to class I molecules on the surfaces of antigen-presenting cells (21), we may be measuring CD8⁺ T-cell proliferation. These possibilities need to be studied further.

CTL activity in immunized mice. Spleen cells from DNA-immunized mice were tested for CTL activity. For stimulation of CTL precursors in vitro, syngeneic spleen cells (2.5×10^6 /ml) were infected with vv/HCV₁₋₉₆₇ at a multiplicity of infection of 5 for 2 h at 37°C, washed five times, and inactivated by gamma irradiation (5,000 rads). Spleen cells (5×10^6 /ml) from pcDNA3-HCVcore- and DNA control-immunized mice were stimulated with recombinant vaccinia virus-infected syngeneic spleen cells for 7 days at 37°C in 5% CO₂ in complete T-cell medium (1:1 mixture of RPMI 1640 and Eagle-Hanks' amino acid medium containing 2 mM L-glutamine per ml, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml, 5×10^{-5} M 2-mercaptoethanol, and 10% fetal calf serum) and then used as the effectors in the CTL assay.

BALB/3T3 clone A31 murine fibroblast cells (*H-2^d*) and J774A.1 murine monocyte-macrophage cells (*H-2^d*) were obtained from the American Type Culture Collection (Rockville, Md.) and infected with vv/HCV₁₋₉₆₇ or vvT7 at a multiplicity of infection of 2 at 37°C in 5% CO₂ for 90 min. After three washes, cells were labeled with Na₂⁵¹CrO₄ (Amersham Corp.) at 37°C in 5% CO₂ for 2 h and used as the target cells (10^4 per well) in a chromium release assay. Additional studies were conducted by coating BALB/3T3 cells with C7-A10 peptide for use as target cells in a chromium release assay by a procedure similar to that described by Shirai et al. (21). Supernatants were harvested at 5 h, and percent cytotoxicity was calculated as $100 \times (\text{experimental release counts per minute} - \text{spontaneous release counts per minute}) / \text{maximum release counts per minute} - \text{spontaneous release counts per minute}$. Maximum ⁵¹Cr release was determined from supernatants of cells lysed by 50% Triton X-100. Spontaneous release was determined from the supernatant of target cells incubated without effector cells. Initially, the CTL activities of spleen cells were tested with different effector-to-target ratios. Representative data for titrations of 100:1, 50:1, and 25:1 effector-to-target ratios are shown in Fig. 3. These results demonstrated that the release of chromium from target cells was an effector-cell-dependent phenomenon, and marked antigen-specific cytotoxicity of targets expressing HCV core protein epitopes was observed in cultures containing 100:1 effector-to-target ratios. The results of three sets of experimental and negative control CTL assays are shown as the means of 12 values obtained with an effector-to-target ratio of 100:1 (Table 1). Effector cells from mice immunized with recombinant HCV core DNA induced approximately 26% lysis of vv/HCV₁₋₉₆₇-infected J774A.1 target cells and 22% lysis of vv/HCV₁₋₉₆₇-infected BALB/3T3 target cells. In contrast, when the same effector cells from mice immunized with recombinant HCV core DNA were incubated with negative control targets, only minimal levels of cytotoxicity were detected (6% lysis against vvT7-infected J774A.1 cells, 3% lysis against vvT7-infected BALB/3T3 cells, and 3% lysis against native BALB/3T3 cells). In addition, spleen cells harvested from mice immunized with control DNA induced only minimal lysis (~4%) of the targets expressing HCV core protein epitopes. When C7-A10 peptide-

FIG. 3. Cytolytic activities of splenic lymphocytes from mice immunized with recombinant DNA encoding the HCV core protein (pcDNA3-HCVcore) at different effector-to-target ratios. The spleen cells were stimulated with recombinant vaccinia virus (vv/HCV₁₋₉₆₇)-infected, gamma-irradiated syngeneic spleen cells and used as the effectors against vv/HCV₁₋₉₆₇-infected, vvT7-infected, C7-A10-coated, and native BALB/3T3 target cells. Bars indicate standard errors.

coated BALB/3T3 cells were used as targets, spleen cells from mice immunized with HCV core DNA induced 29% specific lysis. On the other hand, minimal lysis of C7-A10 peptide-coated BALB/3T3 cells was observed for spleen cells from mice immunized with control DNA. The results presented here clearly demonstrate that HCV core-specific memory CTL responses were induced in mice immunized with the polynucleotide vaccine.

Spleen cells showing CTL activity were further tested by immunomagnetic depletion of CD4⁺ or CD8⁺ lymphocytes with BioMag particles (PerSeptive Diagnostics, Cambridge, Mass.). Depletion of CD8⁺ cells resulted in reduced CTL activity (Fig. 4), whereas depletion of CD4⁺ cells did not change CTL activity. This observation is in agreement with an earlier report (21) that *H-2^d*-restricted CD4⁺ CD8⁺ CTL recognize the C7-A10 epitope of the HCV core protein presented by major histocompatibility complex class I molecules.

In this report, we have demonstrated that intramuscular inoculation of mice with recombinant DNA encoding the HCV core protein induces HCV core-specific humoral and cellular immune responses. At present, it is unclear whether CTL activity is important for protection against the establishment of HCV infection. HLA class I-restricted CTL specific for HCV core and envelope proteins have been identified in subjects with HCV-associated chronic hepatitis (13, 14), as well as in asymptomatic HCV-seropositive individuals (21). However, HCV-specific CTL do not appear to be sufficient to eliminate the virus once chronic infection has been established. Indeed, CD8⁺ major histocompatibility complex class I-restricted CTL recognizing epitopes of the NS3 protein persist for months in the livers of HCV-infected chimpanzees without resolution of viral infection (6). Whether the induction of HCV-specific CTL by vaccination prior to viral infection can provide protection is unknown.

TABLE 1. CTL responses to the HCV core protein in BALB/c (*H-2^d*) mice following immunization with polynucleotide vaccine

Polynucleotide vaccine ^a	% Lysis ^b					
	J774A.1-vv/HCV ₁₋₉₆₇	J774A.1-vvT7	BALB/3T3-vv/HCV ₁₋₉₆₇	BALB/3T3-vvT7	BALB/3T3-C7-A10	BALB/3T3
pcDNA3-HCVcore	26.4 (1.2)	5.8 (1.0)	22.2 (1.4)	3.1 (3.0)	29.4 (0.6)	2.6 (2.1)
DNA control (pcDNA3)	0.2 (0.7)	3.6 (2.8)	1.1 (3.1)	2.4 (1.7)	0.0 (0.4)	0.3 (4.5)

^a Spleen cells from DNA-immunized mice were stimulated for 7 days with vv/HCV₁₋₉₆₇-infected, gamma-irradiated syngenic spleen cells as described in the text.

^b Data shown (effector-to-target ratio of 100:1) are the means for 12 mice receiving three immunizations with the recombinant polynucleotide vaccine (pcDNA3-HCVcore) or DNA control (pcDNA3). The numbers in parentheses are standard errors. J774A.1 cells infected with recombinant vaccinia virus vv/HCV₁₋₉₆₇ and vvT7 were used as the experimental and negative control target cells, respectively. BALB/3T3 cells were either infected with recombinant vaccinia virus (vv/HCV₁₋₉₆₇) or coated with C7-A10 peptide for use as HCV core protein-specific target cells. BALB/3T3 cells alone or infected with an unrelated recombinant vaccinia virus (vvT7) were used as negative control target cells.

Vaccines designed to prevent chronic virus infections have proven to be a challenge and likely will require the employment of new strategies to succeed (15). For persistence, a virus must establish infection and evade eradication by host immune responses, including CTL. Prior immunization with a vaccine designed to induce CTL has been shown to confer complete protection against infection by lymphocytic choriomeningitis virus (15). This finding suggests that protection against viruses that cause persistence or latency is possible.

The potential use of DNA encoding viral protein antigens has been explored recently as a new approach for subunit vaccine development. Immunization of experimental animals with recombinant plasmid DNA encoding influenza virus proteins has been shown to elicit specific antibody and T-cell responses without triggering immune responses to the associated plasmid and has resulted in protection from subsequent challenge (8, 19, 23). DNA vaccines encoding human immunodeficiency virus type 1 envelope glycoprotein (24) and bovine herpesvirus 1 glycoproteins (5) induce neutralizing antibodies to the homologous viruses in experimental animals.

These studies indicate that DNA immunization can elicit protective immune responses and that it is a promising area for the development of subunit vaccines.

Our results showing DNA immunization stimulation of humoral and cellular immune responses to the HCV core protein, including CTL, are encouraging since the core protein is the most conserved of HCV structural proteins. To address the possible immunoprophylactic efficacy of recombinant plasmid DNA encoding the HCV core protein, further studies in an animal model permissive for HCV infection are needed.

We thank Tammy Grant for preparation of the manuscript.

This research was supported by grant NOI-AI-45250 from NIAID.

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FIG. 4. Representative CTL activity following depletion of spleen cells from mice immunized with recombinant DNA encoding the HCV core protein by using anti-murine CD4⁺ or CD8⁺ antibodies. The results shown were obtained at an effector-to-target ratio of 100:1 with vv/HCV₁₋₉₆₇-infected J774A.1 target cells. Bars indicate standard errors.

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