DNA Prime–Canarypox Boost with Polycistronic Hepatitis C Virus (HCV) Genes Generates Potent Immune Responses to HCV Structural and Nonstructural Proteins

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DNA vaccination was employed to study immune responses to hepatitis C virus (HCV) proteins. As an immunizing strategy, we studied immune responses of BALB/c (H-2d) and C57BL/6 mice (H-2b) to HCV genes delivered intramuscularly as a polycistronic construct capsid/E1/E2/NS2/NS3 (pRC/C-NS3) encoding 5 structural and nonstructural proteins. We also evaluated canarypox virus containing the same HCV genes as a means for potentiating immune responses to naked DNA. Our results indicate that mice that received a polycistronic pRC/C-NS3 with canarypox booster had enhanced antibody and cellular responses to HCV proteins. Immunodominant CD8+ T cell responses to several HCV structural and nonstructural proteins, characterized by cytotoxicity and interferon (IFN)–γ production or IFN-γ production without significant cytotoxicity, were observed in both strains of mice. The combination of naked DNA with a nonreplicating canarypox booster encoding HCV polycistronic pRC/C-NS3 genes appears to diversify and enhance T cell responses to HCV proteins.

Hepatitis C virus (HCV) has a worldwide seroprevalence of 1%–3% and is responsible for most cases of non-A, non-B hepatitis. At least 50% of transfusion recipients with acute HCV infection develop chronic or recurrent liver disease that may progress to cirrhosis or hepatocellular carcinoma [1]. Unlike hepatitis B virus, in which plasma-derived or recombinant protein vaccines encoding the surface antigen evoke strong immune responses and protection, properties such as the high variability of genotypes and quasi species [2] make it unlikely that a recombinant protein–derived vaccine would be effective for protection against diverse strains of HCV. Cell-mediated immune responses such as CD8+ cytotoxic T lymphocytes (CTL) may play an important role [3–5] and are better correlated with protection against HCV [6].

To develop a vaccine against HCV, it is important to define a strategy that evokes strong immune responses to variable and conserved regions of the virus. Many CTL epitopes have been defined for all HCV gene products [7]. A polycistronic immunogen that includes CTL of many specificities, some of which are derived from the more conserved nonstructural proteins, may be reactive with most genotypes and quasi species. The multiplicity of epitopes targeted would reduce the effect of vaccine-induced escape variants. It is known that good CTL responses are generally not obtained with protein immunogens formulated with currently licensed adjuvants. CTL responses are, however, elicited well by DNA-based immunization and by pox virus vectors [8, 9].

Injection with plasmid DNA has been shown to be a novel means of immunization that induces antigen-specific immune responses through the delivery of nonreplicating transcription units, which drive the synthesis of specific foreign proteins within the inoculated host [10, 11]. The in vivo–synthesized viral protein can enter both the major histocompatibility complex (MHC) class I and class II antigen–processing pathways. Thus, DNA vaccines induce a broad range of immune responses, including antibodies, cytotoxic T cells, T cell proliferation, and protection against disease [9, 10, 12–14].

The HCV viral genome is a linear, single-positive-strand RNA, processed as a single open-reading frame, with subsequent cleavage into several structural and nonstructural proteins. The translation product is cleaved by host and viral proteases, resulting in at least 10 viral proteins, in the following order: NH2 capsid/E1/E2-p7/NS2/NS3/NS4A/NS4B/NS5A/NS5B/COOH [15]. Genetic immunization with individual coding regions of the core protein and E1 and of core, E1, and E2 together has revealed humoral immune responses to core, E1, and E2 in mice [16–20]; however specific cellular (cytotoxic) T lymphocyte responses were detected only in mice injected with constructs encoding the core [16, 20, 21]. DNA-based immunization has also been attempted with individual nonstructural
HCV genes that induced immune responses to HCV NS3, NS4, and NS5 proteins [22]. In other studies, immunization with peptides from HCV core and nonstructural RNA polymerase NS5 regions has revealed additional immunogenic peptides that, in some cases, appear to be cross-reactive and recognized by CTL in mice and humans [23, 24].

In this study, we compared the immunogenicity of a large polycistronic plasmid vector encoding HCV capsid/E1/E2/NS2/NS3 (pRC/C-NS3) delivered either as plasmid DNA, as recombinant canarypox (ALVAC) expressing the same HCV genes, or in combination, using an HCV DNA-prime and a canarypox boost. Cellular immune responses were studied by CTL induction and by interferon (IFN)-γ production by enzyme-linked immunospot (ELISPOT) assay. The frequency and specificity (Biowhittaker, Walkersville, MD) and were in sterile PBS after ethanol precipitation. Levels of endotoxin were with Qiagen endotoxin removal buffers. Plasmid DNA was resuspended with Qiagen Giga prep columns (Qiagen, Chatsworth, CA) and sequencing across the gene inserts, and expression was analyzed in vitro by transient transfections. In vitro expression of pRC/CMV-HCV plasmids was detected in tissue culture by transient transfections in COS-1 cells (American Type Culture Collection, Rockville, MD) with corresponding constructs. COS-1 cells at 2 x 10⁴ cells/well in 6-well plates were transfected with plasmid pRC/C-NS3 with lipofectin reagent (GIBCO BRL, Gaithersburg, MD) according to the manufacturer’s instructions. Control cells were transfected with pRC/ATG vector only. After a 5-h incubation with serum-free OPTI-MEM (GIBCO BRL) at 37°C, 5% CO₂, an equal volume of OPTI-MEM containing 20% fetal calf serum (FCS) was added. Cells were reincubated at 37°C for another 48 h and then washed with PBS and lysed with 100 µL of SDS gel-loading buffer/ well containing 100 mM Tris at pH 6.8, 100 mM dithiothreitol, 2% SDS, 10% glycerol, and bromophenol blue. The lysates were scraped into microcentrifuge tubes, boiled for 10 min, sheared by sonication, and centrifuged. The supernatant proteins were separated in 10%-12% SDS-polyacrylamide gel and transferred to PGC nitrocellulose membranes for Western blotting. The nitrocellulose membranes were soaked overnight at 4°C in a blocking buffer containing 5% nonfat milk and PBS. Ratios of 1:500-diluted rabbit anti-HCV NS3 or 1:500-diluted serum from an N3/93-infected blood donor, selected for a high titer of broadly reactive antibodies to HCV proteins, or monoclonal antibodies (MAbs) to individual E1 and E2 proteins were applied to the membrane and incubated at room temperature for 1 h, followed by incubation in 1:3000-diluted peroxidase-conjugated goat anti-rabbit IgG or goat anti-human or anti-mouse IgG (Amersham, Cleveland) for 1 h at room temperature. The blots were developed with ECL reagent (Amersham).

Poxvirus constructs. HCV genes from HCV-BK (genotype 1b) were inserted into 2 poxvirus vectors: ALVAC, a vaccine strain of canarypox [26], and the L-variant of WR, a laboratory strain of vaccinia virus [27, 28]. The ALVAC-based recombinants include HCV sequences encompassing capsid through NS3 (capsid/E1/E2/NS2/NS3), which were placed under the control of the synthetic vaccinia early/late H6 promoter [29]. This expression cassette was inserted into a canarypox donor plasmid flanked by genomic sequences from which a nonessential gene was specifically deleted. Recombination between the donor plasmid and the ALVAC virus resulted in the recombinant ALVAC-capsid/E1/E2/NS2/NS3. This recombinant virus was used to stimulate mouse splenocyte effector cells and also as a booster for DNA-based immunization.

The vaccinia (WR)-based recombinants were constructed for use in CTL assays and were generated for each of the HCV genes capsid, E1, E2, NS2, and NS3. The capsid gene was expressed under the control of the vaccinia H6 promoter. The remaining genes were expressed under the control of an early entomopox promoter. Expression cassettes were inserted immediately downstream from the KIL host range gene [30] in the WR strain of vaccinia virus. The inserts were resequenced and were found to correspond to the sequence of the parental clones.

HCV protein expression by these recombinant pox viruses was determined by SDS-PAGE and by Western blots 2 days after infection of COS-1 cells.

Plasmid DNA immunization. Groups of 20–24 BALB/c and...
C57BL/6 mice were injected intramuscularly with 100 µg of each pRC/C-NS3 in a 50-µL volume in both quadriceps. Mice were boosted with the same amount of DNA at 4 and 8 weeks after priming. At week 12, half of the animals in each group were boosted intravenously (iv) with 5 × 10^7 pfu of ALVAC (canarypox vector) containing the C-NS3 genes. Control mice were injected with pRC/ C (not containing a gene insert) in saline and likewise received iv 5 × 10^7 pfu of ALVAC parent construct lacking the HCV genes. Splenocytes for CTL and ELISPOT assays were recovered from mice at 4 and 8 weeks after the ALVAC boost.

Assessment of humoral immune responses. Serum obtained by retro-orbital bleeding was tested at weeks 4, 8, 12, and 16 after immunization for antibody responses to recombinant HCV antigens, using the Ortho HCV 3.0 ELISA adapted for this study by use of goat anti-mouse IgG (BRL) instead of anti-human IgG. Mouse serum from unimmunized animals and mice immunized with empty-plasmid vector pRC/C served as controls. Seroconversion and antibody titers were evaluated using serial dilutions of serum samples. The cutoff for seroconversion was defined as the mean optical density (OD) ± 3 SD for 5 serum samples obtained from mice immunized with control plasmid DNA (pRC/C) lacking the HCV gene.

Preparation of effector cells. Single-cell spleen cells were isolated mechanically between the ground glass surface of 2 slides and by lysing red blood cells with 1.66% ammonium chloride. All cells were cultured at 37°C in 5% CO_2 for 5 days in complete tissue culture medium (TCM: RPMI 1640 supplemented with 10% (v/v) FCS, 1.5 mM L-glutamine, 50 µM 2-ME, 100 U/mL penicillin, and 100 U/mL streptomycin). Spleen cells from DNA-vaccinated BALB/c mice (5 × 10^7/mL) were restimulated in vitro with irradiated (3000 rads) syngeneic spleen cell stimulators (10^6/mL) after pulsing these for 1 h at 37°C with ALVAC (C-NS3) construct at an MOI of 10. Cultures were set up in 24-well plates, and interleukin-2 was not added in this assay.

Preparation of stimulator/target cells. Exponentially growing P815 (H-2^d) mastocytoma cells were infected with vaccinia (WR) virus constructs encoding individual HCV capsid, E1, E2, NS2, NS3 and polycistronic C-NS3 genes at an MOI of 10 for 1 h at 37°C in infection media (RPMI containing 2% FCS). Control P815 target cells were likewise infected with vaccinia virus parent (WR-P) construct not encoding any HCV gene and vaccinia expressing β-gal (WR β-Gal). The cells were incubated overnight for 16 h and labeled for 1 h with 100 μG of [51Cr]NaCrO_4/10^6 P815 cells. Cells were washed 3 times with RPMI 1640 and used as target cells at 5 × 10^4/mL. One hundred microliters of labeled targets was added to each well of a U-bottom 96-well plate. Effector cells (100 µL) were added to the target cells in triplicate wells at various effector-to-target (E:T) ratios. For each target, 6 replicate wells with medium alone and 6 with 5% Triton-X were included for spontaneous release and total release, respectively. The plates were spun for 5 min at 149 g in a bench-top centrifuge and were incubated at 37°C for 6 h. Supernatants (100 µL) from each well were counted in a 1250 Microbeta gamma counter (Wallac, Gaithersburg, MD). Percentage of specific cytotoxicity was calculated as [(average experimental ^51Cr release – spontaneous ^51Cr release)/(average total ^51Cr release – spontaneous ^51Cr release)] × 100. The ^51Cr release assays were performed in parallel with the ELISPOT assays to serve as a control for virus-specific killing.

determination of IFN-γ-secreting cells and cell phenotype by ELISPOT and FACS analysis. The ELISPOT assay was set up as described earlier [31], with a few modifications. In brief, in vitro-stimulated spleen cells (10^7 – 5 × 10^8) were cultured in 100 µL of TCM in triplicate wells in nitrocellulose-bottomed Millititer HA plates (Millipore, Bedford, MA) precoated with 75 µL of a 10 µg/mL monoclonal rat anti-mouse IFN-γ antibody (clone XMG1.2, Pharmingen). Vaccinia virus–infected P815 stimulator cells (10^6) were added after irradiation (10,000 rads). Plates were incubated overnight at 37°C and then washed and incubated with 100 µL of a 5 µg/mL solution of biotin-labeled rat anti-mouse IFN-γ antibody for 2 h. The plates were washed and incubated at room temperature with streptavidin–alkaline phosphatase (1:800) for 15 min. After additional washings, 100 µL of enzyme-substrate solution (3,3-diaminobenzidine-tetrahydrochloride dihydrate) was added, and spots were allowed to develop for 10–15 min. The color reaction was stopped by washing the plates with distilled water. After drying, the spots were counted under low magnification (×40) by using a stereo microscope. Antigen-induced responses were considered positive if the number of spots was greater than the mean ± 3 SD of those in wells stimulated with vaccinia parent for each individual animal group. Statistical significance was determined using Student’s t test.

For determining cell phenotype of IFN-γ-producing cells, in vitro–stimulated effector spleen cells were incubated for 16 h with irradiated (10,000 rads) P815 tumor cells after infection with vaccinia virus constructs encoding HCV proteins. Golgi Plug (Phar- mingen, San Diego, CA) was added during the last 6 h of incubation, to concentrate the levels of intracellular cytokines. The cells were then washed and stained for cell surface CD4– or CD8–fluorescein isothiocyanate (FITC; 1 µg/million cells; Phar- mingen), fixed, permeabilized, and stained for intracellular IFN-γ–PE (0.25 µg; PE-XMG1.2), by use of the Cytofix/Cytoperm Plus kit (Pharmingen) according to the manufacturer’s instructions. Cells were analyzed in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). The quadrant markers for the bivariate dot plots were set on the basis of the negative staining controls, using isotype-matched immunoglobulin controls (FITC-rat IgG2a and PE-rat IgG1, Pharmingen).

Results

Expression of HCV structural proteins after cell transfection. Cell lines were transfected with plasmid pRC/C-NS3 encoding HCV capsid, E1, E2, NS2, and NS3. Antigen expression in transiently transfected cells was determined by immunoblots of cell lysates from transfected COS-1 cells that were probed with sera or MAbs reactive to HCV proteins. Figure 1A shows the detection of the 18–21-kDa capsid protein in cells transfected with plasmids encoding C-NS3. The expression of NS3 in plasmid pRC/C-NS3 was detected as a band of 70-kDa protein (figure 1A). These bands were present only in the recombinant plasmid lanes and not in the control plasmid lanes (pRC/ATG). Although expression of E1 and E2 proteins was not detectable by Western blot in pRC/C-NS3–transfected
COS-1 cells (data not shown), immune responses against these proteins were generated after immunization, as described below.

COS-1 cells that were infected with vaccinia (WR) and canarypox (ALVAC)-encoding HCV genes expressed a 21-kDa band in cells infected with WR/Cap, WR/C-NS3, and ALVAC/C-NS3. In WR- and ALVAC/C-NS3-infected cells, we observed bands of molecular mass of 30 and 70 kDa, representing glycosylated E1 and E2 proteins, respectively. A band of 70-kDa protein was observed in WR/NS3-, WR/C-NS3-, and ALVAC/C-NS3-infected cells (figure 1B).

**Efficacy of priming with plasmid DNA construct HCV pRC/C-NS3 and boosting with recombinant canarypox (ALVAC C-NS3) in the potentiation of immune responses.** Groups of BALB/c and C57BL/6 mice were immunized intramuscularly with polycistronic pRC/C-NS3. Mice were boosted iv with canarypox 4 weeks after the last DNA immunization. Immune

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**Figure 1.**  
*Panel A.* Expression of hepatitis C virus (HCV) proteins, using recombinant plasmids. Plasmid capsid/E1/E2/NS2/NS3 (pRC/C-NS3) were transfected into COS-1 cells with lipofectin reagent (GIBCO BRL, Gaithersburg, MD). Cell lysates were harvested 48 h later, separated by SDS-PAGE, and analyzed by immunoblotting. The plasmids and monoclonal antibodies (MAbs) used for immunoblotting are indicated above the gel. The positions of the HCV capsid and NS3 are indicated by arrows on the right, and molecular weights (in kilodaltons) are indicated on the left.  
*Panel B.* Expression of HCV proteins in recombinant canarypox viruses. COS-1 cells were seeded into 35-mm petri dishes and infected with recombinant vaccinia (WR) or canarypox virus (ALVAC) containing individual (capsid, NS3) or polycistronic (C-NS3) HCV genes at 10 pfu/cell. Control wells were likewise infected with WR (parent) or ALVAC (parent) not containing any HCV genes. After a 16-h infection, the COS-1 cells were lysed with 1 × SDS loading buffer, separated by 12% SDS-PAGE, and analyzed for HCV proteins by immunoblotting. The recombinant viruses and MAbs used are indicated above the gel. The positions of HCV capsid, E1, E2, and NS3 are indicated by arrows at the right, and the molecular weights (in kilodaltons) are indicated on the left.
seroconversion rates (80% for BALB/C and 60% for C57BL/6 mice) were increased with canarypox boosting. The antibody responses shown are the total IgG responses to recombinant HCV proteins.

HCV-specific cell-mediated immune responses. CD8+ T cell responses induced by DNA/canarypox immunization regimens were monitored using IFN-γ ELISPOT and 51Cr-release assays. The protective relevance of the highly sensitive ELISPOT assay is supported by the observation that, during certain viral infections, IFN-γ can inhibit viral replication in vivo in the absence of CTL [3]. In both assays, we used vaccinia virus–encoded individual HCV genes to infect target cells to study CD8+ T cell responses to endogenously processed HCV proteins. Vaccinia vectors are considered more useful than peptides, because the vector permits production of the whole protein (figure 1B) and therefore can elicit responses to many peptides and to all possible haplotypes.

IFN-γ secretion and effector cell phenotype. In both the BALB/c and C57BL/6 strains of mice (table 1), immunization with plasmid DNA construct–encoding HCV pRC/C-NS3 genes induced significant IFN-γ production in response to stimulator cells infected with vaccinia-encoded HCV C-NS3 proteins, as compared with control mice that received empty-plasmid DNA vector (pRC/C). The IFN-γ response was further potentiated in both strains after recombinant canarypox ALVAC (C-NS3) boosting. On dissection to examine responses to individual HCV proteins, maximal IFN-γ secretion was observed to envelope glycoprotein E1 in BALB/c mice (90,680 ± 3302/10⁶ cells) by effector T cells (table 1). IFN-γ secretion was also observed in response to PB15 cells expressing HCV capsid (1120 ± 38/10⁶ cells), E2 (1680 ± 27/10⁶ cells), NS2 (505 ± 7/10⁶ cells), and NS3 (1117 ± 33/10⁶ cells) proteins (table 1). The IFN-γ ELISPOT responses in C57BL/6 mice after immunization with pRC/C-NS3 were obtained to HCV proteins capsid, E1, E2, NS2, and NS3 (table 1). Maximal IFN-γ secretion was observed in response to HCV NS2 (81,820 ± 3530/10⁶ cells) and to NS3 (88,320 ± 9900/10⁶ cells) after ALVAC HCV C-NS3 boosting. Recombinant canarypox boosting also enhanced the number of spot-forming cells >3-fold for

responses were determined 4 and 8 weeks after the canarypox boost.

In vivo HCV-specific humoral antibody responses. As shown in figure 2, specific antibodies to HCV recombinant antigens were detected in the serum of DNA-immunized mice. In both BALB/c and C57BL/6 mice immunized with pRC/C-NS3 plasmid DNA, however, antibody responses to HCV proteins were detected only after canarypox (ALVAC C-NS3) boosting. No HCV-specific antibodies were detected in mice vaccinated with control empty-plasmid DNA vector (figure 2) or in mice immunized with ALVAC (C-NS3) alone in the absence of DNA immunization (data not shown). The greatest OD values were obtained in most responding mice 4 weeks after receiving ALVAC (C-NS3) boost. Both the level of antibody responses and seroconversion rates (80% for BALB/C and 60% for C57BL/6 mice) were increased with canarypox boosting. The antibody responses shown are the total IgG responses to recombinant HCV proteins.

**Figure 2.** Antibody responses in BALB/c and C57BL/6 mice immunized with DNA-canarypox constructs encoding hepatitis C virus (HCV) capsid/E1/E2/NS2/NS3. Anti-HCV humoral antibody responses in immunized mice (BALB/c [serum diluted 1:60] and C57BL/6 [serum diluted 1:120]) 4 weeks after ALVAC HCV (C-NS3) boost, as determined by Ortho HCV ELISA in pooled mouse serum.

Table 1. Interferon (IFN)-γ enzyme-linked immunospot responses in mice immunized with hepatitis C virus (HCV) plasmid DNA with and without canarypox (ALVAC) boost.

<table>
<thead>
<tr>
<th>Mouse strain, immunized group</th>
<th>Capsid</th>
<th>E1</th>
<th>E2</th>
<th>NS2</th>
<th>NS3</th>
<th>C-NS3</th>
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<tr>
<td>BALB/c</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>HCV (pRC/C-NS3)</td>
<td>113 ± 9</td>
<td>8524 ± 17</td>
<td>105 ± 7</td>
<td>ND</td>
<td>20 ± 28</td>
<td>9933 ± 28</td>
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<tr>
<td>HCV (pRC/C-NS3) + ALVAC (C-NS3)</td>
<td>1120 ± 38</td>
<td>90,680 ± 3302</td>
<td>1680 ± 27</td>
<td>505 ± 7</td>
<td>1117 ± 33</td>
<td>118,680 ± 2628</td>
</tr>
<tr>
<td>pRC/control DNA</td>
<td>77 ± 9</td>
<td>67 ± 42</td>
<td>22 ± 10</td>
<td>94 ± 5</td>
<td>8 ± 5</td>
<td>31 ± 18</td>
</tr>
<tr>
<td>pRC/control DNA + ALVAC (parent)</td>
<td>6 ± 3</td>
<td>22 ± 5</td>
<td>ND</td>
<td>70 ± 0</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>C57BL/6</td>
<td></td>
<td></td>
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<tr>
<td>HCV (pRC/C-NS3)</td>
<td>795 ± 21</td>
<td>4010 ± 35</td>
<td>1285 ± 148</td>
<td>5760 ± 566</td>
<td>74,460 ± 6360</td>
<td>119,460 ± 8750</td>
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<td>HCV (pRC/C-NS3) + ALVAC (C-NS3)</td>
<td>2720 ± 141</td>
<td>13,570 ± 353</td>
<td>11,070 ± 212</td>
<td>81,320 ± 3530</td>
<td>88,320 ± 9900</td>
<td>196,320 ± 12,720</td>
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<tr>
<td>pRC/control DNA</td>
<td>10 ± 6</td>
<td>9 ± 12</td>
<td>12 ± 8</td>
<td>38 ± 6</td>
<td>11 ± 2</td>
<td>44 ± 12</td>
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<tr>
<td>pRC/control DNA + ALVAC (parent)</td>
<td>ND</td>
<td>15 ± 6</td>
<td>8 ± 3</td>
<td>17 ± 4</td>
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<td>14 ± 10</td>
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*NOTE.* ND, none detected.

*No. of cells after stimulation with stimulator cells infected with vaccinia-encoded HCV proteins.*
also compared the efficacy of both priming and boosting with amounts of recombinant IFN-γ.

HCV capsid protein (795–2720/10^6 cells) and E1 envelope glycoprotein (1285–11,070/10^6 cells) in HCV DNA-primed mice (table 1). Control target cells infected with vaccinia parent (WR-P) induced <100 IFN-γ spots and have been subtracted from the WR-encoded HCV protein analysis in each group. Vaccinia expressing β-Gal likewise induced low-level IFN-γ production (125 ± 13/10^6 cells).

The phenotype of the IFN-γ-secreting cells was determined by intracellular cytokine staining and flow cytometry. CD8^+ T cells secreted IFN-γ in response to stimulator cells expressing HCV C-NS3 proteins (figure 3B) and, predominantly, in response to E1 envelope glycoprotein of HCV (figure 3D) in BALB/c mice. No CD4, IFN-γ-secreting cells were observed (data not shown). The intracellular staining for IFN-γ was highly specific and could be blocked by competition with large amounts of recombinant IFN-γ (data not shown).

As a control for the DNA prime–pox boost approach, we also compared the efficacy of both priming and boosting with recombinant canarypox vector encoding HCV C-NS3 proteins for the generation of CTL and IFN-γ immune responses in BALB/c mice. Compared with DNA and DNA-pox immunization with C-NS3 genes, weak IFN-γ ELISPOT responses to all HCV proteins were observed (table 2). The envelope glycoprotein E1 ELISPOT response by canarypox immunization was 1105 ± 85 IFN-γ-secreting cells, versus 8525 ± 17 obtained by plasmid DNA immunization and 90,680 ± 3302 obtained by DNA prime–canarypox boost (table 1).

Overall, the ELISPOT was found to be a sensitive assay for characterizing CD8^+ T cell activation to naturally processed viral epitopes and consequent IFN-γ production, because fewer cells were required and more quantitative results could be obtained. The possibility of concomitant activation of both CD4 and CD8 T cells for the production of IFN-γ was prevented by using P815 mastocytoma (H-2d) and EL-4 (H-2b) cells, which express only class I but not class II MHC molecules.

**CTL responses.** The CTL assays were set up in parallel with the ELISPOT assays, using vaccinia-encoded HCV proteins. Several E:T ratios were studied. In BALB/c mice that received HCV plasmid DNA pRC/C-NS3 without canarypox boosting (HCV DNA prime and boost; figure 4A), the CTL activity was demonstrated only against the envelope E1 glycoprotein region (24% specific lysis at an E:T ratio of 50:1). Mice that received the HCV pRC/C-NS3 construct and canarypox boost (DNA prime–canarypox boost; figure 4B) had higher and broader CTL specificities to HCV proteins (E1, 44%; E2, 22%; and NS2, 15% specific lysis at E:T ratio 50:1). Lysis of target cells expressing wild-type vaccinia (WR-P) was <5% (figure 4). Unlike IFN-γ responses, the CTL responses to HCV capsid (8% ± 1%) and NS3 (11% ± 3.6%) were not substantially enhanced after canarypox boosting.

As compared with BALB/c mice, the C57BL/6 (H-2d) mice showed stronger CTL responses to HCV structural and nonstructural proteins after immunization. CTL responses of 16.4% (E1), 32.6% (NS2), and 56.0% (NS3) were obtained in mice immunized with HCV plasmid DNA (HCV DNA prime and boost; figure 5A) at an E:T ratio of 50:1. Boosting with HCV recombinant canarypox (HCV DNA prime–canarypox boost; figure 5B) enhanced CTL responses to several HCV proteins (E2, 34.6%; NS2, 53.1%; and NS3, 62.4%). CTL responses to

<table>
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<th>Immunized group</th>
<th>No. of IFN-γ-secreting cells/10^6 spleen cells</th>
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<tr>
<td>Capsid</td>
<td>E1</td>
</tr>
<tr>
<td>Canarypox</td>
<td></td>
</tr>
<tr>
<td>HCV (C-NS3)</td>
<td>55 ± 14</td>
</tr>
<tr>
<td>Canarypox parent</td>
<td>30 ± 0</td>
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**Table 2.** Interferon (IFN-γ) enzyme-linked immunospot responses in BALB/c mice immunized with canarypox-encoding hepatitis C virus (HCV) genes.

NOTE. ND, none detected.

a No. of cells after stimulation with P815 cells infected with vaccinia-encoded HCV proteins.
Figure 4. Cytotoxic T lymphocyte (CTL) responses in BALB/c mice immunized with plasmid DNA construct encoding hepatitis C virus (HCV) capsid/E1/E2/NS2/NS3: effect of canarypox boost. BALB/c mice were immunized with HCV plasmid pRC/C-NS3 DNA. Half the animals were boosted with canarypox. CTL responses were analyzed 8 weeks after canarypox boost, by use of P815 target cells infected with vaccinia-encoded HCV proteins. A, HCV DNA prime and boost show specific CTL lysis by effector cells at different effector:target ratios from BALB/c mice after HCV DNA prime and boost against P815 (H-2d) target cells infected with vaccinia (WR)-encoded HCV gene products capsid, envelope E1, envelope E2, NS2, and NS3. B, HCV DNA prime–canarypox boost likewise shows specific lysis of target cells after HCV DNA prime–canarypox boost immunization.

HCV capsid (6.9%) and E1 envelope glycoprotein (16.4%) were not enhanced after canarypox boosting (figure 5). The observed CTL activity was determined to be mediated by CD8+ T cells, as indicated by inhibition of lysis after treatment of effector spleen cells with Lyt 2.2 (anti-CD8) antibodies. Treatment with anti-CD4 antibody (clone GK 1.5) did not result in a significant reduction in specific lysis (data not shown). The specificity of the CTL responses to HCV proteins was evidenced by lysis of target cells expressing HCV C-NS3 and lack of CTL responses to wild-type vaccinia (WR-P) and target cells expressing recombinant vaccinia virus encoding the β-Gal protein (figure 6). Lysis of Yac-1 cells was obtained in groups of mice that received HCV plasmid DNA and was enhanced in mice receiving canarypox boosters, providing evidence for NK cell activation in the immunized animals (figure 6). The increase in NK cell activity could be due to canarypox or the possibility that the 2 DNA plasmids, including pRC/control, contain immunostimulatory sequences.

Immunization of mice with recombinant canarypox expressing HCV C-NS3 proteins (in the absence of plasmid DNA) did not result in detectable immune responses, as evidenced by the absence of specific CTL lysis in groups of mice that received canarypox boosters in the absence of plasmid DNA (data not shown).

Figure 5. Cytotoxic T lymphocyte (CTL) responses in C57BL/6 mice immunized with hepatitis C virus (HCV) plasmid DNA encoding capsid/E1/E2/NS2/NS3 proteins: effect of canarypox boost. C57BL/6 mice were immunized with HCV plasmid pRC/C-NS3 DNA. Half the animals were boosted with canarypox. CTL responses were analyzed 8 weeks later. A, HCV DNA prime and boost show specific CTL lysis by effector cells at different effector:target ratios derived from C57BL/6 mice after HCV DNA prime and boost against EL-4 (H-2b) target cells infected with vaccinia (WR)-encoded HCV gene products capsid, envelope E1, envelope E2, NS2, and NS3. B, HCV DNA prime–canarypox boost likewise shows specific lysis of target cells after HCV DNA prime–canarypox boost immunization.
nor reveal any significant lysis (<10% specific lysis) to any of the HCV proteins, even after ≥1 boosts (data not shown). Similar results were obtained in mice immunized intraperitoneally with the recombinant canarypox constructs (data not shown).

Discussion

HCV is a highly heterogeneous virus, with CTL epitopes present within both conserved and variable regions of the genome [7, 32]. It is well recognized that CD8+ T lymphocytes mediate important effector mechanisms of immunity against intracellular pathogens such as viruses. These T cells recognize small peptides, which are presented on the surface of infected cells by class I MHC molecules [33]. DNA-based immunization, compared with immunization with soluble recombinant proteins, has the ability to induce strong T-helper cell responses as well as CD8+ CTL activity, presumably because of intracellular processing of viral peptides within muscle and antigen-presenting cells. In contrast to synthetic peptide immunization, in which only a limited number of epitopes can be used, all naturally occurring T-helper and CTL epitopes of each encoded protein are available for recognition by T cells [10].

One issue in vaccine development, particularly for HCV vaccines, is whether several antigens can be combined in the same vaccine, thus achieving better protection. The potential to design a vaccine encompassing several epitopes may be a major advantage of genetic immunization. As an immunizing strategy, we studied humoral and cellular responses to HCV genes delivered in a polycistronic construct encoding 5 structural and nonstructural HCV antigens: capsid/E1/E2/NS2/NS3. As a part of this strategy, we also evaluated canarypox virus containing the HCV genes as a means for further potentiating immune responses to naked DNA. The generation of protective cell-mediated responses by DNA prime and poxvirus booster immunizations has precedence in studies of mice that used the modified vaccinia virus Ankara [34] and studies of macaques that used recombinant fowlpox virus [35]. Inoculation of these highly attenuated [36] and efficacious poxvirus-based vectors has shown successful immunization against many viruses, including human immunodeficiency virus type 1, by inducing strong antibody and memory CD8+ CTL responses [8, 37] or by other as yet undefined mechanisms.

Our results indicate that, although HCV-specific cellular immune responses are induced by naked polycistronic HCV capsid/E1/E2/NS2/NS3 plasmid DNA, the inclusion of canarypox booster potentiates both the antibody- and cell-mediated immune responses to HCV antigens in both the BALB/c and C57BL/6 strains of mice (table 1; figures 4–6). Immune responses to several HCV structural and nonstructural proteins were induced in mice immunized with this approach, as compared with mice that received only plasmid DNA or only recombinant canarypox vectors expressing the same HCV genes. The immunodominance pattern of HCV protein recognition (suggested by strong CTL and IFN-γ production) differed in the 2 strains of mice. The envelope E1 glycoprotein dominated immune responses in BALB/c (H-2d) mice, and the nonstructural NS2 and NS3 proteins were predominantly recognized in C57BL/6 (H-2b) mice. The distinct mouse haplotypes, preferential use of different accessory cells for antigen presentation, and the regulatory activities of cytokines themselves may influence the observed immune responses.

Genetic immunization has been attempted with HCV genes in mice. Investigators have studied cellular and/or humoral immune responses to HCV nucleocapsid, envelope E2, or nonstructural NS3, NS4, and NS5 genes delivered alone [16, 17, 19, 22] or delivered with nucleocapsid as chimeric vectors after fusion with hepatitis B virus (HBV) surface antigens [18]. Attempts to combine HCV capsid and envelope proteins in the same construct [20] were shown to decrease both the rate and titer of antibody seroconversion. In these studies, only mice injected with plasmid DNA that included capsid showed CTL responses. Absence of cellular responses to envelope in that study [20] may be due to lack of a sensitive assay for measuring weak CTL activities, such as the ELISPOT assay.

In the present study, we performed CTL assays in parallel with the IFN-γ ELISPOT assays to serve as a control for virus-specific killing. It is interesting that T cell recognition of the naturally processed form of capsid in C57BL/6 mice, and of capsid and NS3 in BALB/c mice, was associated with IFN-γ release but with no direct or weak cytolytic activity, even after canarypox boosting (figures 4 and 5). Whether this denotes 2 functionally distinct T cell populations mediating different functions (IFN-γ production vs. CTL) is presently not known.
Epitopes of this phenotype, however, might be of interest, especially for immune therapy for chronic HCV infection by associating the benefit of localized lymphokine release with low or absent direct cytopathicity to infected hepatocytes. Several lines of evidence have suggested the important role of IFN-γ in the control of viral replication [38]. Passive transfer of class I restricted CTLs led to clearance of the expression of HBV antigens in HBV transgenic mice. The mechanism involved appeared to be nonlytic and mediated by IFN-γ and TNF-α [3].

The observation that DNA priming and canarypox boosting with multiple HCV capsid/E1/E2/NS2/NS3 genes enhance immune responses to structural (capsid and envelope) and nonstructural HCV proteins has important implications. It has been reported that, during natural HCV infection, structural proteins such as capsid and NS3 stimulate strong T cell responses that appear enhanced during viral clearance [39-41]. The protective efficacy of HCV envelope E1 and E2 glycoproteins has been evaluated by Choo et al. [42], who showed that chimpanzees immunized with these recombinant proteins were protected against a homologous HCV virus challenge. Humoral responses were observed to E2, but cross-protection against heterologous strains of HCV was not tested in this study. HCV envelope E1 glycoprotein recently was shown to be useful as a therapeutic vaccine for chronic HCV disease in chimpanzees [43]. Although it is likely that candidate vaccines against HCV will include the envelope gene product, it is clearly desirable that these vaccines elicit broad cross-reactive immunity capable of conferring protection against different strains and quasi species of HCV. It is encouraging, therefore, that the DNA prime–boost immunization approach with an HCV polystricronic construct encoding multiple HCV genes can stimulate immune responses to several HCV proteins, including the envelope glycoproteins.

The potency or magnitude of immune responses generated by HCV DNA prime–boost immunization is greater than that of HCV DNA prime–boost and greater than that of canarypox prime–boost. Immunization with recombinant canarypox encoding the same HCV genes alone did not stimulate significant cellular (table 2) or humoral (data not shown) immune responses in recipient mice, even after additional boosters. Our own experience with DNA vaccination in HBV has revealed efficacy in the DNA/canarypox–based immunotherapy for chronic infection (present authors’ unpublished data), and complete protection against challenge of newborn chimpanzees was induced by DNA-based immunization. Preliminary experiments in chimpanzees inoculated with mixtures of HCV pRC/capsid + pRC/NS3 DNA and canarypox constructs have revealed an increase in the CTL precursor frequency responses to HCV proteins included in the DNA constructs (authors’ unpublished data).

In summary, immunization with plasmid DNA and boosting with canarypox containing HCV capsid/E1/E2/NS2/NS3 genes induced cellular immune responses in mice to HCV structural and nonstructural proteins, including the envelope glycoproteins. The significance of these responses as protective immune responses against HCV infection needs to be assessed and is currently under study in chimpanzees.

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