DNA Immunization with Hepatitis C Virus (HCV) Polycistronic Genes or Immunization by HCV DNA Priming-Recombinant Canarypox Virus Boosting Induces Immune Responses and Protection from Recombinant HCV-Vaccinia Virus Infection in HLA-A2.1-Transgenic Mice

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We studied immune responses to hepatitis C virus (HCV) genes delivered as DNA encoding the entire HCV protein coding genome in two polycistronic plasmids encoding HCV capsid-E1-E2-NS3 and HCV NS3-NS4-NS5 in HLA-A2.1-transgenic mice. Immune responses to HCV DNA prime and recombinant canarypox virus boost were also studied with the above constructs. At 8 weeks after a canarypox virus boost, the DNA prime/canarypox virus boosting regimen induced potent cellular immune responses to HCV structural and nonstructural proteins on target cells expressing the HLA-A2.1 allele. High frequencies of gamma interferon-secreting cells, as detected by enzyme-linked immunospot assay, were obtained in response to several endogenously expressed HCV proteins. We also observed cytotoxic-T-lymphocyte reactivity in response to endogenously expressed HCV proteins in fresh spleen cells without in vitro expansion. Upon challenge with a recombinant vaccinia virus expressing HCV proteins at 2 months postimmunization, the HCV DNA prime/canarypox virus-immunized mice showed a complete reduction in vaccinia virus titers compared to HCV DNA prime/boost and mock-immunized controls. Immune responses were still detectable 4 months after canarypox virus boost in immunized mice. Interestingly, at 10 months postimmunization (8 months after canarypox virus boost), the protection in HCV DNA prime/boost-immunized mice against recombinant HCV-vaccinia virus challenge was higher than that observed in HCV DNA prime/canarypox virus boost-immunized mice.

About 80% of human hepatitis C virus (HCV) infections become chronic with lifelong viremia. It is estimated that there are more than 200 million chronically HCV-infected carriers in the world, 4 million of whom are in the United States (3). Treatment of the carrier state is at present not very satisfactory. HCV is a notoriously difficult target for immunization: it exists in numerous genotypes and within an infected host in numerous quasispecies (5). It is therefore desirable to develop a strategy that will induce strong immune responses to several variable and conserved regions of the virus.

Immunization against HCV will involve the generation of cell-mediated immunity to mediate killing or downregulation of infected cells (7, 11). An effective way of generating cell-mediated immunity is by DNA-based immunization. Numerous approaches are available for enhancing DNA-based immune responses, including the coadministration of cytokine expressing plasmids (9, 14), the use of immunostimulatory DNA sequences (15), the use of Salmonella vectors (32), and targeting of DNA to dendritic cells (33). In our previous studies with DNA-based immunization against HBV (18) and HCV (20), we found that the use of a recombinant poxvirus as a booster months after immunization with DNA produced a much stronger booster effect than that obtained when DNA was used as a booster. In the present study we evaluated plasmid DNA immunization with polycistronic gene constructs encoding the whole protein coding HCV genome in HLA-A2.1-transgenic mice. These transgenic mice contain a cross between β₂-microglobulin-transgenic mice on a B6 background with HLA-A2.1-transgenic mice, resulting in doubly transgenic mice. This model represents an attractive alternative for initial vaccine development studies. Several investigators (4, 8, 28) have found that these mice recognize the same HCV-derived peptides that are recognized by human HLA-A2.1-restricted cytotoxic T lymphocytes (CTL).

Immunization was attempted with HCV capsid-E1-E2-NS2-NS3 (C-NS3) and HCV NS3-NS4-NS5 (NS3-NS5) proteins delivered either as plasmid DNA or in combination with an HCV DNA-prime and a recombinant canarypox virus (ALVAC; expressing the same HCV genes) boost. Cellular immune responses were studied by CTL induction and by gamma interferon (IFN-γ) production by enzyme-linked immunospot (ELISPOT) assay. The frequency and specificity of CD8+ T-cell responses for individual HCV gene products were quantified by using recombinant vaccinia viruses expressing individual HCV genes to detect antigen-specific responses in HLA-A2.1-transgenic mice. We show that, compared to mice immunized with HCV whole gene plasmid DNA encoding the polyprotein C-NS3 and NS3-5 DNA prime/boost, immunization with HCV DNA prime/canarypox virus boost induces...
stronger cellular immune responses to HCV proteins. These responses were detectable in fresh spleen cells without in vitro stimulation. Challenge with recombinant vaccinia viruses expressing HCV proteins encoded by the HCV DNA vaccine also resulted in a decrease in vaccinia virus titers compared to titers in mock-immunized controls challenged with the same recombinant vaccinia viruses in the ovaries where vaccinia preferentially replicates. Interestingly, long-term followup of protection (i.e., at 8 months postimmunization) in these mice revealed that HCV DNA prime/boost-immunized mice showed stronger cell-mediated immunity and resistance to recombinant vaccinia virus challenge than that produced by DNA prime/canarypox virus boost immunization. These results have implications both for prophylactic and therapeutic vaccine design and for the control of virus replication.

**MATERIALS AND METHODS**

**Mice.** HLA-A2.1 mice containing the HLA-A2.1 transgene on a B6 background were developed at The Scripps Clinic by Linda Sherman. These mice were bred and housed in the New York Blood Center pathogen-free animal research facility. These mice express the a1 and a2 domains from the HLA-A2.1 molecule and the o3 domains from the murine H-2Kb molecule. All procedures with the animals were conducted in accordance with institutional animal care and use committee-approved protocols.

**Plasmid constructs for immunization.** HCV cDNA sequences were derived from pRC/2B, a plasmid containing the entire open reading frame of the HCV-BK strain (genotype 1b [17]) inserted into the vector pRC/CMV (Invitrogen). Partial HCV genes were cloned into expression vector pRC/ATG, which was generated by inserting a double-stranded oligonucleotide containing the Kozak ATG consensus sequence produced by annealing the oligonucleotides 5'-AGCTGCCACCATGTC-3' and 5'-GCGCGCCATGCGTGGAC-3' into the HincIII and NotI sites of the polylinker of pRC/CMV. To express HCV genes, each coding sequence was amplified by PCR and cloned into pRC/ATG by using the NotI and XbaI restriction sites. The constructs did not contain the HCV 5'-untranslated region. Recombinant plasmid DNA was prepared with Qiagen Gigaprep columns (Qiagen, Chatsworth, Calif.) and Qiagen endotoxin removal buffers. Plasmid DNA was resuspended in sterile phosphate-buffered saline after ethanol precipitation. The levels of endotoxin were determined by using the amoebocyte lysate QCL-1000 kit (BioWhittaker, Walkersville, Md.) to be <20 endotoxin units/mg of DNA.

**In vitro expression of plasmid constructs.** In order to ensure that plasmid DNA constructs were intact and functional, the plasmids were sequenced across the 3' domain of H-2Kb and the 2 domains from the HLA-A2.1 molecule.

**Poxvirus constructs.** The poxvirus constructs were provided by Virogenetics Corp. (now Aventis Pasteur). HCV genes from HCV-BK (genotype 1b) were inserted into two poxvirus vectors: ALVAC (a vaccine strain of canarypox [29]) and the L-variant of WR (a laboratory strain of vaccinia virus [19, 21]). The ALVAC-based recombinants include HCV sequences encompassing capsid through NS3 (i.e., capsid-E1-E2-NS2-NS3; hereafter referred to as C-NS3) or NS3 through NS5 (i.e., NS3-NS4-NS5; hereafter referred to as NS3-NS5). The sequences were placed under the control of the synthetic vaccinia virus early/late H6 promoter (22). These expression cassettes were inserted into a canarypox virus 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 C-NS3 and the recombinant ALVAC NS3-NS5. The recombinant viruses were used to stimulate mouse splenocyte effector cells and also as a booster for DNA-based immunization.

The vaccinia virus (WR)-based recombinants were constructed for use in CTL assays and were generated for each of the HCV genes: capsid, E1, E2, NS2, NS3, NS4, and NS5. The capsid gene was expressed under the control of the vaccinia virus H6 promoter. Recombinant vaccinia viruses were also constructed expressing C-NS3 and NS3-NS5, with both inserts in both constructs under the control of the vaccinia virus H6 promoter. The remaining genes were expressed under the control of an early entomopoxvirus promoter. Expression cassettes were inserted immediately downstream from the KIL host range gene (10) in the WR strain of vaccinia virus. The inserts were resequenced and found to correspond to the sequence of the parental clones.

HCV protein expression by these recombinant poxviruses was determined and found to be positive by Western blots 2 days after infection of COS-1 cells (20).

**Tumor cell lines.** The tumor cell lines used included the following: EL-4 murine thymoma cells originally derived from C57BL/6 mice, EL-4 cells stably transfected with the A2.1/Kb chimeric gene (EA2Kb) or Jurkat human T-cell leukemia cells that are HLA-A2.1 negative, and stable transfectants of Jurkat cells expressing A2.1 or A2.1/Kb (a gift from Linda Sherman). EA2Kb cells are stable transfectants and express the product of the HLA-A'0201/Kb chimeric gene (the a1 and a2 domains from HLA-A'0201 and the o3 domain of H-2Kb [12]). EA2Kb cells were grown in RPMI 1640 containing 10% fetal calf serum (FCS), 4 mM l-glutamine, 5 × 10^-5 M 2-mercaptoethanol, and 50 μg of glutamine/ml supplemented with 20 μg of G418 sulfate/ml. The tumor cell lines were used as targets in the murine cytotoxic assays and as stimulator cells in the ELISPOT assays.

**Immunization.** Groups of 20 to 24 HLA-A2.1 mice were injected intramuscularly (i.m.) with 50 μg of each plasmid construct HCV C-NS3 (pRC/C-NS3) and HCV NS3-NS5 (pRC/NS3-NS5) in a 50-μl volume in both quadriceps. Mice were boosted with the same plasmids at 3 weeks postpriming. At week 6, half of the animals were boosted intravenously (i.v.) with 5 × 10^5 PFU each of ALVAC (canarypox virus vector) containing the C-NS3 and NS3-NS5 genes (Fig. 1). Control mice were injected with control plasmid DNA (pRC/C, not containing a gene insert) in saline and likewise received 5 × 10^5 PFU i.v. of the ALVAC parental construct lacking the HCV genes. Splenocytes for the CTL and ELISPOT assays were recovered from mice at 4, 8, 16, and 36 weeks after the final ALVAC boost.

**CTL assay.** (i) Preparation of effector cells. Effector cells were prepared as described before (19). Briefly, spleen cells were isolated mechanically between the ground glass surface of two slides and by lysing red blood cells with 1.66% ammonium chloride. Spleen cells from immunized mice (5 × 10^6/ml) were restimulated in vitro with irradiated (3,000 rads) syngeneic spleen cells (10^6/ml) after pulsing these for 1 h at 37°C with ALVAC C-NS3 and ALVAC NS3-NS5 constructs at a multiplicity of infection of 10. All cells were cultured in 24-well plates.
plates at 37°C in 5% CO₂ for 5 days in complete tissue culture medium (RPMI 1640 supplemented with 10% [vol/vol] FCS, 1.5 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U of penicillin/ml, and 100 U of streptomycin/ml).

(ii) Preparation of stimulator or target cells. Exponentially growing chimeric EA2Kb (H-2b), J2AKb, or human HLA-A2 cell lines were infected with vaccinia virus (WR) constructs encoding individual HCV capsid, E1, E2, NS3, NS4, NS5, or polycistronic C-NS3 or NS3-NS5 genes at a multiplicity of infection of 10 for 1 h at 37°C in infection medium (RPMI containing 2% FCS). Control target cells were likewise infected with vaccinia virus parent [WR (P)] construct not encoding any HCV gene and vaccinia virus expressing β-galactosidase (WR β-Gal). The cells were incubated overnight for 16 h and labeled for 1 h with 100 μCi of [3H]Na2CrO4, CrO3, or CrCl3. Cells were washed three times with RPMI 1640 and used as target cells at 5 × 10⁵/ml. Then, 100 μl of labeled targets was added to each well of a U-bottom 96-well plate. Effector cells (10⁵ cells in 100 μl) were added to the target cells in triplicate wells at various effector/target (E/T) ratios. For each target, six replicate wells with medium alone and with 5% Triton X-100 were included for spontaneous release and total release, respectively. The plates were spun for 5 min at 500 rpm in a benchtop centrifuge and incubated at 37°C for 5 h. Supernatants (100 μl) from each well were counted in a 1250 Microbeta gamma counter (Wallac, Gaithersburg, Md.). The percent specific cytotoxicity was calculated as follows: [(average experimental ⁵¹Cr release – spontaneous ⁵¹Cr release) / average total ⁵¹Cr release] × 100. The ⁵¹Cr release assays were performed in parallel with ELISPOT assays.

Blocking of CTL activity by antibodies. Hybridoma culture supernatants of BB7.2 (HLA-A2.1) or purified monoclonal anti-CD8 (anti-Lyt-2.2; BD Pharmingen, San Diego, Calif.) or anti-CD4 (OK1.5 hybridoma; Pharmingen, San Diego, Calif.) antibodies were added to the 96-well CTL assay plates to determine the effect of these antibodies on CTL activity.

Determination of IFN-γ-secreting cells by ELISPOT assay. The ELISPOT assay was set up as described previously (19) with a few modifications. Briefly, in the direct ex vivo assay and in the expanded ELISPOT assay, fresh (unstimulated) and in vitro-stimulated spleen cells (1 × 10⁵ to 5 × 10⁵), respectively, were cultured in 100 μl of complete tissue culture medium in triplicate wells (with nitrocellulose at the bottom of each well) in Millititer HA plates (Millipore Co., Bedford, Mass.) precoated with 75 μl of a 10-μg sample of monoclonal rat anti-mouse IFN-γ antibody (clone XMG1.2; Pharmingen/ml). Vaccinia virus-infected EA2Kb stimulator cells (10⁵) 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 (1:1000) for 15 min. After additional washings, 100 μl of enzyme-substrate solution (DAB; 3,3′-diaminobenzidine-tetrahydrochloride dihydrate) was added, and spots were allowed to develop for 10 to 15 min. The color reaction was stopped by washing the plates with distilled water. After they dried, the spots were counted under low magnification (×40) under a stereo microscope. Antibody-induced responses were considered positive if the number of spots was greater than the mean plus three standard deviations than in wells stimulated with vaccinia virus parent for each individual animal group.

Cytokine gene expression. Splenocytes of mice at 8 weeks after canarypox virus boost were stimulated with synergistic stimulator cells (ratio, 5:1) infected with vaccinia virus parent, recombinant vaccinia virus expressing HCV (C-NS3), recombinant vaccinia virus expressing β-Gal proteins. The splenocytes were analyzed for T-cell receptor (TCR) αβ, CD4, CD8, and IL-2, IFN-γ, and tumor necrosis factor alpha (TNF-α) cytokine mRNA gene expression by the RNase protection assay (Pharmingen) in accordance with the manufacturer’s instructions. Mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and L32 genes were used for normalization.

Protein study and plaque assay. At 8 weeks (2 months) and 36 weeks (8 months) after ALVAC boost, mice were challenged intraperitoneally (i.p.) with 10⁶ PFU of each of the recombinant vaccinia viruses expressing HCV C-NS3 [rVV-(C-NS3)] and HCV NS3-NS5 [rVV-(NS3-NS5)] or of control recombinant vaccinia virus expressing β-Gal [rVV-(β-Gal)]. Mice were sacrificed 5 days later. The ovaries (where vaccinia virus preferentially replicates in mice) were harvested, homogenized, sonicated, and assayed for recombinant vaccinia virus titers by plating 10-fold dilutions on BSC-40 indicator cells and staining them with 0.075% (wt/vol) crystal violet (1).

RESULTS

Immune responses to HCV plasmid DNA encoding multiple genes. We immunized HLA-A2.1 mice i.m. with mixtures of plasmids pRC/C-NS3 and pRC/NS3-NS5. Mice were boosted with plasmid DNA (i.m.) or with recombinant canarypox virus (i.v.) encoding the same HCV genes 3 weeks after the last DNA immunization (Fig. 1). Immune responses were determined at 4, 8, and 16 weeks after the last DNA or canarypox virus boost. The results are described below.

Analysis of cytokine gene expression. Mouse splenocytes were analyzed for TCR αβ, CD4, CD8, and for IL-2, IFN-γ, and TNF-α cytokine mRNA gene expression at 8 weeks after canarypox virus boost by the RNase protection assay. Splenocytes were stimulated with stimulator cells infected with recombinant vaccinia virus parent or recombinant vaccinia virus expressing HCV C-NS3 or recombinant vaccinia virus expressing β-Gal proteins. Mouse GAPDH and L32 genes were used for normalization. Mice immunized with HCV DNA prime/boost (lane 3) and HCV DNA prime/canarypox virus boost (lane 7) showed increased mRNA levels of IFN-γ gene expression after in vitro stimulation with target cells expressing HCV proteins. The increase in the levels of IFN-γ was specific to HCV and was not obtained with target cells expressing β-Gal protein (lanes 1 and 5) or target cells infected with recombinant vaccinia virus parent (lanes 2 and 6). Lanes 4 and 8 represent unstimulated splenocytes from HCV DNA prime/boost and HCV DNA prime/canarypox virus boost mice, respectively.

FIG. 2. Cytokine gene expression. Mouse splenocytes were analyzed for TCR αβ, CD4, and CD8 and for IL-2, IFN-γ, and TNF-α cytokine mRNA gene expression at 8 weeks after canarypox virus boost by the RNase protection assay. Splenocytes were stimulated with stimulator cells infected with recombinant vaccinia virus parent or recombinant vaccinia virus expressing HCV C-NS3 or recombinant vaccinia virus expressing β-Gal proteins. Mouse GAPDH and L32 genes were used for normalization. Mice immunized with HCV DNA prime/boost (lane 3) and HCV DNA prime/canarypox virus boost (lane 7) showed increased mRNA levels of IFN-γ gene expression after in vitro stimulation with target cells expressing HCV proteins. The increase in the levels of IFN-γ was specific to HCV and was not obtained with target cells expressing β-Gal protein (lanes 1 and 5) or target cells infected with recombinant vaccinia virus parent (lanes 2 and 6). Lanes 4 and 8 represent unstimulated splenocytes from HCV DNA prime/boost and HCV DNA prime/canarypox virus boost mice, respectively.
tested for their ability to recognize viral antigens presented by the A2.1 transgenic product. Vaccinia virus expressing individual HCV genes was used to infect target cells to study CD8+ T-cell responses to endogenously processed HCV proteins.

IFN-γ secretion by fresh splenocytes after DNA vaccination: canarypox virus-induced IFN-γ secretion in response to HCV proteins. At 8 weeks (2 months) after the canarypox virus boost, the immunization regime comprising HCV DNA prime/canarypox virus boost stimulated strong IFN-γ secretion in response to several HCV proteins encoded by the transgene product compared to mice that did not receive the canarypox virus boost. Table 1 shows (as determined by ELISPOT assay) direct ex vivo IFN-secreting T-cell responses to HCV capsid (5 ± 2/10⁶ cells), E1 (65 ± 6/10⁶ cells), E2 (22 ± 6/10⁶ cells), NS2 (283 ± 77/10⁶ cells), NS3 (2,588 ± 282/10⁶ cells), NS4 (20 ± 6/10⁶ cells), and NS5 (1,788 ± 21/10⁶ cells) proteins obtained from fresh splenocytes of DNA prime/canarypox virus-immunized A2.1 mice. The IFN-γ ELISPOT responses obtained in HCV DNA prime/boost mice were lower and could only be detected in response to HCV NS2 (2 ± 1/10⁶ cells) and NS3 (16 ± 1/10⁶ cells) proteins (Table 1). Stronger results were obtained in the in vitro expanded assay (Table 2).

Recall memory responses after DNA vaccination. Recall memory responses were studied after a 5-day in vitro expansion of mouse splenocytes with irradiated syngeneic cells expressing HCV antigens. Expansion resulted in an overall increase in IFN-γ-secreting cells to several HCV proteins. In mice immunized with the HCV DNA prime/canarypox virus boost, IFN-γ secretion in response to HCV C-NS3 was 76,345 ± 2,120/10⁶ cells, and in response to NS3-NS5 it was 58,345 ± 675 IFN-γ-secreting cells/10⁶ splenocytes. The IFN-γ responses to individual HCV proteins in this group was HCV E1 (10 ± 21/10⁶ cells), E2 (795 ± 170/10⁶ cells), NS2 (11,454 ± 707/10⁶ cells), NS3 (11,245 ± 258/10⁶ cells), and NS4 (25 ± 14/10⁶ cells). Mice immunized with HCV DNA prime/boost also showed an increase in IFN-γ secretion in response to HCV E2 (125 ± 21/10⁶ cells), NS2 (160 ± 84/10⁶ cells), and NS3 (1,930 ± 82/10⁶ cells) proteins. Overall, the IFN-γ secretion in response to HCV C-NS3 was 4,430 ± 282 spots/10⁶ cells, and it was 1,310 ± 99 spots/10⁶ cells in response to HCV NS3-NS5 antigens (Table 2). Control target cells infected with vaccinia virus parent [WR (P)] induced <100 IFN-γ spots, and these have been subtracted from the WR-encoded HCV protein analysis in each group. Vaccinia virus expressing β-Gal likewise induced low levels of IFN-γ production (125 ± 13/10⁶ cells).

We also performed ELISPOT experiments with untransfected Jurkat cells, Jurkat cells expressing HLA-A2.1, and Jurkat cells expressing A2Kb and CIRA2 stimulator cells. Our data showed the absence of an HCV-specific ELISPOT IFN-γ response with untransfected HLA-A2.1-negative Jurkat cells. A2Kb and CIRA2.1 stimulator cells that lack mouse class I molecules gave HCV-specific reactivity and were comparable to one another (Table 3). The slightly decreased reactivity seen with these cells versus the EA2Kb cells can possibly be explained by the ability of murine CD8 to react more effectively with the murine α3 domain of the A2.1/Kb molecule than with the human α3 of the natural human A2.1 molecule (12).

Long-term recall memory responses after DNA vaccination. ELISPOT immune responses were studied in mice at 10 months (8 months after a canarypox virus boost). In mice immunized with HCV DNA prime/boost, IFN-γ secretion in response to HCV C-NS3 was 5,445 ± 23/10⁶ cells, and in response to NS3-NS5 it was 4,505 ± 35 IFN-γ-secreting cells/10⁶ splenocytes. Mice immunized with the HCV DNA prime/canarypox virus boost, on the other hand, showed a slightly decreased level of IFN-γ secretion of 3,675 ± 14/10⁶ splenocytes in response to HCV C-NS3 and a level of 2,955 ± 14 IFN-γ-secreting cells/10⁶ splenocytes in response to NS3-NS5 (Table 4). As with previous experiments, control target cells infected with vaccinia virus parent [WR (P)] were subtracted from the WR-encoded HCV protein analysis in each group.

CTL responses: direct ex vivo cytotoxic activity in fresh and in vitro expanded splenocytes of HLA-A2.1 mice. The CTL assays were set up in parallel with the ELISPOT assays by using EA2Kb target cells infected with recombinant vaccinia virus-expressing HCV proteins. Several E:T ratios were studied. We detected CTL (42% specific lysis) in fresh unstimulated spleen cells in response to HCV NS3 protein in mice that received the HCV DNA prime/canarypox virus boost (Fig. 3B).

### Table 1. Direct ex vivo IFN-γ ELISPOT responses are enhanced in HLA-2.1-transgenic mice immunized with plasmids encoding pRC/HCV(C-NS3) plus pRC/HCV(NS3-NS5) genes 8 weeks after canarypox virus boost

<table>
<thead>
<tr>
<th>Immunized group</th>
<th>Capsid</th>
<th>E1</th>
<th>E2</th>
<th>NS2</th>
<th>NS3</th>
<th>NS4</th>
<th>NS5</th>
<th>C-NS3</th>
<th>NS3-NS5</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV DNA (C-NS3 + NS3-NS5)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2 ± 1</td>
<td>16 ± 1</td>
<td>ND</td>
<td>12 ± 1</td>
<td>26 ± 2</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>HCV DNA + ALVAC (C-NS3 + NS3-NS5)</td>
<td>5 ± 2</td>
<td>65 ± 6</td>
<td>22 ± 6</td>
<td>283 ± 77</td>
<td>2,588 ± 282</td>
<td>20 ± 6</td>
<td>1,788 ± 21</td>
<td>3,288 ± 212</td>
<td>738 ± 71</td>
</tr>
</tbody>
</table>

*That is, the number (mean ± standard deviation) of IFN-γ-secreting cells per 10⁶ fresh spleen cells after stimulation with EA2Kb cells infected with the indicated vaccinia virus-encoded HCV proteins. ND, none detected.

### Table 2. In vivo expanded IFN-γ ELISPOT responses are enhanced in HLA-2.1-transgenic mice immunized with plasmids encoding pRC/HCV(C-NS3) plus pRC/HCV(NS3-NS5) genes 8 weeks after canarypox virus boost

<table>
<thead>
<tr>
<th>Immunized group</th>
<th>E1</th>
<th>E2</th>
<th>NS2</th>
<th>NS3</th>
<th>NS4</th>
<th>NS5</th>
<th>C-NS3</th>
<th>NS3-NS5</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV DNA (C-NS3 + NS3-NS5)</td>
<td>ND</td>
<td>125 ± 21</td>
<td>160 ± 84</td>
<td>1,930 ± 82</td>
<td>ND</td>
<td>ND</td>
<td>4,430 ± 282</td>
<td>1,310 ± 99</td>
</tr>
<tr>
<td>HCV DNA + ALVAC (C-NS3 + NS3-NS5)</td>
<td>10 ± 21</td>
<td>795 ± 170</td>
<td>11,545 ± 707</td>
<td>11,245 ± 258</td>
<td>25 ± 14</td>
<td>ND</td>
<td>76,345 ± 2,120</td>
<td>58,345 ± 675</td>
</tr>
</tbody>
</table>

*See Table 1, footnote a.
The CTL responses increased in magnitude (NS3 specific lysis, 90%) and were also observed in response to HCV NS2 protein (51%) after in vitro expansion for 5 days at an E:T ratio of 50:1 (Fig. 4B). The CTL were specific to endogenously synthesized HCV proteins. In mice that received HCV plasmid DNA pRC/C-NS3 plus pRC/NS3-NS5 without canarypox virus boosting (HCV DNA prime and boost), the response of the CTL to the HCV NS3 region (63% specific lysis at E:T ratio of 50:1) was demonstrated only after in vitro expansion (Fig. 4A). In contrast to the IFN-γ responses, CTL responses to HCV capsid, NS4, and NS5 (=20%) were not substantially enhanced even after canarypox virus boosting.

**Activity of CTL from HLA-A2.1-transgenic mice is blocked by antibodies to CD8 and HLA-A2.1.** To clarify which molecules play an important role in lysis of target cells expressing HCV proteins and whether these CTL were restricted by the HLA-A2.1 molecule, we performed the CTL assay in the presence of anti-CD4, anti-CD8, or anti-HLA-A2.1 antibody in vitro. The observed CTL activity was determined to be mediated by CD8⁺ T cells, as indicated by the inhibition of lysis after the treatment of effector spleen cells with Lyt-2.2 (anti-CD8) antibodies (Fig. 5). Treatment with anti-CD4 antibody (clone GK1.5) did not result in a significant reduction in specific lysis (Fig. 5). The specificity of the CTL responses to HCV proteins was evidenced by lysis of the EA2Kb target cells expressing HCV C-NS3 and the lack of CTL responses to EA2Kb target cells infected with wild-type vaccinia virus [WR (P)] and target cells expressing recombinant vaccinia virus encoding the β-Gal protein (Fig. 5). These results indicate that the transgenic mice can generate HCV-specific HLA-A2.1-restricted CTL and ELISPOT responses, and a major portion of this response is directed toward the A2.1-restricted epitopes. The absence of specific lysis of untransfected Jurkat cells (HLA-A2.1 negative) indicated that a high level of specific lysis required the expression of A2.1/Kb molecules, as well as the specific HCV peptide.

**DNA vaccine confers protection against infection with recombinant vaccinia virus expressing HCV C-NS3 and HCV NS3-NS5 antigens.** To examine the efficacy of immunization in protection against virus infection, HLA-A2.1 mice immunized with HCV DNA prime/canarypox virus boost or with HCV DNA prime/boost were challenged with recombinant vaccinia virus expressing HCV whole gene proteins delivered as two separate recombinant vaccinia virus constructs. Mice were challenged i.p. with 10⁷ PFU each of recombinant HCV C-NS3 and HCV NS3-NS5 viruses at 2 and 8 months after canarypox virus boost. Mice were sacrificed 5 days later, and the ovaries were harvested for vaccinia virus plaque assay.

At 8 weeks postimmunization, mice immunized with HCV DNA prime/canarypox virus boost and challenged with recombinant vaccinia virus expressing HCV proteins (rHCV) revealed no rHCV vaccinia virus replication. This finding corresponded to at least an 8-log decrease in vaccinia virus titers compared to the same group of mice challenged with recombinant vaccinia virus expressing β-Gal. In mice immunized with DNA prime/boost, a 2-log decrease in rHCV vaccinia virus titers was obtained compared to mice challenged with recombinant vaccinia virus encoding β-Gal (Fig. 6).

At 8 months postimmunization, we observed a 3-log decrease in the rHCV vaccinia virus titer in ovaries of mice immunized with HCV DNA prime/canarypox virus boost compared to mock-immunized mice challenged with the same rHCV. Mice immunized with HCV DNA prime/boost, showed a 5-log decrease in vaccinia virus titers compared to mice challenged with recombinant vaccinia virus expressing β-Gal. Mice immunized with control or empty DNA (pRC/control) and canarypox virus (ALVAC parent) did not yield any significant decrease in recombinant HCV-vaccinia virus titers in their ovaries compared to the same group of mice challenged with recombinant vaccinia virus expressing β-Gal protein (Fig. 7).

**DISCUSSION**

DNA vaccines are receiving considerable attention for their ability to induce cellular immune responses. They have induced immune responses to, and in some cases even protected against, various types of infection, such as influenza and malaria (25, 27, 31). Cellular responses to HCV genes delivered as DNA constructs encoding structural and nonstructural HCV antigens, especially when combined with poxvirus-based boosting, have been shown to be an effective approach to inducing HLA class I-dependent cellular immune responses (18).

Because vaccine development in animal models depends on the ability to mimic human HLA-restricted T-cell responses, transgenic mice that express the intact human class I molecule HLA-A2.1 provide an important model system with which to examine both the ability to induce cellular immune responses. They have in-
In the present study, we investigated the potential for vaccination of HLA-A2.1 mice by using naked DNA encoding polycistronic HCV structural and nonstructural genes either by itself or when combined with boost with canarypox virus expressing the same HCV genes to generate CD8-specific responses. Since mice are refractory to challenge with HCV, we also investigated the potential of these vaccination regimes to protect against “challenge” with vaccinia virus expressing HCV proteins (1). The advantage of DNA-based vaccines is their ability to induce immune responses to endogenously synthesized gene products. This is important since the ability to kill targets expressing the HCV proteins is a critical parameter in the killing of virus-infected cells.

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FIG. 3. Direct ex vivo CTL responses in HLA-A2.1 mice immunized with plasmid DNA construct encoding HCV C-NS3 and HCV NS3-NS5: effect of canarypox virus boost. HLA-A2.1 mice were immunized with HCV plasmid pRC/C-NS3 and pRC/NS3-NS5 DNA. Half of the animals were boosted with canarypox virus. CTL responses with unstimulated spleen cells were analyzed 8 weeks after canarypox virus boost with EA2Kb target cells infected with vaccinia virus-encoded HCV proteins. Panel A (HCV DNA prime and boost) shows specific CTL lysis by effector cells at different E:T ratios from HLA-A2.1 mice after HCV DNA prime and boost against EA2Kb target cells infected with the vaccinia virus (WR)-encoded HCV gene products capsid, envelope E1, envelope E2, NS2, and NS3. Panel B (HCV DNA prime/canarypox virus boost) likewise shows the specific lysis of target cells after HCV DNA prime/canarypox virus boost immunization.

FIG. 4. In vitro-expanded CTL responses in HLA-A2.1 mice immunized with HCV plasmid DNA encoding C-NS3 and NS3-NS5 proteins: effect of canarypox virus boost. HLA-A2.1 mice were immunized with HCV plasmid pRC/C-NS3 and pRC/NS3-NS5 DNA. Half of the animals were boosted with canarypox virus. CTL responses were analyzed 8 weeks after a canarypox virus boost with in vitro-stimulated spleen cells with EA2Kb target cells infected with vaccinia virus-encoded HCV proteins. Panel A (HCV DNA prime and boost) shows specific CTL lysis by effector cells at different E:T ratios from HLA-A2.1 mice after HCV DNA prime and boost against EA2Kb target cells infected with the vaccinia virus (WR)-encoded HCV gene products capsid, envelope E1, envelope E2, NS2, and NS3. Panel B (HCV DNA prime/canarypox virus boost) likewise shows the specific lysis of target cells after HCV DNA prime/canarypox virus boost immunization.
since CD8<sup>+</sup> CTL are acknowledged as a major effector mechanism in the termination of viral infections. In HCV infections, HLA class I-restricted HCV-specific CD8<sup>+</sup> CTL are detectable in the liver infiltrating lymphocytes and, to a lesser extent, in peripheral blood. In keeping with our previous results in C57BL/6 and BALB/c mice (18), the HLA-A2.1 mice showed higher cellular immune responses to HCV proteins after immunization with HCV DNA prime/canarypox virus boost (Tables 1 to 3 and Fig. 3 and 4) compared to mice immunized with naked polycistronic HCV C-NS3 DNA alone. Detection of the functional CD8<sup>+</sup> CTL response usually requires in vitro re-stimulation to increase the precursor frequencies to detectable levels. After immunization with HCV DNA prime-canarypox virus boost regimen, the IFN-γ secretion responses to HCV capsid, E1, E2, NS2, NS3, NS4, and NS5 proteins were sufficiently high to be detectable in fresh, unstimulated splenocytes (Table 1). In the HCV DNA prime and DNA boost mice, IFN-γ secretion was detected in response to HCV NS2, NS3, and NS5 proteins, although to a lesser extent (Table 1). Direct ex vivo CTL response was also obtained to HCV NS3 protein in mice immunized with HCV DNA prime/canarypox virus boost regimen (Fig. 3). IFN-γ secretion in response to HCV E2, NS2, and NS3 proteins was still detectable in fresh, unstimulated splenocytes at 16 weeks postimmunization in HLA-A2.1 mice that received the HCV DNA prime/canarypox virus boost (data not shown). An immunodominant NS3 peptide, 1406-1415, containing the HLA-A2.1 motif (KLSGLGINAV) that induced strong IFN-γ secretion (but not CTL), was identified (data not shown). Direct ex vivo simian immunodeficiency virus-specific cytotoxicity has recently been reported in macaques vaccinated with a DNA prime/modified vaccinia virus Ankara boost regimen (2).

To test whether DNA vaccination induced memory CTL that were protective against infection of a peripheral organ, mice immunized with HCV DNA prime and boost and with HCV DNA prime/canarypox virus boost were challenged i.p. after 8 weeks with recombinant vaccinia virus expressing the HCV genes, and the virus titers were measured in the ovaries (1). The measurement of protection of a peripheral organ against recombinant vaccinia infection is likely to be a surrogate for protection against HCV challenge in primates. Anti-
viral protection by CTL against infection of solid, nonlymphoid tissue is more demanding, since the memory CTL need to be activated and have to home through solid tissue to the site of infection (liver in the case of HCV) to exert antiviral effector functions. Mice immunized by HCV DNA prime/canarypox virus boost were also protected from challenge with recombinant vaccinia virus expressing HCV C-NS3 and NS3-NS5 proteins. Immunization with HCV DNA encoding the same HCV genes alone stimulated lower cellular immune responses and protection from recombinant HCV vaccinia virus challenge (Fig. 6).

The generation of protective cell-mediated responses by DNA prime and canarypox virus booster immunizations has precedence in studies in mice infected with the modified vaccinia virus Ankara (26) and in macaques infected with recombinant fowlpox virus (24). Inoculation of these highly attenuated (30) poxvirus-based vectors has shown at least partially successful immunization against many viruses, including human immunodeficiency virus type 1, by inducing strong antibody and memory CD8+ CTL responses (8, 16, 23) or by other, as-yet-undefined mechanisms.
Surprisingly, when the HLA-A2.1 mice were challenged 8 months after a canarypox virus boost with recombinant vaccinia virus expressing HCV C-NS3 and HCV NS3-NS5, the mice immunized with HCV DNA prime and DNA boost showed greater resistance to recombinant vaccinia virus challenge compared to mice that received HCV DNA prime/canarypox virus boost (Fig. 7). Clearly, the superior protective efficacy obtained with the HCV DNA prime/canarypox virus boost mice at 8 weeks after canarypox virus boost was no longer apparent. It seems that the immune responses evoked by HCV DNA prime/canarypox virus boost appear early and are more robust soon after immunization but, in the present study, tended to fade faster long term (Table 4). Protective immune responses instead appear to be longer lasting in HLA-A2.1 HCV DNA prime/DNA boost mice. More studies are needed to address this issue, along with a careful evaluation of effector and memory immune responses 6 to 8 months postimmunization. Although the strength and breadth of a vaccine-induced CD8 T-cell response and proper assessment of these responses by assays that measure the levels of functionally active lymphocytes are critical to the development of an effective HCV vaccine, it seems logical that the two most important factors required to obtain potent, long-lived antiviral protective CTL memory are the induction of initial CTL responses with a large clonal burst size and/or to ensure that the responses can persist. These results have obvious implications for prophylactic and therapeutic immunization.

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REFERENCES


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