Bicistronic Woodchuck Hepatitis Virus Core and Gamma Interferon DNA Vaccine Can Protect from Hepatitis but Does Not Elicit Sterilizing Antiviral Immunity

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The immunity elicited against nucleocapsid of hepatitis B virus (HBV) and closely related woodchuck hepatitis virus (WHV) has been shown to be important in resolution of hepatitis and protection from infection. Further, activity of gamma interferon (IFN-γ), which may directly inhibit hepadnavirus replication, promotes antiviral defense and favors T helper cell type 1 (Th1) response, which is seemingly a prerequisite of HBV clearance. In this study, to enhance induction of protective immunity against hepadnavirus, healthy woodchucks were immunized with a bicistronic DNA vaccine carrying WHV core (WHc) and woodchuck IFN-γ (wIFN-γ) gene sequences. Three groups, each group containing three animals, were injected once or twice with 0.5 mg, 0.9 mg, or 1.5 mg per dose of this vaccine. In addition, four animals received two injections of 0.6 mg or 1 mg WHc DNA alone. All animals were challenged with WHV. The results showed that four of nine animals injected with the bicistronic vaccine and one of four immunized with WHc DNA became protected from serologically evident infection and hepatitis. This protection was not linked to induction of WHc antigen-specific antibodies or T-cell proliferative response and was not associated with enhanced transcription of Th1 cytokines or 2',5'-oligoadenylate synthetase. Strikingly, all animals protected from hepatitis became reactive to nucleocapsid of hepatitis B virus (HBV) and closely related woodchuck hepatitis virus (WHV). This study shows that the bicistronic DNA vaccine encoding both hepadnavirus core antigen and IFN-γ is highly effective in steering HBV-specific T-cell reactivity. It has been postulated on the basis of data from other microbial infections (2), as well as chimpanzee and woodchuck models of hepatitis B (21, 38), that induction of a prevailing Th1 response and IFN-γ should be advantageous in the control of HBV infection. In this context, it has been shown that IFN-γ is highly effective in steering T-cell response toward Th1 type (2). In addition, IFN-γ strongly enhances class I and II major histocompatibility complex (MHC) expression and hence augments antigen presentation by both endocytic and exocytic pathways (2, 17).

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immunocompromised patients, and infants born to HBV-infected mothers (64), and breakthrough infections have been reported (35). Also, the HBsAg-based vaccines have a very limited or no beneficial effect in patients with chronic hepatitis B, who normally have poor anti-HBV humoral and cellular immune responses (15, 27). It is of note that an attempt to augment an antibody response against WHV surface antigen (WHsAg) by inducing T helper type 2 cells in chronic WHV infection led to severe liver damage, particularly when high levels of antibodies to WHsAg (anti-WHs) were produced (23). This suggested that a similar approach to treatment of chronic hepatitis B would be futile.

The protective effect of immunization with HBV core antigen (HBcAg), an inner structural protein of the Dane particle, was first demonstrated in chimpanzees (26, 51, 52). After injections with liver-derived or a recombinant HBcAg, antibodies against HBcAg (anti-HBc) were induced and apparent complete protection from HBV was observed in some animals (26, 51). A similar outcome has been seen in woodchucks immunized with native WHV core antigen (WHcAg) or recombinant core protein prior to WHV challenge (59, 61). The immunization prevented emergence of serologically evident, i.e., serum WHsAg-reactive, infection in some woodchucks, and the results suggested that priming of a specific Th1-type response, rather than antibodies to WHcAg (anti-WHc), could be important in establishing protective immunity. However, the presence of virus and its replication status were not assessed after challenge of these seemingly protected animals using currently available nucleic acid amplification assays with high sensitivity.

Immunizations with DNA encoding selected structural or nonstructural viral proteins have been shown to induce specific humoral and cellular immune responses (reviewed in reference 16). This is because the protein encoded by the delivered DNA is de novo synthesized and can enter both class I and class II MHC presentation pathways and prime CD8⁺ and CD4⁺ T cells simultaneously. The immune responses elicited by DNA vaccination have been shown to be protective against pathogens in a variety of experimental systems (25, 33, 49, 62, 68, 69, 73). Moreover, it was uncovered that increased efficacy of DNA vaccination and skewing toward a preferable T helper cell response can be achieved by codelivery of plasmids encoding molecules acting as adjuvants, such as cytokines, chemokines, and costimulatory factors (4, 7, 28, 32).

A number of DNA immunization protocols have been tested with highly variable success in animal models of hepapnavirus infection (12, 31, 32, 49, 53, 55, 58, 60, 66, 67, 72). In woodchucks, to boost and to shift toward a preferable Th1 antiviral response, coadministration of plasmids encoding WHcAg and IFN-γ (63) or interleukin-12 (IL-12) (19), a cytokine promoting maturation of Th1 cells and production of IFN-γ, has been tested. As a result, it was shown that the protective immunity against WHV infection could be meaningfully enhanced by that induced by WHc DNA alone. However, protection was never achieved in all animals vaccinated, and it remained unclear whether the protection was complete, since the presence of virus was assessed by methods with relatively low sensitivity.

In the present study, in an attempt to enhance the effectiveness of the DNA vaccine encoding WHcAg by inducing simultaneous production of IFN-γ to prime the Th1 cell response and, at the same time, to fully control the codelivery of the antigen- and cytokine-encoding sequences, a bicistronic vector capable of concomitant expression of WHcAg and woodchuck IFN-γ (wIFN-γ) was constructed. This expression vector was investigated in parallel with the plasmid encoding WHcAg alone for the ability to mount complete (sterilizing) protection against the hepapnavirus. This was achieved by challenging the vaccinated woodchucks with a liver pathogenic dose of WHV containing 1.1 × 10^10 DNAase-protected virus genome equivalents (vge) and by ultrasensitive PCR-nucleic acid hybridization (PCR-NAH) assays to detect WHV genomes and their replication activity (45, 46). We also examined the abilities of the bicistronic plasmid to elicit WHcAg-specific humoral and T-cell immune responses and to upregulate expression of Th1 cytokines and IFN-γ in peripheral lymphoid cells and hepatic tissue. We have found that immunization with the bicistronic DNA was more effective than immunization with WHc DNA alone in preventing serum WHsAg-positive WHV infection. However, all vaccinated animals found to be free of the serologically detectable infection and hepatitis acquired low levels of replicating virus after challenge with a massive dose of WHV, indicating that the protection was not absolute. The findings reported here raise concern about the actual prophylactic potency of the vaccination with DNA encoding hepapnavirus internal protein. They indicate that this approach cannot protect the animal from acquiring serologically occult hepapnavirus carriage, of which one of the long-term potential consequences is development of hepatocellular carcinoma (46, 56). They also reveal that prevention against hepatitis and protection from infection are not synonymous in the case of hepapnavirus infection.

MATERIALS AND METHODS

Animals. Sixteen healthy, adult woodchucks housed in the Woodchuck Hepatitis Research Facility at Memorial University, St. John’s, Newfoundland, Canada, were used in this study. The possibility of prior exposure of the animals to WHV was excluded on the basis of negative serological results for WHV infection markers, i.e., WHsAg and anti-WHc, and the absence of WHV DNA tested by a nested PCR-NAH assay (sensitivity, ≤10 vge/ml) using DNA from randomly selected serum, peripheral blood mononuclear cell (PBMC), and liver biopsy samples, as reported previously (11, 45, 46). Animal experimental protocols were approved by the Institutional Presidents’ Committee on Animal Bioethics and Care.

Construction of pC-WHc and pWHc-wIFNγ expression vectors. Full-length WHV core (WHc) gene sequence (567 bp in length: GenBank accession number J02442) (18) was amplified by PCR and inserted into the EcoRI site of the pCI expression vectors. To express simultaneously and sequentially and directionally inserting into the pcDNA3.1 eukaryotic expression vector enzyme. Thus, the transcription of the WHc-IRES-wIFN-γ cassette was amplified with sense primer 5'-GCCGGATCCATGAAATACACAAGTT-GCGCGGATCCATGAAATACACAAGTT and antisense primer 5'-GCCGGATCCATTATTTTGGATGCTCTCGAC with incorporated BamHI and KpnI sites (shown underlined) using recombinant wIFN-γ as the template, which was previously generated in this laboratory (70). Encephalomyocarditis virus internal ribosome entry site (IRES) sequence was amplified from pIRE2-EGFP vector (Clontech, Palo Alto, CA) with sense primer 5'-GGTGGAGGCTTATATAAGCAGAGC and antisense primer 5'-GCCGGATCCATTATTTTGGATGCTCTCGAC with inserted BglII site. The final construct, designated pC-WHc, was confirmed by sequencing. To express simultaneously WHcAg and woodchuck IFN-γ, a bicistronic vector containing both WHc gene and full-length wIFN-γ DNA was constructed. Briefly, wIFN-γ DNA was amplified with sense primer 5’-GCCGGATCCATTATTTTGGATGCTCTCGAC with incorporated BamHI and KpnI sites (shown underlined) using recombinant wIFN-γ as the template, which was previously generated in this laboratory (70). Encephalomyocarditis virus internal ribosome entry site (IRES) sequence was amplified from pIRE2-EGFP vector (Clontech, Palo Alto, CA) with sense primer 5'-GGTGGAGGCTTATATAAGCAGAGC and antisense primer 5’-GCCGGATCCATTATTTTGGATGCTCTCGAC with inserted BglII site. The final construct, designated pC-wIFNγ, was generated by sequentially and directionally inserting into the pcDNA3.1 eukaryotic expression vector (Invitrogen, Carlsbad, CA) full-length wIFN-γ DNA, then IRES, and finally WHc sequence released from pC-WHc by digestion with EcoRI restriction enzyme. Thus, the transcription of the WHc-IRES-wIFNγ was driven by a cytomegalovirus immediate-early promoter and translation of downstream wIFN-γ was IRES dependent (see Fig. 1A). Larger quantities of both pC-WHc and, at the same time, to fully control the codelivery of the antigen- and cytokine-encoding sequences, a bicistronic vector capable of concomitant expression of WHcAg and woodchuck IFN-γ (wIFN-γ) was constructed. This expression vector was investigated in parallel with the plasmid encoding WHcAg alone for the ability to mount complete (sterilizing) protection against the hepapnavirus. This was achieved by challenging the vaccinated woodchucks with a liver pathogenic dose of WHV containing 1.1 × 10^10 DNAase-protected virus genome equivalents (vge) and by ultrasensitive PCR-nucleic acid hybridization (PCR-NAH) assays to detect WHV genomes and their replication activity (45, 46). We also examined the abilities of the bicistronic plasmid to elicit WHcAg-specific humoral and T-cell immune responses and to upregulate expression of Th1 cytokines and IFN-γ in peripheral lymphoid cells and hepatic tissue. We have found that immunization with the bicistronic DNA was more effective than immunization with WHc DNA alone in preventing serum WHsAg-positive WHV infection. However, all vaccinated animals found to be free of the serologically detectable infection and hepatitis acquired low levels of replicating virus after challenge with a massive dose of WHV, indicating that the protection was not absolute. The findings reported here raise concern about the actual prophylactic potency of the vaccination with DNA encoding hepapnavirus internal protein. They indicate that this approach cannot protect the animal from acquiring serologically occult hepapnavirus carriage, of which one of the long-term potential consequences is development of hepatocellular carcinoma (46, 56). They also reveal that prevention against hepatitis and protection from infection are not synonymous in the case of hepapnavirus infection.
DMEM. After incubation in a humidified, 5% CO2 atmosphere for 37°C, the culture medium (9) had just been replaced with serum-free, antibiotic-free medium, the mixture was added to WCM-260 cell culture in which complete hepatocytes, previously derived and characterized in our laboratory (13, 30), and then combined together. After incubation for 20 min at ambient temperature, the cells were fixed for 3 min with cold ethanol ether (48), exposed to rabbit monoclonal antibody (MAb) specific for the heavy chain of woodchuck class I MHC molecule (42, 43), were used. To simultaneously detect WHAg and class I MHC antigen in WCM-260 hepatocytes transfected with the pWHc-wIFNγ construct, the cells prepared and incubated with rabbit anti-WHa, as described above, were exposed for 30 min on ice to B1Bb9 MAb. Then, the cells were exposed to fluorescein isothiocyanate-conjugated goat anti-mouse IgM or IgG antibodies (Jackson Immunoresearch Labs, Inc.) for 30 min on ice and examined by confocal microscopy.

### DNA immunization and WHV challenge

In the first phase of the study, four healthy woodchucks were immunized twice with pC-WHe alone (Table 1, pC-WHe group). Two of the animals were injected with 0.6 mg and two other animals were injected with 1.0 mg of pC-WHe DNA per injection. For a control, one woodchuck (C1/M) was injected twice with 1 mg DNA of pC empty vector (Table 1). For each injection, half of the plasmid DNA was administered subcutaneously at 10 to 15 sites on the back and the other half was intramuscularly (Table 1). For each injection, half of the plasmid DNA was administered subcutaneously at 10 to 15 sites on the back and the other half was intramuscularly injected into the quadriceps of the hind leg at 3 to 5 sites in animals under general anesthesia. Approximately 2 months after the first immunization, a booster DNA injection of the same dose was given.

In a parallel experiment, nine woodchucks were injected with the pWHc-wIFNγ DNA (Table 1, pWHc-wIFNγ group). The animals were divided into three subgroups, and each of these animals received 0.5 mg, 0.9 mg, or 1.5 mg of the total plasmid DNA using the same mode of DNA administration as for the pC-WHe group. The pWHc-wIFNγ doses of 0.9 mg and 1.5 mg were calculated to contain DNA encoding WHAg at amounts equivalent to those contained in pC-WHe doses of 0.6 mg and 1.0 mg, respectively. Two months after primary immunization, a booster DNA injection with the same dose as for the primary injection was given to selected woodchucks (Table 1). After 2 to 3 months, woodchucks belonging to pC-WHe and pWHc-wIFNγ groups were challenged by intravenous injection with WHV/tm2 inoculum at 1.1 /H11002 1010 DNase-protected virions (GenBank accession number AF286936) (11). In addition, one woodchuck (C2/F) received 1.3 mg DNA of the wIFNγ plasmid (pC-wIFNγ) for each of the
two injections prior to challenge with WHV (Table 1). Also, C3M woodchuck, which did not receive any plasmid injection before WHV challenge, was examined in parallel as a control (Table 1).

Furthermore, four other healthy woodchucks, each injected with 1.1 × 10^{10} DNase-protected vge of WHV, were monitored as unvaccinated controls in a parallel study in which the same sample collection scheme and evaluation were used as indicated for the DNA-vaccinated animals (S. A. Gujar, C. S. Guy, and T. I. Michalak, unpublished data).

Sample collection. Sera were collected biweekly from the time of DNA immunization until 6 months after challenge with WHV and then monthly. PBMC were obtained biweekly after DNA immunization and weekly following challenge with WHV for 2 months, then biweekly for 4 months, and monthly thereafter.

The cells were isolated by density gradient centrifugation on Ficoll-Paque (Pharmacia Biotech, Baie d’Urfé, Quebec, Canada) as described elsewhere (30). Liver biopsy specimens were obtained by surgical laparotomy (46). The first biopsy sample was taken before DNA immunization, the second was taken 6 or 7 weeks after challenge with WHV, and the third was taken at autopsy. In some animals, the second biopsy specimen was obtained 2 weeks after the challenge, the third was obtained 6 weeks later, and the fourth was obtained at autopsy. At autopsy, in addition to liver tissue, samples of serum, PBMC, and lymphatic organs were collected and preserved, following procedures described before (24, 46).

Serological and WHV DNA detection assays and cccDNA and mRNA detection assays. Serum WHsAg, anti-WHc, and anti-WHs were determined by specific enzyme-linked immunosorbent assays as described previously (46). For detection of WHV DNA in serum, PBMC, and liver biopsy specimens and in autopsy lymphatic and hepatic tissue samples, DNA was extracted by the proteinase K-phenol-chloroform method (46). The isolated DNA was subject to direct and, if required, nested PCR analysis using WHV core gene-specific primers, controls described previously (46). For detection of WHV covalently closed circular DNA (cccDNA) by specific PCR, mung bean nuclease treatment, primers, and conditions previously established were used (30, 45).

The presence of WHV mRNA was examined using total RNA extracted with TRizol (Invitrogen). Samples were treated with DNase I following instructions of a DNA-free kit (Ambion, Austin, TX) before reverse transcription to cDNA (10, 46, 71). In all cases, DNA isolations were carried out in parallel with mock samples containing water instead of test nucleic acid samples. In addition, DNA from serum or DNA or RNA from PBMC or liver tissue of a healthy animal and from a woodchuck with WHsAg-positive chronic hepatitis were included as positive and negative controls, respectively. In the case of RT-PCR, test RNA not transcribed in the RT process was used to rule out potential carryover of negative and positive controls, respectively. In the case of RT-PCR, test RNA not transcribed in the RT process was used to rule out potential carryover of

Real-time RT-PCR. To quantify expression of wIFN-γ and woodchuck TNF-α (wTNF-α), IFN-α-responsive woodchuck 2,5-oligoadenylate synthetase (wOAS), woodchuck CD3 (wCD3), β-actin and WHV RNA, real-time RT-PCR assays were established using LightCycler Faststart Master SYBR I kit (Roche Diagnostics, Laval, Quebec, Canada) and a LightCycler (Roche Diagnostics). Briefly, cDNA derived from approximately 25 ng of total RNA was used as the template for each RT mixture. For PCR amplifications, the following primer sets were used: for wIFN-γ, sense primer 5'-AGGAGCATGAGGATACATCA and antisense primer 5'-CGAGCAACGAGTGAAGGAG; for wTNF-α, sense primer 5'-TGGACCTGAAGTATGCCT and antisense primer 5'-TCTCACAAATCCTTCA; for wOAS, sense primer 5'-TCAGGCAAAGGACAC and antisense primer 5'-ACTTCTCTTTCGGACATGCT; for wCD3, sense primer 5'-TCATGGTCTGCTGCTAT and antisense primer 5'-GCTGGCTTCCTCGGTAGGCTC; and for woodchuck β-actin, sense primer 5'-CACCCGTAGGAAATGCACC and antisense primer 5'-ATTCCTGCTCCTGCTAG GTC. WHV RNA was quantified using sense primer 5'-ATGGACCACTTTACCTCTATC and antisense primer 5'-GCTGGCTTCCTCGGTAGGCTC; and for woodchuck β-actin, sense primer 5'-CACCCGTAGGAAATGCACC and antisense primer 5'-ATTCCTGCTCCTGCTAG GTC. WHV RNA was quantified using sense primer 5'-ATGGACCACTTTACCTCTATC and antisense primer 5'-GCTGGCTTCCTCGGTAGGCTC; and for woodchuck β-actin, sense primer 5'-CACCCGTAGGAAATGCACC and antisense primer 5'-ATTCCTGCTCCTGCTAG GTC. WHV RNA was quantified using sense primer 5'-ATGGACCACTTTACCTCTATC and antisense primer 5'-GCTGGCTTCCTCGGTAGGCTC; and for woodchuck β-actin, sense primer 5'-CACCCGTAGGAAATGCACC and antisense primer 5'-ATTCCTGCTCCTGCTAG GTC.

RESULTS

WHAg and IFN-γ expression in cells transfected with pC-WHe or pWH-wIFNy. The transfection competence of pC-WHe and the bicistronic WHc-wIFNγ plasmids (Fig. 1A) were examined in transient-transfection experiments using WCM-260 hepatocytes as the targets. As shown in Fig. 1B, WHAg was detected in the cells transfected with either pC-WHe or pWH-wIFNy. On the other hand, expression of wIFN-γ was monitored both directly by detecting the cytokine mRNA by RT-PCR and indirectly by detection of enhanced expression of the class I MHC antigen that requires the presence of functionally active cytokine protein. The results showed that wIFN-γ mRNA was readily detected in WCM-260 cells transfected with WHc-wIFNγ and pC-wIFNy, but not with pC-WHe (Fig. 1C) and that the display of class I antigen was significantly increased on Hepa-1 hepatocytes transfected with pWH-wIFNy but not on those transfected with pC-WHe alone (Fig. 1B). In addition, the cells transfected with a control pC-wIFNy plasmid also showed upregulated expression of class I MHC antigen, while they remained WHAg nonreactive (Fig. 1B).

These data implied that neither transfection with Lipofectamine 2000 reagent nor expression of WHAg enhanced
class I MHC antigen display. Taken together, the results clearly showed that the observed increase in the class I MHC display was a specific consequence of synthesis of functionally active IFN-γ/H9253 in cells transfected with pWHc-wIFNγ/H9253 or pC-wIFNγ/H9253.

**WHcAg-specific antibody and T-cell responses after immunization with WHc DNA.** In the first phase of the study, four naïve woodchucks were immunized with pC-WHc DNA (Table 1, group pC-WHc). Two of the animals (A/M and B/M) injected twice with 0.6 mg of pC-WHc did not produce anti-WHc (Fig. 2A). However, two other animals (C/M and D/M), which received two 1-mg doses of DNA, developed anti-WHc 4 to 5 weeks after the first injection with the plasmid (Fig. 2A). The level of anti-WHc elicited was on average ~25% higher in C/M than that in D/M. As expected, a control woodchuck injected with empty pCI vector was anti-WHc negative (Fig. 3D and Table 1). WHV-specific lymphoproliferative response was not measured in these animals.

In the subsequent experiments, to test whether introduction of wIFN-γ DNA into the WHc DNA construct would strengthen induction of immune responses toward WHcAg and augment protection against hepadnavirus, three groups of woodchucks, each of which contained three animals, were injected with 0.5, 0.9, or 1.5 mg per dose of pWHc-wIFNγ/H9253 DNA (Table 1, group pWHc-wIFNγ). Of these nine animals, three responded by producing anti-WHc (Fig. 3 and Table 1). The antibodies appeared at 3 and 5 weeks after first immunization in animals 1/F and 4/F, respectively, and in 1 week after the booster injection in animal 8/M, independently of the dose of pWHc-wIFNγ given. Therefore, in contrast to the pC-WHc group, there was no relation between the amount of pWHc-wIFNγ given and induction of
anti-WHc. Animal C2/F injected with pC-wIFNγ was anti-WHc negative (Fig. 3D and Table 1).

In four animals vaccinated with pWHc-wIFNγ, WHV-specific T-cell proliferative response was measured prior to and after challenge with WHV. Two of the animals, 2/F immunized twice with 0.5 mg and 8/M injected twice with 1.5 mg of the plasmid, mounted a specific T-cell response, as shown in Fig. 4. As expected, this response was directed against proteins encoded by WHV core gene, i.e., rWHcAg and rWHeAg, and WHc97-110 peptide, but not toward rWHxAg or WHsAg, confirming antigen-restricted specificity of the T-cell response induced by pWHc-wIFNγ. Also, it is of note that a significantly higher magnitude of WHc-specific T-cell response was detected in animal 2/F (maximum SI of 13.7) than in animal 8/M (maximum SI of 4.2). Overall, the results revealed that immunization with pWHc-wIFNγ DNA was able to elicit a WHc-specific T-cell response. However, this response was not induced uniformly among the animals immunized, suggesting that unidentified host factors played a decisive role in its development. In addition, animal C3/M, which was not vaccinated but infected with the same WHV inoculum as the vaccinated animals, developed a WHV-specific T response detectable from 10 weeks postinfection (Fig. 4). The same pattern of the virus-specific T-cell response was observed in four other animals investigated in the parallel study. In all of them, the T-cell response directed toward WHV antigens appeared between 6 and 12 weeks postinfection (data not shown). Anti-WHc were detected in C3/M in 1 week (Fig. 3D) and in the above four control woodchucks in 4 to 6 weeks (data not shown) after injection with WHV.

Immunization with WHc DNA can protect from serologically evident WHV infection and hepatitis. Animals immunized with pC-WHc or pWHc-wIFNγ DNA were challenged with a massive dose of WHV containing 1.1 × 10^{10} vge to determine the protective effectiveness of the vaccination strategy tested. Of the four woodchucks that were injected with pC-WHc DNA, animal C/M developed anti-WHc and remained serum WHsAg nonreactive during the 12-month follow-up after inoculation with virus (Fig. 2B and Table 1). The liver biopsy specimens obtained at 6 weeks after challenge and 12 months later at autopsy showed entirely normal histology. In contrast, animal D/M, which also developed anti-WHc following DNA vaccination, established serum WHsAg-positive, histologically evident chronic hepatitis lasting until the end of the 12-month observation period. The two remaining woodchucks, A/M and B/M, immunized with 0.6-mg doses of pC-WHc developed transiently serum WHsAg-positive infection and self-limited acute hepatitis (Fig. 2A and Table 1). Anti-WHc antibodies appeared in these two animals after WHV challenge and remained at high levels throughout the 12-month observation period. Liver samples collected after the acute phase of infection showed residual inflammatory alterations persisting to the end of follow-up of these animals. These features were similar to those found in remote resolved hepatitis, as previously reported (11, 24, 46). The C1/M control animal immunized twice with 1 mg of empty pCI vector and infected with WHV developed classical serum WHsAg-positive infection and self-limited acute hepatitis (Fig. 2A and Table 1). Anti-WHc antibodies appeared in these two animals after WHV challenge and remained at high levels throughout the 12-month observation period. Liver samples collected after the acute phase of infection showed residual inflammatory alterations persisting to the end of follow-up of these animals. These features were similar to those found in remote resolved hepatitis, as previously reported (11, 24, 46). The C1/M control animal immunized twice with 1 mg of empty pCI vector and infected with WHV developed classical serum WHsAg-positive, self-limited acute hepatitis, which was followed by residual minimal inflammatory alterations in liver parenchyma (Fig. 3D and Table 1).

As shown in Fig. 3 and summarized in Table 1, four of nine animals immunized with pWHc-wIFNγ remained serum WHsAg negative after challenge with WHV. Three of them (1/F, 4/F, and 8/M) acquired anti-WHc due to DNA vaccina-
tion, while one (7/M) remained anti-WHc negative and produced the antibodies in 1 week after challenge. In addition, a very brief episode of WHs antigenemia, identifiable only in a single serum sample, was detected in animals 3/M and 9/M. The remaining three animals (2/F, 5/F, and 6/M) developed serum WHsAg-positive infection lasting between 3 and 20 weeks. All of these five animals became anti-WHc reactive in 1 to 4 weeks after inoculation with WHV (Fig. 3 and Table 1).

Anti-WHs reactivity was detected in two of the woodchucks (Table 1).

Importantly, histological examination of two or three liver tissue samples collected from each animal during follow-up lasting for up to 45 weeks after inoculation with WHV showed consistently normal morphology in five of nine woodchucks (Table 1). Among them were four animals (1/F, 4/F, 7/M, and 8/M) which remained serum WHsAg nonreactive after inoculation with WHV and one (9/M) which had a 1-week-long episode of borderline WHs antigenemia (Fig. 3C). Three (7/M, 8/M, and 9/M) of those five woodchucks were immunized once or twice with 1.5 mg of pWHc-wIFNγ/H9253 DNA and then inoculated with WHV. In contrast, animals that established serum WHsAg-positive infection (2/F, 3/M, 5/F, and 6/M) developed, as expected, acute hepatitis, which subsided to minimal residual inflammatory changes persisting to the end of the observation period. A control animal (C2/F) immunized twice with 1.3 mg of pC-wIFNγ/H9253 DNA and then inoculated with WHV showed a similar outcome.

FIG. 3. Serological markers of WHV infection, WHV DNA detection, and liver histology in woodchucks immunized with pWHc-wIFNγ DNA or control plasmid DNA. Three groups of woodchucks, each group consisting of three animals, were immunized with 0.5 mg/dose (A), 0.9 mg/dose (B), or 1.5 mg/dose (C) of pWHc-wIFNγ DNA at the time points indicated by the vertical, solid black lines. In addition, two woodchucks that received either pCI (1.0 mg twice) or pC-wIFNγ (1.3 mg twice) and one animal that was not vaccinated are shown as controls in panel D. The animals were challenged with 1.1 × 10^{11} vge of WHV at week 0. WHsAg, anti-WHc, and WHV DNA and liver samples were evaluated, and the results are presented as described in Materials and Methods and in the legend to Fig. 2.
with WHV had classical serum WHsAg-positive self-limiting acute hepatitis (Fig. 3D and Table 1). It is of note that woodchuck 2/F, which developed a robust WHc-specific T-cell proliferative response but no anti-WHc antibodies after DNA vaccination and displayed a swift and strong lymphoproliferative response to WHcAg and related antigens following challenge with WHV (maximum SI of 29) (Fig. 4), failed to show the protection (Fig. 3A). In this animal, T-cell proliferation response against multiple WHV antigens, including rWHxAg and WHsAg, was detected around 7 weeks after challenge with WHV, thus approximately at the same time as in animal C3/M (Fig. 4) and four other control woodchucks infected with a massive dose of WHV but not immunized with WHc DNA (data not shown). In contrast to animal 2/F, animal 8/M, which developed a relatively weak WHc-specific T-cell response and anti-WHc following vaccination, was free from WHs antigenemia and protected from hepatitis.

**Transcription of IFN-γ, TNF-α, and OAS in livers and lymphoid cells after vaccination with pWHc-wIFNγ.** To recognize whether induction of antiviral and Th1 cytokines may play a role in protection against WHV infection and hepatitis, the levels of wIFN-γ, wTNF-α, and wOAS mRNAs were quantified. **FIG. 4.** Profiles of the WHV-specific cellular immune response in woodchucks immunized with pWHc-wIFNγ DNA. Animal 2/F was immunized twice with 0.5 mg and animal 8/M was immunized twice with 1.5 mg of pWHc-wIFNγ DNA at the time points indicated by the arrows. In addition, woodchuck C3/M, which was not vaccinated but was infected, served as a control. The animals were challenged with $1.1 \times 10^{10}$ vge of WHV at week 0. The proliferative T-cell responses to the WHV antigens indicated in the symbol keys were measured by [3H]adenine incorporation assay as described in Materials and Methods. The stimulation index was calculated by dividing the mean cpm obtained after stimulation with test antigen by the mean cpm detected in the absence of a stimulant. A stimulation index equal to or greater than 3.1, marked by the horizontal line, was considered positive.
fied by real-time RT-PCR in hepatic tissue and PBMC samples collected before DNA immunization and after challenge with WHV. As shown in Fig. 5A, intrahepatic transcription of wIFN-γ/H9253 and wTNF-α/H9251 was not noticeably different prior to and after exposure to virus in all four animals protected from serologically evident infection and hepatitis, except for animal 8/M, which had an unexplained increase in wTNF-α/H9251 mRNA prior to WHV challenge. This was accompanied by relatively stable levels of wOAS and CD3 mRNA in individual animals, except in animal 8/M (Fig. 5A). In contrast, enhanced transcription of intrahepatic wIFN-γ (2.5- to 5.7-fold increase) and wTNF-α (4.2- to 6.3-fold increase) mRNAs in comparison to the levels prior to WHV challenge was seen in woodchucks not protected from WHV infection (2/F, 5/F, and 6/M) (Fig. 5B). Similar increases were seen in control woodchucks that received pCI or pC-wIFNγ before injection with WHV (Fig. 5C). These increases were observed in the acute phase of infection and were accompanied by upregulated expression of wOAS and CD3 mRNAs. In animals 3/M and 9/M, which showed a brief episode of WHs antigenemia, the mRNA levels tested

FIG. 5. Profiles of relative expression of wIFN-γ, wTNF-α, wOAS, and CD3 genes in liver biopsy samples collected prior to and after challenge with WHV from protected (A), nonprotected (B), and control (C) woodchucks that were immunized with pWHc-wIFNγ or control DNA. The time of challenge with 1.1×10^10 vge of WHV is indicated by an arrow and corresponds to week 0. The mRNA levels of indicated woodchuck genes were quantified by specific real-time RT-PCR. The results are shown as the number of copies of a given gene per reaction after normalization against 3×10^4 copies of woodchuck β-actin, which was quantified in parallel in each test sample.
between 2 and 8 weeks postinfection were only moderately elevated or remained at the prechallenge values. Analysis of the expression of the same genes in serial PBMC samples did not reveal measurable differences between the protected and nonprotected animals or between the pre- and postchallenge phases (not shown). Overall, the data implied that protection from WHV infection and hepatitis induced by pWHc-wIFNγ was not correlated with measurable changes in the expression of Th1 cytokines or IFN-γ in either peripheral lymphoid cells or hepatic tissue samples.

Woodchucks otherwise successfully immunized with pWHc-wIFNγ replicate virus at low levels after challenge with WHV. Although 5 of the 13 animals immunized with pC-WHc or pWHc-wIFNγ were protected from serologically apparent WHV infection and hepatitis, serial serum samples from these animals collected after inoculation with WHV revealed low levels of WHV DNA when analyzed by PCR-NAH assays. This unexpected finding was investigated in detail using approaches previously established for identification of occult hepadnavirus persistence (10, 11, 24, 30, 45, 46). Thus, the analysis of serial serum samples obtained after inoculation with WHV and at autopsy demonstrated WHV DNA at levels usually not exceeding 10^2 vge/ml, including in the initial phase of infection corresponding to acute hepatitis in symptomatic animals (Table 1). In all five animals protected from hepatitis, WHV DNA was also detected in sequential PBMC samples collected after challenge and in lymphatic organs gathered at autopsy (not shown). The estimated levels of WHV genomes ranged between 0.005 and 0.5 vge/10^4 lymphoid cells, calculated as described previously (46).

In the animals protected from serologically evident infection and hepatitis, less than 2 × 10^3 WHV vge per μg of total DNA was detected in liver samples collected after challenge and at the time of autopsy, compared to an estimate of up to 2 × 10^7 vge per μg of total DNA detected in the acute phase of hepatitis in the nonprotected animals. At the end of follow-up of the nonprotected woodchucks, WHV DNA in the liver dropped to loads comparable to those found in the protected animals, i.e., about 10^3 vge per μg of total DNA, with the exception of animal D/M, which developed serum WHsAg-positive chronic hepatitis. Importantly, WHV cccDNA was also detectable in samples of autopsy liver tissue (not shown).

Further, to determine the magnitude of WHV replication in the livers of the protected woodchucks, WHV pregenomic RNA was quantified by real-time RT-PCR in liver biopsy specimens collected from animals 1/F, 4/F, 7/M, and 8/M between 2 and 8 weeks after challenge and at autopsy. As shown in Fig. 6A, less than 100 copies of WHV mRNA per 3 × 10^4 copies of β-actin mRNA was found in these liver samples. The WHV mRNA levels remained stable (in 1-log10-unit range) for up to 45 weeks after challenge. In woodchucks not protected from infection, i.e., 2/F, 3/M, 5/F, 6/M, and 9/M, 1- to 4-log-unit-higher WHV RNA levels than those occurring in the livers of the protected animals were identified at 2 to 8 weeks postinfection, except for animal 3/M. These hepatic loads progressively declined, reaching less than 100 copies of WHV RNA

![FIG. 6. Detection of WHV RNA in liver tissue samples collected after challenge with WHV from protected (A), nonprotected (B), and control (C) woodchucks after vaccination with pWHc-wIFNγ or control DNA. The time of challenge with WHV is indicated by an arrow and corresponds to week 0. The WHV RNA levels were quantified by real-time RT-PCR and normalized against β-actin, as described in the legend to Fig. 5. It is of note that WHV RNA was consistently detected in all liver samples acquired from animals that were otherwise protected after immunization with pWHc-wIFNγ DNA from serum WHsAg-positive infection and hepatitis.](http://jvi.asm.org/...
per 3 × 10^5 copies of β-actin mRNA at the end of the observation period. In control woodchucks, which received p-CI or p-C-IFN (prior to injection with WHV (Table 1), a 2- to 3-log-unit-higher liver WHV RNA load was detected at 2 to 8 weeks postinfection, similar to the loads in nonprotected animals after DNA vaccination, and then the WHV RNA level dropped to below 100 copies per 3 × 10^6 copies of β-actin mRNA at the end of the observation period (Fig. 6C). Comparable results were obtained in animal C3/M and four unvaccinated woodchucks injected with massive doses of WHV (data not shown). Interestingly, animal 9/M, which showed unaltered liver histology after inoculation with WHV (Fig. 3C), carried WHV RNA at a high level at week 7 after challenge with WHV, and this level declined to less than 100 copies at week 15 and thereafter.

**DISCUSSION**

It has been shown that immunizations of WHV-naïve woodchucks with native purified or recombinant WHcAg or with plasmids encoding this antigen were able to protect some animals against experimental WHV infection, as determined by the absence of serum WHsAg and WHV DNA by PCR assays with detection limits of 5 × 10^7 to 10^8 vge/ml (31, 32, 59, 61). It was also demonstrated that coadministration of plasmids encoding WHcAg and woodchuck IFN-γ or IL-12 or with the gene encoding WHcAg fused with CTL-associated antigen 4 enhanced the efficacy of the protection compared to that elicited by WHc DNA alone (19, 32, 63). In the present work, we confirmed that vaccination with WHc DNA can prevent the acquisition of serologically detectable WHV infection and hepatitis. We also showed that the protective efficacy of this vaccine can be improved by delivery of DNA encoding WHcAg and wIFN-γ incorporated into a single plasmid vector. This result indicates that the bicistronic vector approach, in which the expression of the cytokine-encoding sequence is driven independently of that of the viral antigen, represents a valid strategy for cytokine-facilitated enhancement of immunity protecting against hepadnavirus hepatitis. In other studies, the bicistronic plasmids encoding HBV pre-S2 and IL-2 elicited a stronger Th1 response than HBV pre-S2 DNA alone when given to mice (8). Similarly, immunization with HIV gp120/ granulocyte-macrophage colony-stimulating factor (GM-CSF) bicistronic DNA was able to enhance gp120-specific CD4+ T-cell response in mice in comparison with coapplication of plasmids encoding each of these two molecules separately (1). An increased T-cell response was also observed when a bicistronic plasmid encoding HCV NS 3, 4, and 5 proteins and GM-CSF were injected into rats (6). Therefore, the current data support the view that immunization with the DNA constructs encoding viral antigen and a cytokine acting as an adjuvant can significantly enhance specific immune response to the antigen of interest.

In the present study, immunization with the bicistronic pWHc-wIFNγ DNA vaccine protected four of nine (44.5%) animals from serum WHsAg-positive WHV infection. The protection was not related to the number of the DNA injections given, since a single dose was just as effective as two injections. However, of the three woodchucks that received the greatest amount of the bicistronic DNA, two animals did not develop serologically evident infection and all three were free from hepatitis after challenge with the virus (Table 1 and Fig. 3C). Of the remaining six woodchucks injected with the intermediate or low doses of pWHc-wIFNγ, two animals receiving a single injection of 0.9 mg or 0.5 mg of DNA became protected (Table 1, pWHc-wIFNγ group). In contrast, of four animals immunized with pC-WHc alone, only animal C/M injected twice with 1 mg of the plasmid remained serologically negative and free from hepatitis after challenge with virus (Table 1, pC-WHc group). These data showed that the protection induced by pWHc-wIFNγ was related to the amount of the DNA administered, but not to the number of injections given, and that pWHc-wIFNγ was clearly more potent than pC-WHc in preventing serologically evident infection and hepatitis.

Of the woodchucks immunized with pWHc-wIFNγ that acquired protection, three animals produced anti-WHc prior to challenge (Table 1). However, animal 7/M, which did not develop anti-WHc, was also protected from WHsAg-positive infection and hepatitis. An even stronger indication that anti-WHc had little or no contribution to the protective immunity in our study, came from the experiment with woodchucks immunized with pC-WHc. Specifically, two animals, each injected twice with 1-ng doses of pC-WHc, produced anti-WHc. However, while animal C/M was protected from serum WHsAg-positive infection and liver injury, animal D/M established serologically and histologically evident chronic hepatitis (Fig. 2B). This is a rare outcome of WHV infection in adult woodchucks, which is observed in no more than 15% of animals infected with massive WHV doses (~10^10 vge) (40, 41). Overall, we did not observe any evident interdependence between the induction of anti-WHc and protection from experimental infection. This finding is consistent with the data from other studies in which a relation between the humoral response to WHcAg and the outcome after challenge with WHV was investigated (19, 32, 63).

There was also no evident relation between WHcAg-specific T-cell proliferative response induced by vaccination with pWHc-wIFNγ and protection from infection. Specifically, woodchuck 2/F, which mounted a readily detectable T-cell proliferation following stimulation with WHV nucleocapsid-related antigens and a very strong specific T-cell memory response within 1 to 2 weeks of WHV inoculation (Fig. 4A), developed WHs antigenemia and hepatitis (Fig. 3A). In contrast, animal 8/M, which showed borderline WHcAg-specific lymphoproliferation after vaccination and no memory response after challenge (Fig. 4B), was protected from serologically positive infection and hepatitis (Fig. 3C). These results differ from those reported by others for woodchucks immunized by coadministration of WHc and IFN-γ plasmids, where all three animals examined showed a lymphoproliferative response to WHcAg and were protected from infection, as determined by assessing serum WHV DNA by PCR with the detection limit of ~10^3 vge/ml (63). However, in the same study, another animal, which had been injected with WHc DNA and did not produce measurable T-cell response to WHcAg, was also protected. Taken together, we tend to believe that immune responses other than those meaningfully involving specific T cells, for which activity is measured after ex vivo stimulation with WHV nucleocapsid-related antigens, play...
a prevailing role in the protection elicited by vaccination with DNA encoding WHV core protein in the presence of IFN-γ as a vaccine adjuvant. In this regard, a contribution of specific CTL or innate immunity primed by vaccination could be considered. However, this hypothesis cannot be easily tested, since there are no assays available at the present time measuring these types of immune responses in woodchucks.

Vaccinated animals that mounted a WHcAg-specific lymphoproliferative response or those that became protected from serologically evident infection and hepatitis did not show any measurable differences in the expression of wIFN-γ or wTNF-α, as well as wOAS, in serial samples of circulating lymphoid cells, compared to those in the nonprotected animals. Similarly, quantification of the mRNA levels of these cytokines in liver samples from the protected and unprotected animals did not provide a lead to a possible mechanism underlying the protection elicited.

Liver tissue samples from the woodchucks protected from serum WHsAg-positive infection were free from inflammatory alterations, as evidenced by histological examinations. The lack of lymphocytic infiltrations and inflammation was confirmed by unaltered expression of intrahepatic CD3, IFN-γ, and TNF-α (24). Although the nature of the protection elicited by pWHc-wIFNγ vaccine remains uncertain, these findings convincingly showed that when this protective response was successfully induced, it was sufficient to entirely prevent histological and molecular signs of hepatitis. In addition, the data for woodchuck 9/M, which developed a brief episode of WHsAg-positive infection and prevention of liver disease caused by this infection may not be identical.

The lack of sterile protection found in our study could be a consequence of at least two coinciding events. First, since WHcAg is an inner structural antigen, immunity raised against this antigen may not be as effective as that directed toward epitopes of virus envelope in preventing the initial attachment and entry of the virus into hepatocytes and lymphoid cells, which are both naturally targeted by virus (30, 45, 46). Therefore, virus replication can be established, at least at a low level, as shown by our study. On the other hand, the immunity raised against the core antigen, particularly in the presence of IFN-γ, can be highly effective in suppressing virus replication, possibly by a noncytopathic pathway (20), and in limiting virus spread to uninfected cells. This mechanism can keep the virus under control, i.e., at the levels which are not pathogenic to the liver (see below) (40, 46). It has been documented in a series of our previous studies that once WHV replication is established, it can be suppressed but never completely eradicated (10, 24, 46). The same appears to be true for humans who recovered from serum HBsAg-positive acute hepatitis B (39, 47, 57). The results from the current study are in agreement with these previous findings.

Second, all animals vaccinated in our study were challenged with a massive dose of WHV normally causing hepatitis in naïve animals, and the woodchucks otherwise protected from serologically evident infection and hepatitis carried low levels of virus in both lymphoid cells and their livers after challenge. We have previously demonstrated that there is a significant difference in the threshold level of WHV required to infect lymphoid cells and the level to infect hepatocytes (presumably 100- to 1,000-fold) (11, 40, 45). It was found that WHV doses lower than or equal to 10⁹ virions cause serologically concealed infection restricted to the lymphatic system, termed primary occult infection. In contrast, WHV at higher doses induces serologically evident infection, involving both the lymphatic system and the liver, and causes hepatitis, which when resolved is followed by indefinitely long residual infection in the liver and lymphoid tissues, termed secondary occult infection (11, 40, 45, 46). Therefore, detection of low levels of WHV in the livers of the otherwise protected animals may suggest that hepatocytes were initially exposed to a high dose of virus (greater than 10⁹ virions) and infected, but the infection was swiftly suppressed, leaving behind residual WHV replication, which normally escapes elimination by immunological and intracellular antiviral mechanisms. Hence, it is possible that the pWHc-IFNγ DNA vaccine, as well as other vaccines based on immunization against hepadnavirus nucleocapsid antigens, can protect the liver, but are unlikely to protect the immune system from infection with low virus doses.

In conclusion, our findings indicate that DNA vaccine delivering simultaneously to the same location both hepadnavirus core protein and IFN-γ-encoding sequences has a potency to suppress virus replication and prevent development of hepatitis. In spite of its inability to induce sterile immunity, this DNA vaccination approach might have therapeutic value in the treatment of chronic hepatitis B where suppression of virus replication may lead to a decrease in hepatic necroinflammation and stop or slow down progression of chronic liver injury.

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