Electroporation Enhances Immunogenicity of a DNA Vaccine Expressing Woodchuck Hepatitis Virus Surface Antigen in Woodchucks

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The development of therapeutic vaccines for chronic hepatitis B virus (HBV) infection has been hampered by host immune tolerance and the generally low magnitude and inconsistent immune responses to conventional vaccines and proposed new delivery methods. Electroporation (EP) for plasmid DNA (pDNA) vaccine delivery has demonstrated the enhanced immunogenicity of HBV antigens in various animal models. In the present study, the efficiency of the EP-based delivery of pDNA expressing various reporter genes first was evaluated in normal woodchucks, and then the immunogenicity of an analog woodchuck hepatitis virus (WHV) surface antigen (WHsAg) pDNA vaccine was studied in this model. The expression of reporter genes was greatly increased when the cellular uptake of pDNA was facilitated by EP. The EP of WHsAg-pDNA resulted in enhanced, dose-dependent antibody and T-cell responses to WHsAg compared to those of the conventional hypodermic needle injection of WHsAg-pDNA. Although subunit WHsAg protein vaccine elicited higher antibody titers than the DNA vaccine delivered with EP, T-cell response rates were comparable. However, in WHsAg-stimulated mononuclear cell cultures, the mRNA expression of CD4 and CD8 leukocyte surface markers and Th1 cytokines was more frequent and was skewed following DNA vaccination compared to that of protein immunization. Thus, the EP-based vaccination of normal woodchucks with pDNA-WHsAg induced a skew in the Th1/Th2 balance toward Th1 immune responses, which may be considered more appropriate for approaches involving therapeutic vaccines to treat chronic HBV infection.

Immunity to hepatitis B virus (HBV) results from an appropriate activation of antiviral B- and T-cell responses during the acute phase of infection that leads to the clearance of the virus. Protective humoral and cellular immunity to HBV also can be achieved following the preexposure vaccination of healthy, HBV-naïve, adult humans with conventional subunit vaccines, which consist of the viral envelope protein (HBsAg) adsorbed onto alum adjuvant. This vaccine also is markedly effective in preventing chronic HBV infection when administered to neonates born to mothers who are chronically infected with HBV. In contrast, individuals who already developed chronic HBV infection exhibit persistent viral replication and associated deficiencies in the immune response against the virus. These include failure to develop protective, virus-neutralizing antibodies against HBsAg (anti-HBs) and reduced or absent antigen-specific T-helper (Th) cells and cytolytic T lymphocytes (CTL), with associated deficiencies in immune response-dependent antiviral cytokines, such as gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) (3, 5, 6, 11, 18, 21, 28, 36, 41). Nevertheless, therapeutic vaccine approaches to modulate deficient or defective humoral and cellular immunity in chronic HBV carriers have come to represent a promising approach for the treatment of established chronic HBV infection.

Vaccines based on plasmid DNA (pDNA) induce humoral and Th1 cellular immune responses that could be effective in the treatment of chronic HBV infection. However, the successful development of such vaccines has been hampered by low-magnitude and inconsistent immune responses that often follow routine pDNA delivery methods (24, 42). Electroporation (EP) enhances the uptake of DNA vaccines by cells, resulting in significantly increased potency and immunogenicity of pDNA vaccines in several animal models, without adverse responses (1, 2, 4, 19, 25, 26, 35, 38, 44, 48, 49). For example, the EP immunization of mice, pigs, sheep, and rhesus macaques with pDNA vectors expressing HBsAg and/or HBV core protein (HBcAg) demonstrated dose-dependent humoral and cellular immune responses to both antigens (including multispecific CTL as an indicator of Th1 bias) that were superior to those induced by standard hypodermic needle injection (HI) of the same vectors (1, 2, 19, 24, 25, 48, 49). Although the exact mechanisms by which pDNA vaccines elicit such effects are not fully elaborated, the capacity to induce strong Th1 cellular...
responses to HBV antigens is considered essential for activating antiviral immunity that could lead to the clearance of HBV infection in chronically infected humans (3, 5). The enhanced potency of HBV pDNA vaccines administered by EP could prove critical in overcoming the typical immune tolerance to viral antigens present in chronic HBV infection. EP-based DNA immunization now has reached the clinical stage, and EP is being actively investigated in several phase I clinical trials for therapeutic and prophylactic pDNA vaccines in indications ranging from cancer to infectious diseases (24, 43). Therefore, it is conceivable that a therapeutic vaccine for chronic HBV infection using EP-based DNA immunization could be rapidly translated into human testing.

The woodchuck hepatitis virus (WHV) is a hepadnavirus of the Eastern woodchuck (Marmota monax) with genomic organization, biological properties, and replicative strategy closely related to those of HBV (12). The experimental infection of woodchucks with WHV is a well-characterized animal model for studies of the pathogenesis of HBV infection and for the preclinical testing of the safety and efficacy of vaccine approaches and drug candidates for the prevention of HBV disease sequelae, including hepatocellular carcinoma (20, 29, 40). As with HBV infection, the resolution of experimental WHV infection in woodchucks is associated with seroconversion to protective, virus-neutralizing antibodies to WHsAg, significant peripheral blood T-cell responses to viral antigens, and a characteristically biased Th1 cytokine storm in the liver (8, 10, 15, 16, 31–33, 46, 47). In contrast, persistent WHV infection as an outcome of experimental WHV infection is associated with clear deficiencies in peripheral blood virus-specific B- and T-cell responses and in intrahepatic Th1 cytokine expression (10, 33, 46, 47). Results using the woodchuck animal model further exemplify the crucial role for humoral and Th1-mediated cellular immune responses in recovery and protection from chronicity in hepadnavirus infection.

Previously, a pDNA vaccine analog expressing the woodchuck hepatitis virus surface antigen (pDNA-WHsAg) was constructed. In preliminary studies, mice and rabbits immunized with this construct using the Ichor TriGrid EP technology developed robust, consistent, and dose-dependent antibody responses to WHsAg (anti-WHs) with a typical Th1 bias (27). In the present study, standard HI and EP as delivery methods for pDNA into skeletal muscle of adult woodchucks was analyzed. The EP-based method was validated first in woodchucks using several vectors expressing different reporter genes. The humoral and cellular immunogenicities of pDNA-WHsAg delivered to normal (WHV-naïve) woodchucks using several vectors expressing different reporter genes. The humoral and cellular immunogenicities of pDNA-WHsAg delivered to normal (WHV-naïve) woodchucks using several vectors expressing different reporter genes.
free of infectious WHV by the intravenous administration of 1 ml of a more concentrated, preadsorbed, protein solution (i.e., 50 µg) to WHV-susceptible woodchucks. This same adsorbed vaccine lot had been used in previous studies of WHV-susceptible woodchucks that were monitored for up to 1 year and was shown not to transmit any infectious WHV (7, 34).

(ii) Immunization protocol. Twenty-five woodchucks, approximately 1 year of age, all seronegative for markers of WHV infection, were used. Woodchucks were assigned to five treatment groups stratified by body weight, gender, and serum biochemical profile. At weeks 0, 4, and 8, five woodchucks (EP low-dose group) were administered WHAg-pDNA (0.5 mg pMS-310 in 0.5 ml PBS) by EP in the left tibialis cranialis muscle. At these times, five woodchucks (EP high-dose group) were administered WHAg-pDNA (1.0 mg pMS-310 in 0.5 ml PBS) by EP injection in the left and right tibialis cranialis muscles (total of 2.0 mg pDNA per dose). Additionally, with the same administration schedule, five woodchucks (non-EP high-dose group) were administered WHAg-pDNA (1.0 mg pMS-310 in 0.5 ml PBS) by HI alone in the left and right tibialis cranialis muscles (total of 2.0 mg pDNA per dose). Lastly, five other woodchucks (positive control group) received the subcut WHAg vaccine (i.e., 10 µg of formalin-inactivated, alum-adsorbed WHAg protein in 0.5 ml PBS) by standard HI. This vaccine was injected, per our standard routine injection procedures, into the left semimembranosus/semitendinosis muscles at weeks 0 and 8 and into the right semimembranosus/serratus muscles at week 4. As a further control at the same time points five other woodchucks (non-EP negative vaccine control group) received saline (0.5 ml PBS) by HI alone into the left and right tibialis cranialis muscles.

Humoral and cellular immune responses were measured in woodchucks following immunizations by using routine serological and immunological assays during the experimental period. Serum and whole blood were obtained from woodchucks under general anesthesia 2 weeks prior to the start of immunization and on day zero (week zero, designated T0) prior to the administration of the vaccine formulations. Serum for measuring antibodies to WHAg (anti-WHAg) was obtained thereafter at weekly intervals until week 14. Whole-blood samples were obtained every other week until week 12 for hematological and biochemical profiling and then also at weeks 1, 5, 9, and 12 for in vitro T-cell responses to WHAg and for the expression of leukocyte surface marker and TH1/TH2 cytokine mRNAs (see below).

Clinical and biochemical evaluation of woodchucks. The general health of woodchucks was evaluated daily by the observation of appearance, general behavior, and food and water intake. Each time woodchucks were anesthetized and bled, the body weight was recorded. Serum biochemical measurements were performed to evaluate liver function, including serum alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase, gamma-glutamyl-transpeptidase, and sorbitol-dehydrogenase (17).

WH Ag and antibody markers. WHAg, anti-WHAg, and antibodies to WHV core antigen (anti-WHc) were measured in serum by qualitative enzyme-linked immunosorbent assays (ELISA) using serum dilutions of 1:50 (WHAg) and 1:100 (anti-WHAg and anti-WHc) (9). The cutoff value of these assays was defined as ≥0.05 optical density units (ODU). In addition, anti-WHAg was quantified in serial dilution using an enzyme-linked immunosorbent assay (ELISA) and represented as a fold increase (FI) by dividing the averaged transcription level in the presence of the stimulator (WHAg) by the averaged transcription level obtained in the absence of stimulator (blank medium). An FI value of ≥3.1 was considered to represent a positive response for WHAg-specific expression (30, 37). Note that the CD8 molecule is expressed on the surface of leukocytes that are mainly CTLs (it also can be expressed on NK cells). An increase in transcription level for this molecule in WHAg-stimulated cultures was interpreted to indicate that a WHAg-specific subset of CD8+ cells accumulated in the PBMC cultures consisting all or in part of WHAg-specific CTLs.

Parameters of humoral and cellular immune responses. The group responder rate (GRR) to immunizations was defined at any given time point as the percentage of woodchucks positive for anti-WHAg, WHAg-specific T cells, or leukocyte surface marker/cytokine mRNA expression.

Statistical analysis. The geometric mean serum SEAP activity levels of woodchucks following the EP of pgWIZ-SEAP were compared to those of woodchucks that received the vector by HI alone. Toxicity possibly associated with the EP-based administration of pgWIZ-SEAP or pMS-310 was assessed by comparing body weight measurements and hematological and clinical biochemical parameters using Student’s t test (two-tailed). The anti-WHAg geometric mean titers (GMTs) of experimental groups were determined before and following immunization, and results expressed as GMTs were compared between the groups at each sampling using Student’s t test (two-tailed). GRRs positive for anti-WHAg responses, WHAg-specific T-cell response, or leukocyte surface marker/cytokine mRNA expression in the experimental groups were compared by Fisher’s test for proportions (two tailed). P values of <0.05 were considered statistically significant.

RESULTS

Reporter gene expression is greatly enhanced following EP-mediated delivery of pDNA. Vectors encoding LacZ or GFP were injected into the left tibialis cranialis muscle of three woodchucks using the EP device with the electrode array, followed by electrical stimulation, and then into the right tibialis cranialis muscle of each woodchuck by HI. Four days later, all three woodchucks receiving the LacZ-expressing vector, pCMV-beta, by EP had distinctive blue coloration of the muscle tissue starting within 20 min of the X-gal detection reaction, which was indicative of marked LacZ expression, and it became maximal by 24 h in the reaction (Fig. 1A, bottom). In contrast, among woodchucks receiving pCMV-beta by non-EP injection, only one had a faint blue discoloration by 24 h (Fig. 1A, top).
Gene expression and immunogenicity studies were carried out using the woodchuck model. Woodchucks were divided into groups and injected with plasmid DNA encoding various reporter genes or WHsAg. The expression of reporter genes in muscle tissue was monitored using fluorescence microscopy. Serum samples were collected at various time points to determine the levels of secreted alkaline phosphatase (SEAP) activity, which was used as an indicator of gene expression levels.

The immunogenicity study involved administering WHsAg DNA vaccine by EP injection. Blood samples were collected to monitor the anti-WHs antibody response. The antibody levels were measured using immunoassays. The results showed that the EP delivery of WHsAg DNA vaccine induced a higher and more rapid antibody response compared to non-EP injection.

In summary, the delivery of plasmid DNA encoding reporter genes or WHsAg by EP injection in woodchucks resulted in efficient gene expression and a significant antibody response. These findings highlight the potential of EP delivery as a promising method for gene therapy and vaccine development.
zations, with a maximum at 9 weeks pi (841 U/ml) and waning thereafter. Anti-WHs response in the EP high-dose and in the positive vaccine control groups were comparable for most time points following the third immunization at week 8, except for week 9 pi, where anti-WHs GMT was significantly higher in the positive vaccine control group ($P < 0.05$).

As indicated above, the kinetics of anti-WHs development differed among the immunized groups, with variable peak GMT anti-WHs responses occurring anywhere between 5 and 12 weeks pi and with these ranging between 154 and 841 U/ml (Fig. 2). The anti-WHs response waned thereafter, and at the end of the study (week 14), in order of magnitude, these were highest in the positive vaccine control group between weeks 5 and 14 ($P < 0.05$). Anti-WHs GMTs in the non-EP high-dose group were significantly lower than those in the EP high-dose group between weeks 9, 12, and 14, and in the positive vaccine control group at week 9, and again between weeks 11 and 14 ($P < 0.05$). The anti-WHs GMT in the EP high-dose group was significantly lower than that in the positive vaccine control group at week 9 ($P < 0.05$).

The results described above demonstrate that the EP delivery of a high dose of pDNA expressing WHsAg clearly was superior to the same vector dose administered by non-EP injection in regard to the magnitude and sustainability of the induced antibody response. Furthermore, responses to EP administration were dose dependent in the range tested, and at the higher dose the anti-WHs response pattern was comparable to that observed following immunization with a subunit WHsAg vaccine. Both types of vaccine (high dose of EP-administered pDNA-WHsAg and conventional WHsAg protein) elicited anti-WHs antibodies in all woodchucks with similar titers and duration throughout most of the study.

WHsAg DNA vaccination by EP induces significant T-cell proliferative responses in woodchucks similar to immunization with conventional WHsAg vaccine. Based on the unique potential for the use of pDNA vaccines as an additional ther-

![FIG. 2. Serum antibody response to WHsAg following non-EP or EP injection of pIMS-310 into the tibialis cranialis muscle of WHV-negative woodchucks. Groups of five animals received administrations at weeks 0, 4, and 8. (A) Non-EP negative vaccine control group. Woodchucks received saline (0.5 ml PBS) in one muscle site by HI injection alone. Anti-WHs titers were determined by ELISA (assay cutoff value, >101 U/ml). (B) Positive vaccine control group. Woodchucks received a conventional protein vaccine (10 µg of alum-adsorbed WHsAg in 0.5 ml PBS) in one muscle site by HI. (C) Non-EP high-dose group. Woodchucks received pIMS-310 (1.0 mg in 0.5 ml PBS) in two separate muscle sites (total of 2.0 mg pDNA per dose) by HI alone. (D) EP low-dose group. Woodchucks received pIMS-310 (0.5 mg in 0.5 ml PBS) in one muscle site (total of 0.5 mg pDNA per dose) by EP. (E) EP high-dose group. Woodchucks received pIMS-310 (1.0 mg in 0.5 ml PBS) in two separate muscle sites (total of 2.0 mg pDNA per dose) by EP. (F) Comparison of anti-WHs GMTs between experimental groups. Arrows indicate the three doses of saline, conventional protein vaccine, or pIMS-310 administered at weeks 0, 4, and 8. Anti-WHs GMTs in the non-EP negative vaccine control group were significantly lower than those in the non-EP high-dose group at week 9, in the EP high-dose group between weeks 6 and 14, and in the positive vaccine control group between weeks 5 and 14 ($P < 0.05$). Anti-WHs GMTs in the non-EP high-dose group were significantly lower than those in the EP high-dose group at weeks 11 and 14, and in the positive vaccine control group at week 9, and again between weeks 11 and 14 ($P < 0.05$). The anti-WHs GMT in the EP high-dose group was significantly lower than that in the positive vaccine control group at week 9 ($P < 0.05$).]
apeutic modality for chronic HBV infection, vaccine potency and immunogenicity was judged here not simply by anti-WHs titer and duration (which is indeed important) but also on the balance of these anti-WHs responses relative to other immune response components, such as the magnitude and type of T-cell responses elicited (Th1 versus Th2), as typified previously in murine species. Regarding overall T-cell activation following immunizations, it was found that in vitro T-cell responses to purified WHsAg protein particles were correlated generally with the anti-WHs responses (Fig. 3). Prior to immunization at T₀, T-cell responses were undetectable with SIs below the assay cutoff (≥3.1). Although serum anti-WHs antibodies were detected in several woodchucks early on, corresponding T-cell responses were not evident in the first week following the initial immunization with pIMS-310 or conventional WHsAg vaccine (since WHsAg is considered a T-cell-dependent antigen, like HBsAg, the apparent dissociation described above likely results because the anti-WHs ELISA is more sensitive than the T-cell proliferation assay being used).

Following the second immunization (week 4), one of five (20%) and two of five (40%) woodchucks from the EP low-dose and EP high-dose groups, respectively, had detectable T-cell responses to WHsAg (SIs ≥ 3.1) (Fig. 3). In contrast, none (0%) of the woodchucks from the non-EP high-dose group had detectable T-cell responses at this time. Three of five (60%) woodchucks from the positive vaccine control group had detectable T-cell responses to WHsAg at this time, whereas no responses were evident in the five (0%) woodchucks from the non-EP negative vaccine control group throughout the study. Thus, the T-cell responses detected were indeed specific to the WHsAg expressed from pIMS-310 or present within the protein vaccine. One week following the third immunization (week 8), T-cell responses to WHsAg were evident in one of five (20%) woodchucks from the EP high-dose group, and in three of five (60%) woodchucks from the positive vaccine control group (Fig. 3). By week 12, four of five (80%) woodchucks from the EP high-dose group...
and two of five (20%) woodchucks each from the EP low-dose and positive vaccine control groups had positive T-cell responses to WHsAg. T-cell responses remained or became undetectable in all (0%) woodchucks from the non-EP high-dose group. Note that the observed animal- and group-associated variability, and the differences in WHsAg-specific T-cell responses, were not a result of individual variation in overall PBMC responsiveness, because overall proliferation to stimulation with polyclonal lymphocyte activators such as ConA (Fig. 3) (and recombinant human IL-2 for T cells and lipopolysaccharide for B cells; data not shown) were the same for all groups, with 100% of animals responding robustly and comparably at each time point.

T-cell responses of immunized woodchucks to WHsAg were analyzed further for fine specificity using selected WHs peptides that represent important protective epitopes (34) within the pre-S1, pre-S2, and S regions of the viral envelope protein (Fig. 3). None of the peptides recalled T-cell responses in any of the woodchucks prior to immunization or immediately following the first immunization, and woodchucks in the non-EP negative vaccine control group remained negative for responses throughout the study period. Woodchucks from the positive vaccine control group developed T-cell responses to WHs peptides S1 and S7/8 following the second immunization at GRRs (for week 5) between 20 and 60%. Both peptides correspond to sequences that are located within the pre-S1 region (L protein) of the WHV envelope (Fig. 3). The conventional WHsAg vaccine consists of 22-nm subviral particles that do contain small amounts of the L protein, whereas vector pIMS-310 contains a DNA sequence encoding only the M and that do contain small amounts of the L protein, whereas vector pIMS-310 contains a DNA sequence encoding only the M and L proteins, whereas vector pIMS-310 contains a DNA sequence encoding only the M and S proteins (pre-S2 and S regions of WHsAg). Also following the second immunization, WHs peptide S11 detected T-cell responses in woodchucks from the EP high-dose and positive vaccine control groups at the same GRR (40%). T-cell responses to this peptide were absent from woodchucks from the non-EP high-dose and EP low-dose groups. WHs peptides S12/13, S18, and S21 induced T-cell responses in 20%, 60, and 40%, respectively, of woodchucks each from the EP high-dose and positive vaccine control groups. Although T-cell responses to these peptides were observed occasionally in woodchucks from the EP low-dose group, the overall responder rates were lower; i.e., WHs peptides S12/13 and S21 recalled T-cell responses in 20% of woodchucks, and S18 in 40% of woodchucks, from this group. T-cell responses to these three peptides were absent from woodchucks from the non-EP high-dose group.

Following the third immunization (week 8), additional woodchucks in the positive vaccine control group developed T-cell responses to WHs peptides S11, S12/13, S18, and S21, with GRRs (at week 9) of 60, 100, and 60%, respectively. T-cell responses in woodchucks from the EP high-dose group indicated GRRs of 40, 40, and 60% for WHs peptides S11, S12/13, and S21, respectively. Woodchucks from the EP low-dose group also had T-cell responses to these peptides but with lower GRRs than those observed for the EP high-dose group, ranging between 20 and 40%. WHs peptides S11, S18, and S21 recalled T-cell responses in one of five (20%) woodchucks from the non-EP high-dose group but no T-cell responses to S12/13. By the end of the study (week 12), T-cell responses to WHs peptides based on GRRs increased slightly in the EP low-dose and EP high-dose groups. GRRs for S18 and S21 finished at 80% in the EP high-dose group and was significantly higher than that in the non-EP high-dose group (0%) (P < 0.05). GRRs for T-cell responses to WHs peptides S11 and S12/13 achieved 60% in the EP high-dose group. The GRRs for the four peptides S11, S12/13, S18, and S21 generally were higher in the EP high-dose group than in the positive vaccine control group (range, 40 to 60%). Although woodchucks from the EP low-dose group had lower GRRs than the EP high-dose group, they were in fact comparable overall to those in the positive vaccine control group.

WHsAg DNA vaccination by EP results in significant expansion of WHsAg-specific CD4+ and CD8+ leukocytes with increased Th1 and decreased Th2 cytokine expression compared to that of conventional WHsAg vaccine. WHsAg-specific T-cell proliferation was significant following both EP pDNA- and protein-based immunizations. These responses were further dissected in terms of T-cell function (Th and CTL) and Th cell skew (Th1 versus Th2). Accordingly, the expression of mRNAs for leukocyte surface markers (CD4 and CD8), Th1 cytokines (IFN-γ and TNF-α), and Th2 cytokines (IL-4 and IL-10) was measured to study the relative expansion of CD4 and CD8 leukocytes and the balance of Th1/Th2 immune responses. In the woodchuck model, this is accomplished by measuring increases in the expression of these mRNAs in PBMCs stimulated in vitro with WHsAg, since reagents are not available to evaluate all of these markers at the protein level. Increases in mRNA expression in immunized woodchucks were discerned first relative to mRNA expression in unstimulated PBMCs from the immunized woodchucks and then controlled further relative to the values for both unstimulated and WHsAg-stimulated PBMCs from the non-EP negative vaccine control group; this group provided relevant baseline measurements at each time point of the study, and overall they showed no evidence of increased mRNA markers at any time during the study period. WHsAg-specific increases were indicated by a 3.1-fold increase in mRNA expression from unstimulated PBMCs (an FI of ≥3.1 was considered an increase, i.e., a positive response).

Prior to immunizations, WHsAg-induced increases in the expression of leukocyte surface marker and cytokine mRNAs were absent in all woodchucks (FIs ≤ 3.1) (Fig. 4). Following the second immunization (week 4), the WHsAg-stimulated samples at week 5 for one of five (20%) woodchucks each from the EP low-dose, EP high-dose, and positive vaccine control groups had increased (positive) IFN-γ mRNA expression (FIs ≥ 3.1) (Fig. 4). CD4 mRNA was increased in one of five (20%) and three of five (60%) woodchucks, respectively, from the EP high-dose and the positive vaccine control groups. Increases in CD8 mRNA and mRNA for other cytokines were not evident at this time point. Following the third immunization (week 8), the WHsAg-stimulated samples at week 9 for two of five (40%) woodchucks from the EP low-dose group and for three of five (60%) woodchucks each from the EP high-dose and positive vaccine control groups had increased IFN-γ mRNA. Interestingly, the same three of five (60%) woodchucks from the EP high-dose group with increases in IFN-γ mRNA also had increased TNF-α mRNA, and two of them (40%) had increased CD8 mRNA. In the positive vaccine control group, one of five (20%) had increased CD8 mRNA and three of five (60%) had increased TNF-α mRNA. Wood-
chucks in the other groups had no WHsAg-specific increases in CD8 or Th1 mRNAs. Three of five (60%) woodchucks from the EP high-dose and positive vaccine control groups had WHsAg-specific increases in CD4 mRNA, whereas one of five (20%) woodchucks from the non-EP high-dose and EP low-dose groups had WHsAg-specific increases in CD4 mRNA. Regarding Th2 cytokines in WHsAg-stimulated cultures, increased IL-4 mRNA was observed in one of five (20%) woodchucks each from the EP high-dose and positive vaccine control groups. Increased IL-10 mRNA was observed in four of five (80%) woodchucks from the positive vaccine control group but only one of five (20%) woodchucks from both the EP and non-EP high-dose groups.

By the end of the study, WHsAg-stimulated samples at week 12 for two of five (40%) woodchucks from the positive vaccine control group had increased CD8, IFN-γ, and TNF-α mRNAs; one of four (25%), two of five (40%), and four of five (80%) woodchucks, respectively, from the non-EP high-dose, EP low-dose, and EP high-dose groups had increased CD8 mRNA. IFN-γ mRNA GRRs in the non-EP high-dose, EP low-dose, and EP high-dose groups were 25, 40, and 100%, respectively, and those for TNF-α mRNA were 0, 40, and 100%, respectively. The difference in GRRs for Th1 cytokine expression was statistically significant between the EP high-dose and non-EP high-dose groups (P < 0.05). The GRRs for the positive vaccine control and non-EP high-dose groups were not significantly different for CD8, IFN-γ, and TNF-α mRNAs. None of four (0%), two of five (40%), and five of five (100%) woodchucks from the non-EP high-dose, EP low-dose, and EP high-dose groups, respectively, had WHsAg-specific increases in CD4 mRNA at this time point, as did three of five (60%) woodchucks from the positive vaccine control group (P < 0.05; EP high-dose group versus non-EP high-dose group). In contrast, WHsAg-stimulated PBMCs from four of five (80%) woodchucks from the positive vaccine control group had increased IL-4 mRNA (P < 0.05; positive vaccine control group versus non-EP high-dose group). In addition, stimulated PBMCs from all five (100%) woodchucks from the positive vaccine control group had increased IL-10 mRNA (P < 0.05; positive vaccine control group versus all pDNA groups). These results indicate a greater Th2 skew in the WHsAg-specific T-cell responses in woodchucks receiving the subunit WHsAg vaccine. Overall, by the end of the study, woodchucks from the EP high-dose group had improved T-cell expression of CD8, IFN-γ, and TNF-α mRNAs in response to stimulation with WHsAg, thus indicating a

![Figure 4](http://jvi.asm.org/.../7.1267768274.jpg)
greater Th1 skew in responses of woodchucks receiving EP immunizations with WHsAg-pDNA.

**DISCUSSION**

The results of this study show that the EP high-dose group had durable anti-WHs antibody and WHsAg-specific T-cell proliferative responses that were associated with the significant expansion of WHsAg-specific CD4+ and CD8+ leukocytes that expressed mainly Th1 cytokines. WHsAg-specific, CD8+ leukocyte accumulation and Th1 cytokine expression were less frequent among woodchucks in the positive vaccine control group and, when detected, were observed mainly in immediate association with the third immunization. WHsAg-specific Th2 cytokine expression typically was more remarkable in the positive vaccine controls and was rare with the non-EP high-dose and EP low-dose groups, and even with the more-immunogenic EP high-dose group. Accordingly, the often similar development of anti-WHs titers and proliferative T-cell responses to WHsAg in the EP high-dose and positive vaccine control groups was characterized by clear differences in WHsAg-specific CD8+ leukocyte expansion and Th1 versus Th2 cytokine mRNA expression. Thus, woodchucks receiving conventional WHsAg vaccine had a diminished expansion of CD8+ leukocytes and reduced expression of Th1 cytokines and the corresponding increased expression of Th2 cytokines, indicating that the high-dose EP of WHsAg-pDNA in a relevant animal model of HBV infection was able to induce a significant shift in the Th1/Th2 balance with a skew toward Th1 immune responses.

The effects of pDNA immunization by EP on anti-WHs responses were dose dependent. EP of the higher vector dose resulted in more woodchucks with detectable antibody response and with higher anti-WHs titers. The alum-adjuvanted WHsAg protein vaccine control and the EP high dose of pDNA were comparable for the overall study in regard to anti-WHs titers elicited and frequency of positive antibody response, but they suggested slightly different kinetics. For example, anti-WHs responses to conventional vaccine were maximal 1 week following the third immunization, whereas those in EP immunizations increased more gradually to their maximum at 4 weeks after the third immunization. In addition, EP at the higher pDNA dose induced more sustained in vitro proliferative responses by WHs-specific T cells, more CD4+ and CD8+ leukocyte activation by WHsAg in cultured PBMCs, and more expression of the Th1 cytokines (IFN-γ and TNF-α) than non-EP immunizations, likely as a result of differential uptake by muscle (Fig. 3 and 4). Furthermore, the EP low-dose group often had improved responses overall compared to those of the non-EP high-dose group, which likely relates to the more efficient delivery by EP of the lower pDNA dose, thus further reinforcing the observed dose dependency relationships. These results are consistent with other reports of the non-EP pDNA vaccination of woodchucks inducing weak and highly variable humoral and cellular immune responses (13, 22, 23, 39, 45).

When enhanced T-cell-proliferative responses to intact WHsAg were observed following pDNA immunizations, they correlated with a broad and sustained set of T-cell-proliferative responses to the select panel of WHs peptides. Importantly for the pDNA vaccines, such profiles closely mimicked those observed in woodchucks following the resolution of acute, self-limited WHV infection (34). This predicts that such T-cell responses (in addition to the anti-WHs responses) elicited by EP of WHsAg-pDNA are protective against WHV challenge in a prophylactic setting. The similar selectivity and specificity of the T-cell responses to the peptide panel following the EP of pDNA and conventional vaccine confirm and extend previous results for woodchucks following immunization with WHsAg protein adjuvanted with alum or cationic liposome-DNA complexes (7, 34). In fact, the serum anti-WHs titers of woodchucks receiving the higher dose of the DNA vaccine by EP (or the protein vaccine) attained levels that are known to be protective against experimental challenge with WHV (14).

DNA vaccine administered by EP was a strong inducer of Th1 cytokines (Fig. 4), which correlate (much more so than Th2 cytokines) with natural recovery and hence protection from chronicity in both adult and neonatal WHV infection (8, 10, 31–33, 46, 47). IFN-γ is produced by a variety of cells, including CD4+ T cells, CD8+ T cells, and NK cells, and generally it is considered a good measure of Th1 cellular immune responses. Consistently with this result, the increased expression of CD4 mRNA was observed in WHsAg-stimulated PBMC cultures that was most pronounced in woodchucks receiving the high-dose pDNA by EP, which induced less expression of Th2 cytokine mRNAs (IL-4 and IL-10) (Fig. 4). Only the woodchucks with substantial pDNA-induced anti-WHs and WHsAg-specific T-cell responses remaining at the later stages postimmunization demonstrated significant accumulation of CD8+ mRNA in stimulated PBMC cultures (i.e., presumably from stimulated CTLs) along with the production of Th1 cytokine mRNAs. The subunit WHsAg protein vaccine induced Th1 immunity in fewer woodchucks, with the diminished expression of the CD8, IFN-γ, and TNF-α mRNAs in PBMC cultures overall, likely because of the greater expression of IL-4 and IL-10 mRNAs as markers of Th2 immunity.

In summary, the present study using pDNA immunization confirms many of the observations for other species, and it now extends them to the woodchuck model on the preferential induction of CTL and Th1 cytokines by DNA vaccines, especially when the cellular uptake of the pDNA is facilitated by EP. This feature of DNA vaccines in combination with EP extends them to the woodchuck model on the preferential induction of Th1 immunity, in fewer woodchucks, with the diminished expression of the CD8, IFN-γ, and TNF-α mRNAs in PBMC cultures overall, likely because of the greater expression of IL-4 and IL-10 mRNAs as markers of Th2 immunity.
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