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

Katherine H. Liu, Mary A. Ascenzi, Christine A. Bellezza, Abraham J. Bezuidenhout, Paul J. Cote, Gloria Gonzalez-Aseguinolaza, Drew Hannaman, Alain Luxembourg, Claire F. Evans, Bud C. Tennant, and Stephan Menne

Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853; Department of Microbiology and Immunology, Georgetown University Medical Center, Washington, DC 20057; Division of Hepatology and Gene Therapy, Center for Investigation in Applied Medicine (CIMA), University of Navarra, 31008 Pamplona, Spain; and Ichor Medical Systems, 6310 Nancy Ridge Drive, Suite 107, San Diego, California 92121

Received 22 November 2010/Accepted 2 March 2011

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 antiviral 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 by conventional HI versus EP were compared to responses generated by a conventional subunit protein vaccine analog using WHsAg adsorbed to alum adjuvant. The immunogenicity study demonstrated in the woodchuck species that EP of pDNA-WHSAg is superior to standard HI of the same vector in producing higher-magnitude immune responses and to the subunit WHsAg vaccine in producing a shift in the Th1/Th2 balance toward Th1 immune responses. Accordingly, model studies of such pDNA vaccine formulations using an EP delivery format are further warranted in WHV-infected woodchucks as potential therapeutic vaccines for the treatment of chronic hepadnavirus infections in humans.

MATERIALS AND METHODS

Experimental animals. Woodchucks were born to WHV-negative females and reared in environmentally controlled laboratory animal facilities at Cornell University. All experiments involving woodchucks were performed according to protocols approved by the Cornell University Institutional Animal Care and Use Committee. A total of 40 healthy adult woodchucks were used.

EP device and parameters. The TriGrid technology from Ichor Medical Systems, Inc. (San Diego, CA), was used for the EP-based delivery of pDNA vectors in situ into woodchuck skeletal muscle. The EP device consists of an integrated applicator containing an autoinjection unit with a replaceable 1.0-mL U-100 insulin syringe (Becton-Dickinson, Franklin Lakes, NV) and the TriGrid electrode array. The electrode array was comprised of four electrodes arranged in two interlocking equilateral triangles to form a diamond shape surrounding a central injection needle. The intraelectrode spacing of the electrode array used in woodchucks was 6 mm. Woodchucks were injected with pDNA under general anesthesia (ketamine at 50 mg/kg body weight and xylazine at 5 mg/kg) using a 23-gauge needle (Becton-Dickinson). pDNA was injected into the skeletal muscle by pulsing the EP device with 400 ms electrical stimulations of 40 ms at a 10% duty cycle. After the completion of pulsing, the EP device was removed and woodchucks were allowed to recover. For the comparison of injection methods, vaccination was performed by HI using the EP device but without the electrode array (referred to herein as HI alone or as non-EP injection).

Vector gene expression. Intracellular and extracellular reporter gene expression. Vectors pCMV-beta and pEGFP were purchased from Clontech (Palo Alto, CA) and used without further modifications. Vector pCMV-beta contains the open reading frame for the lacZ gene, and vector pEGFP contains the open reading frame for the enhanced green fluorescent protein (EGFP) gene, with each reporter gene under the control of the cytomegalovirus (CMV) promoter. Six adult male woodchucks (1 year of age) were used. Three woodchucks were administered a single dose of the LacZ-expressing plasmid (0.5 mg pCMV-beta in 0.5 ml phosphate-buffered saline [PBS]) by EP in the left tibialis cranialis muscle, and then they were given the same vector dose in the right tibialis cranialis muscle by HI alone (i.e., using the EP device but without the electrode array). Three other woodchucks were administered a single dose of the GFP-expressing plasmid (0.5 mg pEGFP in 0.5 ml PBS) by EP in the left tibialis cranialis muscle and then given the same vector dose in the right tibialis cranialis muscle by HI alone. Woodchucks were euthanized 4 days later, and the injected muscle bellies were excised and cut longitudinally into three pieces. For LacZ expression, a commercially available kit (LacZ detection kit for tissues; Invitrogen, San Diego, CA) was used. For GFP expression, muscle tissue was placed on ice and then examined immediately by fluorescence microscopy.

(ii) Secreted reporter gene expression. Vector pgWIZ-SEAP contains the open reading frame of the secreted alkaline phosphatase (SEAP) gene under the control of the CMV promoter (Gene Therapy Systems, San Diego, CA). Six adult male woodchucks (3 months of age) were used, and the animals were stratified by body weight. Three woodchucks were administered a single dose of the SEAP-expressing plasmid (0.5 mg pgWIZ-SEAP in 0.5 ml PBS) by EP into the left tibialis cranialis muscle. Three other woodchucks received the same vector dose by HI alone into the left tibialis cranialis muscle. Blood samples were obtained from all woodchucks prior to the administration of pDNA at time zero (T0) and then at days 2, 4, and 7 of the study for the measurement of serum SEAP activity using the Phospha-Light system from Applied Biosystems (Foster City, CA). Briefly, a 1:50 dilution of serum was incubated with chemiluminescent substrate and luminescent enhancer, and luminescence was measured using the Synergy 2 multimode microplate reader from BioTek (Winooski, VT). Standard curves were generated based on serial dilutions of SEAP (Sigma, St. Louis, MO) in PBS and were used to convert relative luminescence units into enzyme concentrations.

EP immunization with pDNA expressing WHsAg. (i) Vector and vaccine. Vectors pMSs-310 contains a codon-optimized DNA sequence encoding the middle (M) protein of the WHV7 envelope (i.e., pre-S2 and S regions of WHsAg) (8) under the control of the CMV promoter as described previously (27). Vector pIMS-310 was suspended in PBS to provide pDNA doses of 0.5 and 1.0 mg.

The WHsAg used as the conventional vaccine consisted of 22-nm particles purified by zonal ultracentrifugation from the serum of woodchucks infected chronically with WHV (14). The purified WHsAg particles were inactivated with formalin but were not pretreated with enzymes that remove pre-S sequences. Formalin-inactivated WHsAg was adsorbed to alum, as described previously for HBsAg (14), and was adjusted with PBS to provide a vaccine dose of 10 μg per 0.5 ml. Prior to use, the vaccine antigen had been safety tested and shown to be...
free of infectious WHV by the intravenous administration of 1 ml of a more concentrated, preadsorption, 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 WHsAg-pDNA (0.5 mg pM3S-310 in 0.5 ml PBS) by EP in the left tibialis cranialis muscle. Also at these times, five woodchucks (EP high-dose group) were administered WHsAg-pDNA (1.0 mg pM3S-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 WHsAg-pDNA (1.0 mg pM3S-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 vaccine control group) received the subunit WHsAg vaccine (i.e., 10 μg of formalin-inactivated, alun-adsorbed WHsAg protein in 0.5 ml PBS) by standard HI. This vaccine was injected, per our standard routine injection procedures, into the left semimembranosus/semitendinosus muscles at weeks 0 and 8 and into the right semimembranosus/semitendinosus 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 period of immunization. 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 WHsAg (anti-WHs) 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 WHsAg 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).

WHV antigen and antibody markers. WHsAg, anti-WHs, 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 (WHsAg) and 1:100 (anti-WHs and anti-WHC) (9). The cutoff value of these assays was defined as ≥0.05 optical density units (ODU). In addition, anti-WHs was quantified in serum by serial dilution using enzyme-linked immunosorbent assays (ELISA) in three 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 WHsAg (anti-WHs) 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 WHsAg and for the expression of leukocyte surface marker and Th1/Th2 cytokine mRNAs (see below).

Results

Expression of leukocyte surface marker and cytokine mRNAs. The expression of mRNAs for the woodchuck leukocyte surface markers CD4 and CD8, for the Th1 cytokines IFN-γ and TNF-α, and for the Th2 cytokines IL-4 and IL-10 was determined by a real-time reverse transcription-PCR (RT-PCR) assay as described previously (30, 37). Briefly, 105 PBMCs were cultured in duplicate in the presence of blank culture medium or culture medium containing WHsAg (2 μg/ml), and the cells were collected after 2.5 days of culture. PBMCs then were lysed, blank medium- or WHsAg-stimulated samples were combined, and total RNA was isolated using the RNaseasy kit from Qiagen (Valencia, CA). Following treatment with DNase 1 (Invitrogen), RNA was reverse transcribed into cDNA with MultiScribe reverse transcriptase (Applied Biosystems) using oligo(dT). Triplicates of cDNA were amplified on an ABI PRISM 7000 sequence detection instrument (Applied Biosystems) using SYBR green master mix (Applied Biosystems) and woodchuck-specific primers. Woodchuck β-actin mRNA expression was used to normalize target gene expression. Transcription levels of target genes were determined by the formula 2ΔΔCT, where ΔΔCT indicates the difference in the threshold cycle between β-actin and target gene expression. For each target gene, the transcription level obtained in the presence of the stimulator (WHsAg) 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 WHsAg-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 transcriptional level for this molecule in WHsAg-stimulated cultures was interpreted to indicate that a WHsAg-specific subset of CD8+ cells accumulated in the PBMC cultures consisting all or in part of WHsAg-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-WHs, WHsAg-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 pM3S-310 was assessed by comparing body weight measurements and hematological and clinical biochemical parameters using Student’s t test (two-tailed). The anti-WHs geometric mean titers (GMTs) of experimental groups were determined before and following immunization, and GMTs were compared between the groups at each sampling using Student’s t test (two-tailed). GRRs positive for anti-WHs responses, WHsAg-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.

Downloaded from http://jvi.asm.org/ on November 3, 2017 by guest

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. 1B).
Patches that received vector pgWIZ-SEAP by EP were statistically indicated to have significantly higher serum SEAP activity levels in woodchucks (error bars) compared to the control group. The geometric mean serum SEAP activity levels in woodchucks receiving vector pgWIZ-SEAP by EP were higher than those in the control group. Vertical lines indicate standard deviations.

The delivery of a plasmid encoding the reporter gene, LacZ, resulted in LacZ expression in muscle tissue surrounding the injection sites, as shown in Fig. 1A, top. In contrast, muscles from the other two woodchucks had no evidence of LacZ expression at this time. Similarly, using the GFP expressing vector, the muscles of all three woodchucks receiving pEGFP by EP had the characteristic green fluorescent signal indicative of marked GFP expression by fluorescence microscopy (Fig. 1B, bottom). No significant fluorescent green signal was detected in muscles of the three woodchucks receiving pEGFP by non-EP injection (Fig. 1B, top). In addition, the delivery of a plasmid encoding the reporter gene, SEAP, with or without EP resulted in significantly higher serum levels with EP delivery (Fig. 1C). These results validate that the EP delivery of pDNA encoding both intracellular and secreted markers into the woodchuck tibialis cranialis muscle results in much more efficient gene expression than delivery by HI alone.

WHsAg DNA vaccination by EP elicits anti-WHs antibody responses in woodchucks similar to those elicited by immunization with conventional WHsAg vaccine. In the EP versus non-EP immunogenicity study in WHV-naive woodchucks using pDNA expressing WHsAg, the initial immunization at time 0 induced anti-WHs within 4 weeks in two of five (40%) and in four of five (80%) woodchucks in the EP low-dose and EP high-dose groups, respectively; each had transient low-level anti-WHs responses with titers ranging between 102 and 231 (Fig. 2). At the same time, anti-WHs was evident in one of five (20%) and in two of five (40%) woodchucks, respectively, following non-EP injection of WHsAg-pDNA (non-EP high-dose) and standard injection of conventional WHsAg vaccine (positive vaccine control). Woodchucks from the non-EP negative vaccine control group that received saline placebo had no detectable anti-WHs at any time throughout the study period. Thus, the higher dose of pLMs-310 given by EP rapidly induced detectable anti-WHs in more woodchucks than did the same construct dose by non-EP injection; however, at these early times, the difference in anti-WHs GMTs between these two groups was not statistically significant ($P > 0.05$).

Following the second immunization at week 4, all five of the woodchucks (100%) in the EP high-dose group remained or became positive for anti-WHs, and four of five (80%) woodchucks in the EP low-dose and non-EP high-dose groups remained or became anti-WHs positive (Fig. 2). Variation was noted in the magnitude of anti-WHs response among individual woodchucks in each group receiving pDNA as well as in the positive vaccine control group (with 100% new anti-WHs positive) (Fig. 2). Among woodchucks receiving WHsAg-pDNA, the peak GMTs for anti-WHs during the observation period were highest in the EP high-dose group at 7 weeks postimmunization (pi) (333 U/ml), followed by that in the EP low-dose (230 U/ml; week 6) and non-EP high-dose groups (154 U/ml, week 5). The anti-WHs GMT in the positive vaccine control group (463 U/ml; week 6) was higher than that in the EP high-dose group but waned thereafter, and at 8 weeks pi the antibody response was similar to slightly lower than that in the EP high-dose group (235 versus 248 U/ml; $P > 0.05$).

Following the third immunization at week 8, anti-WHs titers increased again in all (100%) woodchucks from the EP high-dose group, with a maximum titer at 12 weeks pi (409 U/ml), which waned thereafter (Fig. 2). Anti-WHs titers increased and then waned in three of five (60%) woodchucks from the EP low-dose group (note that one of these woodchucks had no detectable anti-WHs throughout the study) and in four of five (80%) woodchucks from the non-EP high-dose group. Based on the maximum anti-WHs responses for non-EP high-dose and EP low-dose groups at 5 weeks (154 U/ml) and 6 weeks pi (230 U/ml), respectively, the anti-WHs GMTs for the EP high-dose group were significantly higher ($P < 0.05$) than those for the non-EP high-dose and EP low-dose groups between 11 and 14 weeks pi and 12 and 14 weeks pi, respectively. All five woodchucks (100%) from the positive vaccine control group had increased anti-WHs titers following the protein immuni-

FIG. 1. Expression of reporter genes following non-EP or EP injection of pDNA into the tibialis cranialis muscle of woodchucks. (A) LacZ expression. Three woodchucks received a single dose of vector pCMV-beta (0.5 mg pDNA in 0.5 ml PBS) into the right tibialis cranialis muscle by HI alone (non-EP; top) and the same vector dose into the left tibialis cranialis muscle by EP injection (EP; bottom). LacZ expression in muscle tissue surrounding the injection sites was determined 4 days later by immunohistochemistry; results from one subject are shown. (B) GFP expression. Three woodchucks received a single dose of the vector pEGFP (0.5 mg pDNA in 0.5 ml PBS) into the right tibialis cranialis muscle by HI alone (non-EP; top) and the same vector dose into the left tibialis cranialis muscle by EP injection (EP; bottom). GFP expression in muscle tissue surrounding the injection sites was determined 4 days later by fluorescence microscopy; results from one representative subject are shown. Arrows in the bottom panel indicate the muscle tissue with strong fluorescent green signal. (C) SEAP expression. Woodchucks received a single dose of the vector pgWIZ-SEAP (0.5 mg pDNA in 0.5 ml PBS) intramuscularly by HI or with EP (three animals per group). SEAP activity was determined as described in Materials and Methods. The arrow indicates the single dose of vector pgWIZ-SEAP administered at time 0. The single asterisks indicate that the geometric mean serum SEAP activity levels in woodchucks that received vector pgWIZ-SEAP by EP were statistically significantly different at days 2 and 4 from those in woodchucks that received the same vector by non-EP injection ($P < 0.05$). Vertical lines (error bars) denote standard deviations.
The administration of pLMS-310 to woodchucks by non-EP or EP injection was safe based on comparable patterns of body weight changes among these groups and compared to those in the non-EP negative vaccine control and positive vaccine control groups (data not shown). No hepatic flare reactions were observed in immunized groups based on the assay of liver enzyme activities in blood and complete blood counts (data not shown), and based on these measurements it was concluded that there was no evident toxicity related to DNA vaccine administration by non-EP or EP injection.

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 pLMS-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 pLMS-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 pLMS-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 pLMS-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 pLMS-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 6 and again between weeks 9 and 14 (P < 0.05). Anti-WHs GMTs in the EP low-dose group were significantly lower than those in the EP high-dose group at weeks 12 and 14, 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 \( T_0 \), T-cell responses were undetectable with SIs below the assay cutoff (\( \geq 3.1 \)). 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 \( \geq 3.1 \)) (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.

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 \( \geq 3.1 \)) (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 low-dose and EP high-dose groups in this study. 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 non-EP negative 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 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 40, 60, and 100%, 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 IL-4 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-10 mRNA, while only one of five (20%) woodchucks in the EP low-dose and EP high-dose groups had increased IL-10 mRNA (P < 0.05). 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

---

**FIG. 4.** Group responder rates for leukocyte surface marker and Th1/Th2 cytokine mRNA expression. GRRs for CD4, CD8, IFN-γ, TNF-α, IL-4, and IL-10 mRNA expression are presented. The number of woodchucks was five in each group. Expression was considered positive if the FI was ≥3.1. The single asterisks indicate that the GRRs for the non-EP negative vaccine control group were statistically different from those for the EP high-dose group for CD8, IFN-γ, TNF-α, and CD4 mRNA expression at 12 weeks pi and from those for the positive vaccine control group for IL-4 mRNA expression at 12 weeks pi and for IL-10 mRNA expression at 9 and 12 weeks pi (P < 0.05). The double asterisks indicate that the GRRs for the non-EP high-dose group were statistically different from those for the positive vaccine control group for IL-10 mRNA expression at 9 and 12 weeks pi (P < 0.05). The triple asterisks indicate that the GRRs for the EP low-dose group were statistically different from those for the positive vaccine control group for IL-10 mRNA expression at 12 weeks pi (P < 0.05). The quadruple asterisks indicate that the GRR for the EP high-dose group was statistically different from that for the positive vaccine control group for IL-10 mRNA expression at 12 weeks pi (P < 0.05).
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-adjutanted 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 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 could be used to modulate immune responses in certain disease conditions that are characterized by deficient or tolerant Th1 immune responses, such as chronic HBV infection. It is believed that a so-called cure of chronic HBV infection by therapeutic vaccination will involve the induction of a recovery phenotype similar to that observed following natural recovery from acute infection with HBV. This recovery phenotype should include the emergence of the relevant viral antigen-specific B and T cells from any tolerant or unresponsive state, resulting in an optimal combination of robust antibody, Th1, and CTL responses against HBV. The limited therapeutic potential for licensed HBV vaccines alone (i.e., HBsAg protein in alum) to induce a therapeutic recovery in patients with chronic HBV infection is well known. In the woodchuck, the use of pDNA vaccines expressing WHsAg administered by EP to induce preferentially skewed Th1 immune responses may provide additional strategies for modeling therapeutic vaccination in chronic HBV infection.
REFERENCES