

**Immunogenicity in Mice and Rabbits of DNA Vaccines Expressing
Woodchuck Hepatitis Virus Antigens**

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Abstract

The licensed vaccine against hepatitis B virus (HBV) is an effective means to prevent infection, but is not an effective therapeutic strategy to treat established chronic infections when used alone. In an animal model of chronic HBV infection (the woodchuck experimentally infected with woodchuck hepatitis virus (WHV)), the combination of conventional vaccine and potent antiviral drugs has shown promise as a potential therapeutic intervention. This approach might be improved further through the application of newer vaccine technologies. In the present study, we evaluated electroporation (EP)-based intramuscular (i.m.) delivery of a codon optimized DNA vaccine for the WHV surface antigen (WHsAg) in mice and rabbits. In mice, this immunization procedure compared favorably to vaccination by i.m. injection of the DNA vaccine or i.m. administration of a recombinant WHsAg-alum vaccine, exhibiting characteristics expected to be beneficial for a therapeutic vaccine strategy. These included dose efficiency, consistency, vigorous induction of antibody responses to WHsAg, as well as a Th1 bias. Following scale-up to rabbits, a species that approximates the size of the woodchuck, the EP dosing regimen was markedly more effective than conventional i.m. injection of the DNA vaccine. Taken together, these results provide the foundation for studies of EP-based DNA immunization in the woodchuck in order to further assess its potential as an immunotherapeutic approach for treatment of chronic HBV infection in humans.

Keywords: DNA vaccine; hepatitis; woodchuck; electroporation

Abbreviated title: DNA vaccine for Woodchuck Hepatitis Virus

1. Introduction

Approximately 350 million people worldwide are chronically infected with hepatitis B virus (HBV) and have a high risk of developing liver cirrhosis and primary liver cancer [1]. The recent introduction of virostatic nucleoside and nucleotide analog drugs represents an important development since they can significantly suppress viral replication. However, discontinuation of drug treatment is accompanied frequently by viral recrudescence [2, 3], requiring long-term therapy. Given that these agents do not generally attain the ultimate goal of eradicating the chronic infection and that they are associated with high cost, the occurrence of adverse events, and emergence of drug-resistant mutants [3, 4], anti-viral therapy alone is sub-optimal for patients chronically infected with HBV. It is thought that achieving durable remission or cure of the chronic HBV infection and a reduced risk of disease sequelae requires the restoration of the recovery phenotype, including the emergence of Th cells from any unresponsive state, antibody against HBV surface antigen (anti-HBs), and anti-HBV CTL responses [5-13]. Favorable immune response markers potentially associated with a therapeutic response might include a Th1 skew with IFN- γ -producing CD4 and CD8 T cells.

The only licensed immunotherapeutic option for chronic HBV infection is interferon- α [14]. However, treatment with interferon- α is a relatively long-term therapy that induces durable remission in only a minority of patients and is associated with frequent side effects. Given these considerations, a vaccine immunization approach that could be applied therapeutically in chronic carriers, alone or in conjunction with antiviral drugs, would represent a timely and perhaps more useful therapeutic strategy, the benefit being the potential for long-term remission without the need of long-term therapy. The HBV vaccine currently licensed for prophylactic use did decrease viral loads in a small number of chronic hepatitis B patients during or immediately following a

course of vaccination [15, 16], but it was of little further efficacy for long-term control (or elimination) of the infection [17-19]. Lack of efficacy in the setting of chronic HBV infection is likely due to the inability of the recombinant subunit HBV vaccine to induce sufficient Th1-mediated effector CTL responses in the face of an opposing Th2 response bias [9-11, 20]. In contrast, plasmid DNA vaccines, because of their apparent ability to induce a Th1 skew toward cellular immune responses [21, 22], may represent an appealing alternative strategy for immunotherapy of chronic hepatitis B [23].

In support of this idea, a number of studies have shown the induction of potent CTL responses following DNA immunization with HBV antigens in normal mice [24-28], HLA-transgenic mice [29, 30], and non-human primates [31]. In addition, two recent proof-of-concept clinical studies have demonstrated that conventional i.m. injection of plasmid DNA vaccines in chronic HBV carriers can induce viral specific CD8 T cell responses and even durable reduction in viral titer in some patients [32-34]. Based on these promising results, the further development of plasmid DNA vaccines for therapy appears warranted. In light of the low efficiency of transfection characteristic of conventional DNA injection, the investigation of methods for enhancing intracellular delivery of the DNA are of particular interest [35]. Our long-term goal is to assess whether using electroporation (EP), a potent DNA delivery method [36], could be useful to obtain a stronger, more consistent therapeutic response to HBV DNA vaccination in patients with chronic hepatitis B. We, and others, have recently reported that EP-based delivery of plasmid DNA vaccines expressing HBV surface antigen (HBsAg) or HBV core antigen (HBcAg) into normal laboratory animals markedly enhanced immune responses to these antigens as compared to conventional i.m. injection of the DNA vaccines [37-40]. Based on these results, assessment of EP-based vaccination in an animal model of chronic HBV infection is warranted.

Woodchucks experimentally infected with the woodchuck hepatitis virus (WHV) represent an accepted, well-characterized model for chronic HBV infection [41, 42]. WHV and HBV are closely related viruses as shown by studies in which chimpanzees immunized with a WHV surface antigen (WHsAg)-alum vaccine produce antibody against WHsAg (anti-WHs), with low levels of cross-reactive anti-HBs (indicating that cross-reactive epitopes are in fact present), and they were protected against acute type B hepatitis when challenged with HBV [43]. However, testing of HBV antigen-specific DNA vaccines in woodchucks is of limited value because the woodchuck, as a species, does not recognize sufficient numbers of cross-reactive epitopes on HBV antigens to permit relevant testing. For example, even though adult woodchucks vaccinated with conventional HBsAg-alum vaccine developed high titers of anti-HBs, they did not develop detectable cross-reacting anti-WHs, and were not protected against acute hepatitis following challenge with WHV [43]. Accordingly, in order to proceed with more extensive proof of concept for DNA vaccines in the woodchuck as a relevant disease model (as opposed to using HBV DNA vaccines in limited numbers of chimpanzees), it was necessary to develop the species-specific antigen analogs of WHV (e.g., WHsAg in place of HBsAg). We, therefore, developed WHsAg DNA vaccine candidates for eventual testing in that model. Since definitive studies in woodchucks can often be time and resource intensive (e.g., [13]), initial immunogenicity testing of WHV antigen-specific vaccine candidates was evaluated in normal mice and rabbits to provide the foundation to move into testing in chronic WHV carrier woodchucks. In addition to verifying the immunogenicity of the vaccine candidates and providing data to support vaccine dose selection in the woodchucks, these studies also provided an opportunity to assess qualitative aspects of the immune response. Specifically, there are currently no data with WHV antigens assessing the Th1 versus Th2 balance in the response when

the target antigens are administered as an adjuvanted protein or as a DNA vaccine. Finally, the effect of methods for enhancing antigen expression from DNA vaccines, including improved vector sequences and the use of codon-optimized (CO) antigen sequences were also assessed in order to model the most potent vaccine candidates for use in the woodchucks.

2. Materials and methods

Animals

Female Balb/c mice (8-12 weeks) were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Experiments were approved by Ichor's Institutional Animal Care and Use Committee (IACUC) and were conducted under the guidelines set forth by the National Institutes of Health in the *Guide for Care and Use of Laboratory Animals*. Female New Zealand White rabbits (2.5-3.0 kg) were housed at LAB International (San Diego, CA). Experiments were approved by LAB International's IACUC and were conducted under the regulations set forth by the USDA.

Plasmids

The wild type (WT) gene encoding the middle (M) protein (preS2 and S regions) of WHsAg for the WHV7 isolate was synthesized by GeneArt (Regensburg, Germany) based on a previously published sequence [44] (GenBank accession number NC-004107). A CO version of the gene was also obtained from GeneArt. The optimization modifications did not change the predicted amino acid sequence of the CO expressed protein from that of the WT protein. The recombinant DNA plasmids, designated pIMS-244 and pIMS-247, were produced at Ichor by inserting, respectively, the WT or the CO WHsAg gene into the mammalian expression vector

pVAX-1 (Invitrogen, Carlsbad, CA), which utilizes a shortened version of the CMV enhancer/immediate early promoter and the bovine growth hormone polyA signal to drive gene expression.

The CO sequence of the WHsAg gene was cloned also into pIMS-245, another vector backbone, which was prepared by modifying the gWIZ mammalian expression vector (Genelantis, San Diego, CA) by a reversal of its kanamycin gene through a Msc I + Stu I fragment reversal, and a replacement of its poly A with a modified rabbit β -globin polyA. The resulting pIMS-245 vector contains the full length CMV enhancer, intron A and immediate early promoter elements, combined with a highly efficient synthetic rabbit β -globin polyA signal, to drive high levels of gene expression. The CO WHsAg gene was cloned from the pVAX-1 vector version into the pIMS-245 vector as a Sal I to Xba I fragment (pIMS-310). A CO version of the WHV core antigen (WHcAg) gene sequence from the WHV7 isolate [44] (GenBank accession number NC-004107) was synthesized by GeneArt with Nhe I and Pst I unique restriction sites added at its 5' end and with Not I and Xba I unique restriction sites added at the 3' end. Subsequently, the CO WHcAg sequence was sub-cloned into the pVAX-1 vector as an Nhe I to Not I insert (pIMS-362) and into pIMS-245 as a Pst I to Xba I insert (pIMS-363).

Plasmids were transferred into TOP10 chemically competent *E. coli* (Invitrogen, Carlsbad, CA) and grown on kanamycin selective antibiotic plates. Plasmid batches were prepared using the Qiagen Endofree Plasmid Giga Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and dissolved in 1X calcium and magnesium free PBS (Mediatech Inc., Herndon, VA).

Immunization procedure

Mice and rabbits were anesthetized with isoflurane gas anesthesia. Then, fur was clipped over the administration site followed by aseptic swabbing. Vaccine delivery was performed by an intramuscular (i.m.) administration of plasmid DNA using Ichor's TriGrid™ EP technology as previously reported [39]. The inraelectrode spacing of the TriGrid electrode array used in mice and rabbits was 2.5 mm, and 6.0 mm, respectively.

For EP-based DNA immunization, mice were injected into one *tibialis anterior* (TA) muscle with 20 µl of DNA solution using a 3/10cc U-100 Insulin syringe (Becton-Dickinson, Franklin Lakes, NJ). Rabbits were injected with 0.5 ml into the *vastus lateralis* of one quadriceps muscle using a 1cc syringe (Becton-Dickinson) with a 23 gauge needle. DNA dose was as indicated in the figures. Injection of DNA was followed immediately by electrical stimulation at an amplitude of 250 volts/centimeter of electrode spacing. The total duration of electrical stimulation was 40 mS, applied over a 400 mS interval (a 10% duty cycle). After completion of pulsing, the integrated TriGrid administration device was removed and the animal was transferred to warm recovery. DNA immunization controls consisted of conventional i.m. injection of the antigen-expressing DNA vaccine into the TA (mice) or into the quadriceps (rabbits).

In certain experiments, we included additional control immunizations with a conventional WHsAg protein vaccine that was formalin inactivated and adsorbed onto alum, as described elsewhere [10, 20]. Mice were injected i.m. into the quadriceps with 2 µg of WHsAg vaccine in a 100 µl volume. Rabbits were injected i.m. into the quadriceps with 10 µg of WHsAg vaccine in a 200 µl volume.

Assessment of immune responses

At various times following immunization, blood was collected by retro-orbital bleed under isoflurane gas anesthesia in mice, or by central auricular artery bleed in rabbits. Serum was recovered by centrifugation. Anti-WHs and anti-WHc antibody responses were measured by ELISA. Briefly, serial dilutions of serum samples were added to 96-well plates coated with 100 ng/well of purified WHV antigens; WHsAg was derived in our laboratory by established 4-step ultracentrifugation from the serum of a chronic carrier woodchuck infected with the WHV7 isolate [45], and WHcAg was a gift from Dr. D. Peterson, University of Virginia.

Standard ELISA assays were used for final quantification of the titers of antibodies in all of the test sera from each individual experiment in retrospect. Assays were performed under fully controlled conditions to enable precise calculations and comparisons within and between assays and assay runs. The results were calculated as titers at specified OD readings using appropriate standards, controls, and re-assay of certain samples in order to assure assay consistency for comparisons among experiments. Horseradish peroxidase (HRP)-labeled anti-mouse IgG (R&D Systems, Inc., Minneapolis, MN) was used to determine total mouse IgG responses, and anti-mouse IgG₁ and anti-mouse IgG_{2a}, (Southern Biotechnology Associates, Birmingham, AL) were used for a comparative analysis of isotype responses. Antibody titer was calculated as a reciprocal sample dilution as indicated above using a Dynatech ELISA reader set for dual filter absorbance readings at 630 nm and 490 nm. Antibody titer was calculated as the reciprocal of the sample dilution yielding an OD₄₉₀ of 0.600. For studies depicted in Figures 5 and 6, biotinylated anti-mouse IgG (KPL, Inc., Gaithersburg, MD), or anti-rabbit IgG (KPL), as well as streptavidin-horseradish peroxidase conjugate (Zymed Laboratories, Inc., South San Francisco, CA) and

SureBlue TMB microwell peroxidase substrate (KPL) were used for detection. OD reading at 450 nm was performed using a Model 550 microplate reader (BioRad, Hercules, CA). Antibody titer was calculated as the reciprocal of the sample dilution yielding an OD₄₅₀ of 0.600. One-sided Student's *t* test was used for statistical analysis of the data assuming a lognormal distribution [46]. Statistical significance was set at a value of $p < 0.05$.

3. Results

Comparison of two vaccine candidates for immunogenicity in mice

Two WHsAg DNA vaccine candidates differing by the gene insert were compared for immunogenicity in Balb/c mice. The pIMS-244 vaccine contains a WT nucleotide sequence, while the pIMS-247 vaccine contains a CO sequence of the WHsAg gene. Mice received two immunizations at a monthly interval by EP. Two vaccine doses, 4 and 20 µg of DNA, were tested. Blood samples were collected three weeks after the last immunization for analysis of anti-WHs IgG titers. As shown in Figure 1, antibody titers were induced in all mice. Quantitative analysis of antibody response showed that anti-WHs IgG titers in mice immunized with 20 µg of pIMS-247 (CO) were significantly higher ($p < 0.05$; Student's *t* test) than in mice immunized with 20 µg of pIMS-244 (WT). In addition, titers in mice immunized with 20 µg of pIMS-244 were not significantly different ($p > 0.05$) from titers in mice immunized with 4 µg of pIMS-247. This indicates that pIMS-247 is approximately five times more potent on a dosing basis than pIMS-244. Therefore, pIMS-247 was selected for further studies.

Kinetic analysis of anti-WHs IgG response in mice following DNA or protein vaccination

To further characterize the immune response to the selected vaccine candidate, Balb/c mice were immunized with 4 or 20 µg of pIMS-247 with EP. Controls were immunized by conventional i.m. injection with 4 or 20 µg of pIMS-247. Positive controls were immunized with i.m. injection of 2 µg of purified WHsAg formulated in alum. Three immunizations were administered at monthly intervals. Blood was collected three weeks after each immunization for determination of anti-WHs IgG titers.

As summarized in Table 1, three weeks after the first immunization (day 21), 100% of the animals in the groups immunized with WHsAg/alum or with 20 µg pIMS-247/EP, and 50% of the mice immunized with 4 µg pIMS-247/EP responded with elevated titers (i.e., anti-WHs IgG titers were above preimmune level). In contrast, only one of the animals immunized with 20 µg by conventional i.m. injection of pIMS-247 and none of the animals immunized with 4 µg by conventional i.m. injection of pIMS-247 showed induction of antibody response at this time point. Three weeks after the second immunization (day 49), all the animals immunized with either the DNA vaccine using EP or the protein/alum vaccine displayed elevated anti-WHs IgG titers. At this time point, most, but not all of the animals immunized by direct i.m. injection of the DNA had elevated anti-WHs IgG titers. Three weeks after the third immunization (day 77), 100% of the animals in all groups showed elevated antibody titers.

A quantitative analysis (Figure 2) showed that, at day 21 after the first immunization, elevated anti-WHs IgG geometric mean titers (GMT) were found in cohorts immunized with DNA/EP and protein/alum. However titers were relatively modest at that time point. In contrast, antibody titers in cohorts immunized with conventional i.m. injection of DNA were not significantly different from preimmune levels. Following the second immunization, GMT had

increased in all groups by one to two orders of magnitude as compared to the previous time point. GMT in the cohort immunized with protein/alum was higher by approximately 3 fold than in the group immunized with 20 µg DNA with EP. As indicated in Figure 2, this difference was statistically significant. The GMT in the groups immunized with 20 µg DNA with EP and 4 µg DNA with EP were comparable. The GMT in the group immunized with 20 µg DNA with conventional i.m. injection was significantly lower ($p < 0.05$, Student's *t* test) than the GMT of the groups immunized with EP and comparable ($p > 0.05$) to the GMT of the group immunized with 4 µg DNA with conventional i.m. injection. After the third immunization, the GMT ranking among the different groups remained unchanged. GMT were still significantly higher in the protein vaccine group as compared to the 20 µg DNA/EP group, and in the 4 µg DNA/EP group as compared to the 4 µg DNA/i.m. group. The GMT was also higher in the 20 µg DNA/EP than in the 20 µg DNA/i.m. group; however the difference was not significant. Sixteen weeks after the third immunization, at day 168, all animals still displayed elevated antibody titers, thus showing that the responses were sustained over times with all the vaccine modalities tested.

Analysis of IgG subclasses

The IgG₁ and IgG_{2a} components of the anti-WHs IgG response were also analyzed in this experiment in order to compare the Th1/Th2 skew among the different immunization groups. A high IgG_{2a} / IgG₁ titer ratio is associated with a Th1 response, while a low IgG_{2a} / IgG₁ titer ratio reflects a Th2 response [47]. The anti-WHs IgG₁ and IgG_{2a} GMT are shown in Figure 3. At day 21, both IgG₁ and IgG_{2a} titers were comparable among animals immunized with the protein vaccine and 20 µg DNA/EP, reflecting similar IgG_{2a} / IgG₁ titer ratios amongst the two groups.

Most animals in other groups did not have detectable titers. At day 49, the IgG₁ titers were markedly induced in all immunization groups. As shown in Figure 3, at this and all subsequent time points, anti-WHs IgG₁ GMT was significantly higher in the protein vaccine group than in the DNA/EP immunization groups, and it was significantly higher in the DNA/EP groups than in the corresponding DNA/i.m. immunization groups. In contrast, at day 49 and all following time points, the anti-WHs IgG_{2a} GMT were similar ($p > 0.05$) between the protein vaccine and the 20 µg DNA/EP immunization groups. At day 49, anti-WHs IgG_{2a} GMT were significantly higher in the DNA/EP groups as compared to the corresponding DNA/i.m. groups. However, after the third immunization GMT was significantly higher in the 4 µg DNA/EP as compared to 4 µg DNA/i.m. In contrast, there was no significant difference between the GMT of the 20 µg DNA/EP and 20 µg DNA/i.m. groups.

Overall, these results indicate that after the second immunization, the IgG_{2a} / IgG₁ titer ratios of the protein and DNA groups had diverged. This trend was maintained at day 77, three weeks after the third immunization and at day 168, sixteen weeks after the third immunization. In order to estimate the Th1/Th2 bias in each animal, the IgG_{2a} / IgG₁ titer ratios were calculated in each serum sample at days 49, 77 and 168. As shown in Figure 4, at all three time points, the IgG_{2a} / IgG₁ titer ratios in all the cohorts immunized with DNA were significantly higher than ratios in animals immunized with protein/alum, thereby confirming the trend toward Th2 in the protein group and toward Th1 in the DNA groups. At day 49, the group immunized with protein/alum showed a Th2 skew, while the groups immunized with DNA displayed a mixed Th1/Th2 response. At days 77 and 168, the groups immunized with protein/alum still had a Th2 skew, while the groups immunized with conventional i.m. injection of 4 or 20 µg of DNA, or with 4 µg DNA/EP appeared skewed toward Th1. Interestingly, the group that received 20µg DNA/EP

immunization still displayed a mixed Th1/Th2 response at days 77 and 168, and the IgG_{2a} / IgG₁ titer ratio in this group was significantly lower than in each of the other groups immunized with DNA. However, as indicated in Figure 4, even in this group the IgG_{2a} / IgG₁ titer ratio remained significantly higher than in the group immunized with protein/alum.

Immunogenicity study in rabbits

Although conventional i.m. administration of DNA vaccines generally induces robust immune responses in mice, results in species of larger size (including humans) have generally been suboptimal in terms of potency and consistency [48, 49]. Using a strong delivery method such as EP can help preserve strong immune responses in larger species [50]. Since our goal is to establish an immunization platform suitable for testing in woodchucks, we assessed vaccine immunogenicity in rabbits, a species with approximately the same body size than the woodchuck. New Zealand White rabbits were immunized with 0.4 mg or 2.0 mg of pIMS-247 with EP. Controls were immunized by conventional i.m. injection with 0.4 mg or 2.0 mg of pIMS-247. Positive controls were immunized with 10 µg WHsAg/alum. Initial immunization was administered at day 0. Booster immunizations were administered at weeks 6 and 14. Blood was collected 6-8 weeks after each immunization for determination of anti-WHs IgG titers. As shown in Table 2, following immunization with WHsAg/alum or DNA/EP, rabbits consistently responded with elevated anti-WHs IgG titers after one to two immunizations (i.e., anti-WHs IgG titers were above preimmune level). In contrast, in rabbits immunized with conventional i.m. DNA injection, response was inconsistent, even after three immunizations. As shown in Figure 5, in animals immunized with EP anti-WHs IgG titers were modestly elevated after the first immunization, then dramatically induced after the second immunization. After the third

immunization, there was a trend toward further GMT increase, although this increase was not statistically significant. There was no statistically significant difference between the GMT of the two DNA/EP immunization dose groups. In contrast, GMT in groups immunized with conventional i.m. injection remained at preimmune levels after one or two immunizations, then modestly increased after the third immunization; however, as indicated in Figure 5, the GMT were significantly lower than in the EP immunized animals with an overall difference of approximately two orders of magnitude. Consistent with the results observed in mice (Fig. 2), the GMT were also significantly lower in rabbits immunized with conventional DNA injection than in rabbits immunized with protein/alum. However, in contrast to the one order of magnitude difference in GMT observed with the two different immunization methods in mice (Fig. 2), extrapolation of each immunization procedure to rabbits resulted in GMT that was approximately three orders of magnitude lower with conventional i.m. DNA injection than with protein/alum (Fig. 5). This suggests a marked decrease in the relative immunogenicity of the WHsAg DNA vaccine administered by conventional injection as the procedure is extrapolated from mice to rabbits. In contrast, the ratios of GMT measured in rabbits immunized with DNA/EP and in rabbits immunized with protein/alum were consistent with the results in mice, indicating that EP based delivery can help preserve the immune response to the WHsAg DNA vaccine upon dose scale-up in rabbits.

Combining EP-based delivery and constructs engineered for enhanced gene expression

Improvement in DNA vaccine potency can be achieved by engineering the expression vector to enhance gene expression [35]. We tested whether combining this approach together with EP-based delivery would increase vaccine potency. As described in *Materials and Methods*, we

constructed a vector backbone, pIMS-245, designed for higher gene expression. In separate *in vivo* reporter gene studies, the pIMS-245 backbone was shown to provide a 10-fold increase in expression levels as compared to a pVAX-1 based vector (data not shown). Mice were immunized with pIMS-247, a CO WHsAg DNA vaccine using the pVAX-1 backbone, or pIMS-310, the same CO WHsAg DNA sequence in a vaccine using the pIMS-245 backbone. Mice received three immunizations with 1 µg or 4 µg of DNA, at a monthly interval. Blood samples were collected three weeks after each immunization and analyzed for anti-WHs IgG titers. As shown in Figure 6A, anti-WHs IgG titers in mice immunized with 4 µg of pIMS-310 were significantly higher following the second and third immunizations than in mice immunized with 4 µg of pIMS-247. Similarly, anti-WHs IgG titers in mice immunized with 1 µg of pIMS-310 were significantly higher at these time points than in mice immunized with 1 µg of pIMS-247. In addition, assessment at day 49 showed no statistical difference between titers in mice immunized with 1 µg of pIMS-393 and mice immunized with 4 µg of pIMS-247. This indicates that pIMS-310 is approximately four times more potent on a dosing basis than pIMS-247. The IgG subclass profiles with the two constructs are comparable (data not shown), which suggests that the backbone does not modify the Th1/Th2 skew of the response.

To assess whether the pIMS-245 backbone could be usable for EP-based delivery of other WHV antigens, we assessed immunogenicity of two DNA vaccines for CO WHcAg, including pIMS-362, which is based on pVAX-1, and pIMS-363, which is based on pIMS-245. Two vaccine doses, 1 µg and 4 µg of DNA, were tested. Mice received two immunizations at a monthly interval. Blood samples were collected three weeks after each immunization for analysis of anti-WHc IgG titers. As shown in Figure 6B, both constructs were immunogenic in mice. Anti-WHc IgG titers in mice immunized with 4 µg of pIMS-363 were significantly higher

following the final immunization than in mice immunized with 4 µg of pIMS-362. Similarly, anti-WHc IgG titers in mice immunized with 1 µg of pIMS-363 were significantly higher at this time point than in mice immunized with 1 µg of pIMS-362. In addition, assessment at day 49 showed no statistically significant difference between titers in mice immunized with 1 µg of pIMS-363 and mice immunized with 4 µg of pIMS-362. This indicates that pIMS-363 is approximately four times more potent on a dosing basis than pIMS-362.

4. Discussion

This article reports the characteristics of immune responses induced by EP-based WHsAg DNA immunization in normal mice and rabbits. Studies in mice showed that EP-based immunization induced faster, higher magnitude, anti-WHs IgG response than conventional i.m. injection of the DNA vaccine. EP also appeared to be a more dose efficient procedure than conventional i.m. immunization, maintaining comparable responses even with a five fold reduction in DNA dose (20 µg to 4 µg). In contrast, a significant decrease in antibody titer was observed with conventional i.m. injection when the DNA dose was reduced from 20 µg to 4 µg. In addition, EP-based immunization required fewer immunizations than conventional injection to induce response in 100% of subjects in the mouse model (two vs. three). It is noteworthy that after the third immunization, anti-WHs IgG titers induced using EP were statistically higher than titers induced using conventional i.m. injection only at the lower dose (4 µg), but not at the higher dose (20 µg). The relatively high magnitude response observed using conventional i.m. injection with the higher dose of vaccine after three immunizations in mice suggests that, in the mouse model, the relatively low efficiency of conventional DNA delivery may be overcome

using relatively high DNA dose levels and multiple immunizations. However, it is important to note that as the administration procedure was extrapolated for administration in rabbits, immunogenicity assessment showed that, in contrast to mice, conventional i.m. DNA immunization induced no or minimal response, even after multiple immunizations. This is consistent with previous observations that DNA vaccines tend to be less immunogenic in larger species than in mice [51]. In contrast, in rabbits immunized using EP, immune response to the WHsAg DNA vaccine was preserved with anti-WHs IgG titers that appeared comparable to titers found in mice.

We identified improvements in vector design that were conducive to stronger immunogenicity. Codon optimization appeared to enhance vaccine potency by approximately five times. This effect, which has been previously shown with other viral antigens [52-54], has been linked in several cases to improved gene expression associated with the use of codon-optimized genes [55, 56]. The use of a vector backbone engineered for high gene expression appeared to further enhance vaccine potency, which could be quite advantageous for dose sparing as well as the development of more complex vaccines (for instance including several WHV antigens and/or a genetic adjuvant).

These studies also provided the opportunity to compare the immune responses elicited with a DNA vaccine to those induced by a conventional subunit vaccine. The WHsAg protein vaccine was administered in mice at a dose (2 µg) that is approximately 20- to 30-fold in excess of the minimal dose typically needed to induce detectable antibody in 50-100% of mice (i.e., based on studies with analogous HBsAg vaccines; P. Cote and J. Gerin, unpublished observations). At this dose of WHsAg protein, the vaccine induced a moderately higher magnitude anti-WHs IgG response than DNA immunization with EP. However, analysis of the antibody subclasses

revealed that the response profile with protein/alum and DNA/EP immunizations were quite different. While protein-based immunization induced a predominantly IgG₁ response, DNA immunization induced either a more balanced IgG₁ / IgG_{2a} response or a predominantly IgG_{2a} response, depending on number of immunizations and immunization protocol.

As discussed in the introduction, induction of strong CTL responses and Th1 bias are considered desirable characteristics for a therapeutic vaccine candidate for chronic infection with HBV. It is thought that the limited ability of the licensed HBV vaccine, a protein vaccine based on purified HBV surface antigen (HBsAg) formulated in alum, to induce a therapeutic effect in chronic HBV patients [15, 16] could be due to the induction of a Th2 skew and the inability of this vaccine to induce CTL responses [19]. A comparable conclusion has been reached in the woodchuck model using a protein vaccine based on purified WHsAg formulated in alum [10]. Induction of Th1 skew and robust CTL responses has been previously reported for a number of DNA vaccines, and is considered a general characteristic of this immunization modality [22]. However, exceptions have been reported with predominant induction of IgG₁ over IgG_{2a} with some antigens [57-59]. Since there is no method available to predict such exceptions, it is useful to test IgG subclass induction for any new DNA vaccine candidate. The data presented here show that the WHsAg protein vaccine induced a response with a Th2 skew. In contrast, DNA vaccination either by EP or conventional i.m. injection induced a shift of the response toward Th1. Of note, the shift of the response toward Th1 in the EP group was not as pronounced as in the conventional i.m. group, especially at the higher vaccine dose. Although we currently do not have an explanation for this difference between DNA immunization administered by conventional i.m. delivery or EP, numerous reports have shown the ability of EP-based DNA vaccines to induce both antibody and T cell responses (reviewed in [50]). A balanced Th1/Th2

response may reflect the ability of EP to stimulate both of these arms of the immune response, which could be useful for a therapeutic HBV vaccine, as well as other vaccines.

Taken together, these observations provide the experimental basis to implement EP-based DNA vaccine studies in the woodchuck model. Previous reports have shown that EP-based DNA vaccination induces marked cellular responses against a variety of viral antigens in immunologically well-characterized species such as the mouse [60, 61], as well as larger species such as farm animals [38, 62] and non-human primates [63-66]. Also, the induction of CD8 T cell responses following EP-based DNA immunization against HBsAg, a protein closely related to WHsAg, has been recently reported in mice [39] and non-human primates [40]. Therefore, it is likely that EP-based DNA immunization against WHsAg will be able to induce the analogous responses in woodchucks, thereby potentially enabling antiviral effect and subsequent therapeutic effect against disease progression. Having extrapolated the immunization procedure into rabbits, we are well positioned to select doses capable of eliciting immune responses in woodchucks. We anticipate that favorable results in the woodchuck model would not only provide proof of principle for EP-based DNA therapeutic immunization for hepatitis B, but also valuable information (including dosing, administration protocols and safety data) to help support translation into human subjects. Of note, EP-based DNA immunization has now reached the clinical stage [50]. In particular, EP is being actively investigated with several Phase I clinical trials for therapeutic and prophylactic DNA vaccines in indications ranging from cancer to infectious diseases [50]. Therefore, it is conceivable that a therapeutic vaccine for chronic hepatitis B using EP based DNA immunization could be rapidly translated into human testing.

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Table 1: Numbers of mice in each immunization group displaying anti-WHs IgG titers above preimmune level

	4 µg DNA/i.m.	20 µg DNA/i.m.	4 µg DNA/EP	20 µg DNA/EP	2 µg WHsAg/alum
Day 0	0/5	0/6	0/6	0/6	0/6
Day 21	0/5	1/6	3/6	6/6	6/6
Day 49	4/5	5/6	6/6	6/6	6/6
Day 77	5/5	6/6	6/6	6/6	6/6
Day 168	5/5	6/6	6/6	6/6	6/6

Table 2: Numbers of rabbits in each immunization group displaying anti-WHs IgG titers above preimmune level

	0.4 mg DNA/i.m.	2.0 mg DNA/i.m.	0.4 mg DNA/EP	2.0 mg DNA/EP	10 µg WHsAg/alum
Day 0	0/4	0/4	0/4	0/4	0/4
Day 42	1/4	0/4	4/4	2/4	4/4
Day 98	2/4	0/4	4/4	4/4	4/4
Day 143	2/4	3/4	4/4	4/4	4/4

Figure legends

Figure 1: Immunogenicity of two WHsAg DNA vaccine candidates in mice. Balb/c mice (N=6) were immunized at days 0 and 28 by injecting in one TA muscle with EP 4 μg (gray diamonds) or 20 μg (black diamonds) of pIMS-244 containing WT WHsAg sequence, or 4 μg (gray squares) or 20 μg (black squares) of pIMS-247 containing CO WHsAg sequence. Preimmune levels are indicated by a circle. Three weeks after the last immunization, serum was collected, and anti-WHs IgG titers were assessed. Horizontal bars represent the geometric mean titers. Statistical difference of each pIMS-247 immunized group relative to the 20 μg pIMS-244 group are shown as * ($p < 0.05$) using a one sided Student's *t* test assuming a lognormal distribution.

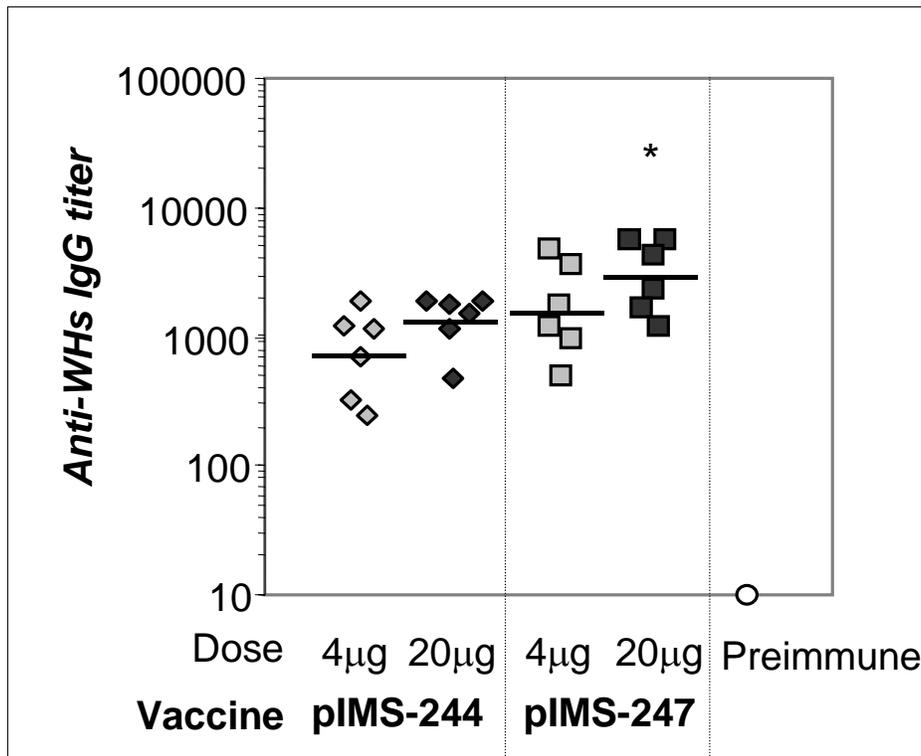


Figure 2: Assessment of anti-WHs IgG responses in mice following immunization with DNA or protein vaccine. Balb/c mice (N=6) were immunized at days 0, 28 and 56 by administering 4 µg or 20 µg of pIMS-247 by conventional injection or with EP in one TA muscle. Positive controls (N=6) were immunized by injecting 2 µg of purified WHsAg formulated in alum in one quadriceps. Serum was collected three weeks after each immunization (days 21, 49 and 77). A final serum collection took place 16 weeks (day 168) after the final immunization. Anti-WHs IgG titers were assessed in individual serum samples. Error bars represent one standard deviation. Statistical difference of each DNA/EP immunized group relative to the corresponding DNA/i.m. group, and of the protein group relative to the 20 µg DNA/EP group are shown as * (p<0.05) and ** (p<0.01) using a one sided Student's *t* test assuming a lognormal distribution.

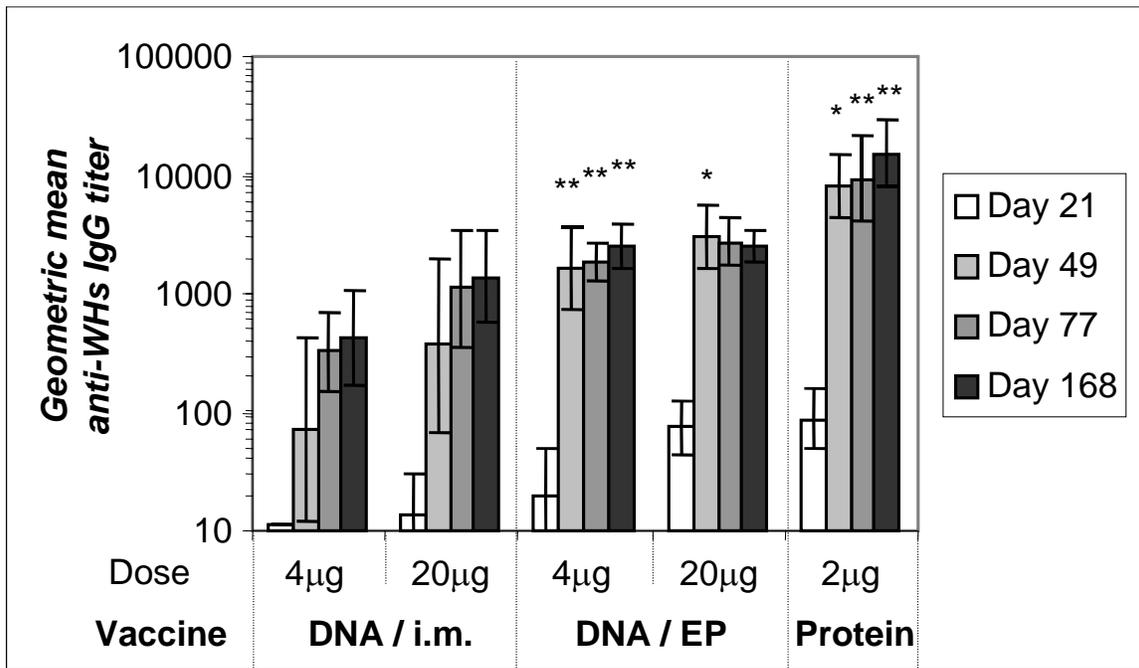


Figure 3: Assessment of anti-WHs IgG subclasses following immunization with DNA or protein vaccine in mice. Balb/c mice (N=6) were immunized at days 0, 28 and 56 by administering 4 µg or 20 µg of pIMS-247 by conventional injection or with EP in one TA muscle. Positive controls (N=6) were immunized by injecting 2 µg of purified WHsAg formulated in alum in one quadriceps. Serum was collected three weeks after each immunization (days 21, 49 and 77). A final serum collection took place 16 weeks after the final immunization (day 168). Anti-WHs IgG₁ (A) and IgG_{2a} (B) titers were assessed in individual serum samples. Error bars represent one standard deviation. Statistical difference of each DNA/EP immunized group relative to the corresponding DNA/i.m. group, and of the protein group relative to the 20 µg DNA/EP group are shown as ** (p<0.01) using a one sided Student's *t* test assuming a lognormal distribution.

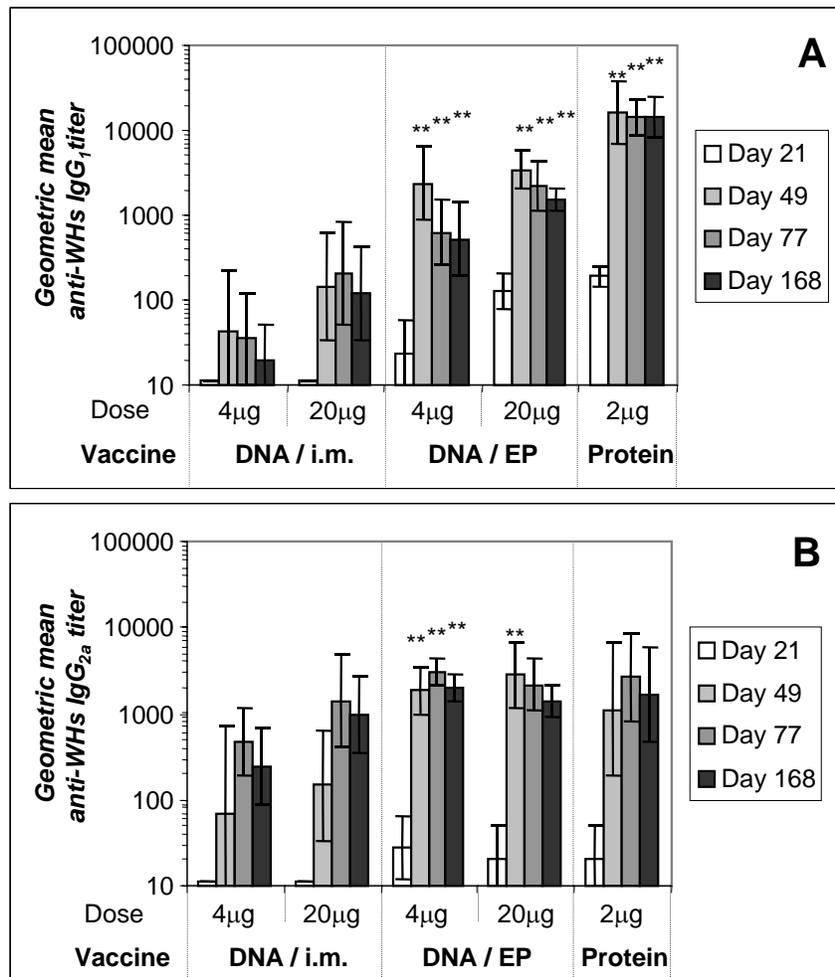


Figure 4: IgG_{2a} / IgG₁ titer ratios following immunization with DNA or protein vaccine in mice. Balb/c mice were immunized and anti-WHs IgG₁ and IgG_{2a} titers were determined as described in Figure 3. IgG_{2a} titer / IgG₁ titer ratios were determined for each individual animal at days 49 (A), 77 (B) and 168 (C). Mice immunized with DNA by conventional i.m. injection are represented with triangles (white triangles: 4 µg of DNA; gray triangles: 20 µg of DNA); mice immunized with DNA using EP are represented with squares (white squares: 4 µg of DNA; gray squares: 20 µg of DNA); mice immunized with WHsAg in alum are represented with black circles. Horizontal bars represent the geometric means. Statistical difference of each DNA immunized group relative to the protein immunized group are shown as * (p<0.05) and ** (p<0.01) using a one sided Student's *t* test assuming a lognormal distribution.

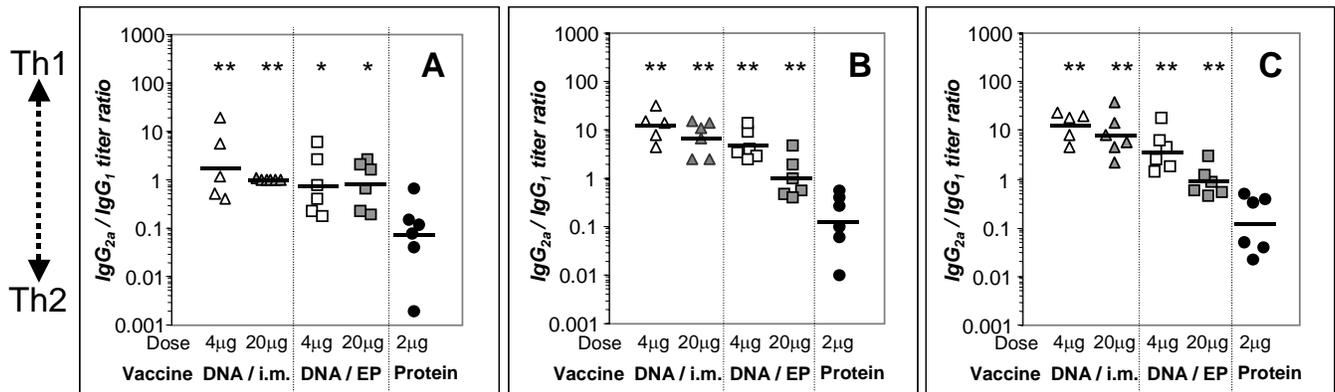


Figure 5: Assessment of anti-WHs IgG responses in rabbits following immunization with DNA or protein vaccine. New Zealand White rabbits (N=4) were immunized at days 0, 42 and 98 by administering in one quadriceps muscle 0.4 mg or 2.0 mg of pIMS-247 by conventional injection or with EP. Positive controls (N=4) were immunized with 10 µg of purified WHsAg formulated in alum in one quadriceps Serum was collected at days 0, 42, 98 and 143. Anti-WHs IgG titers were assessed in individual serum samples. Error bars represent one standard deviation. Statistical difference of each DNA/EP immunized group relative to the corresponding DNA/i.m. group, and of the protein group relative to the 2.0 mg DNA/EP group are shown as ** (p<0.01) using a one sided Student’s *t* test assuming a lognormal distribution.

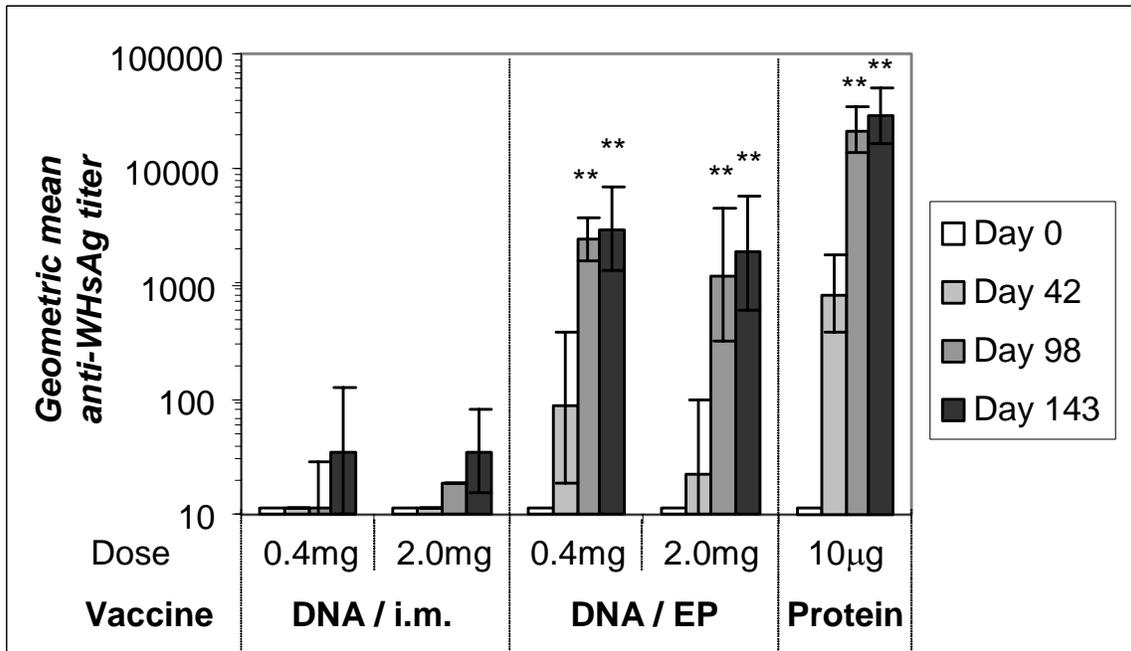


Figure 6: Immunogenicity of WHsAg and WHcAg DNA vaccine candidates based on two different vector backbones in mice. (A) Balb/c mice (N=5) were immunized at days 0, 28 and 56 by injecting in one TA muscle with EP of pIMS-247, a CO WHsAg DNA vaccine based on the pVAX-1 vector backbone, or pIMS-310, a CO WHsAg DNA vaccine based on the pIMS-245 backbone, using doses of 1 µg or 4 µg of DNA. Serum was collected at day 0, then three weeks after each immunization, and anti-WHs IgG titers were assessed. (B) Balb/c mice (N=5) were immunized at days 0 and 28 by injecting in one TA muscle with EP of pIMS-362, a CO WHcAg DNA vaccine based on the pVAX-1 vector backbone, or pIMS-363, a CO WHcAg DNA vaccine based on the pIMS-245 backbone, using doses of 1 µg or 4 µg of DNA. Serum was collected at day 0, then three weeks after each immunization, and anti-WHc IgG titers were assessed.

Error bars represent one standard deviation. Statistical difference of each pIMS-245 based vaccine dose group relative to the corresponding pVAX-1 based vaccine dose group at days 49 and 77 are shown as * ($p < 0.05$) and ** ($p < 0.01$) using a one sided Student's *t* test assuming a lognormal distribution.

