Analysis of Humoral Immunity of Hepatitis D Virus DNA Vaccine Generated in Mice by Using Different Dosage, Gene Gun Immunization, and *In Vivo* Electroporation

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Background: Hepatitis D virus (HDV) DNA vaccine can produce Th1 and cytotoxic T-cell immune responses but only a low anti-HDV antibody titer is generated with a large hepatitis D antigen (L-HDAg) construct. In contrast, DNA vaccine expressing small hepatitis D antigen (S-HDAg) can generate a high titer of anti-HDV antibodies. Whether the low humoral immunity of L-HDAg DNA vaccine is due to inadequate dosage or can be ameliorated by other modes of immunization needs further evaluation.

Methods: Plasmid (p25L) encoding L-HDAg and plasmid (pS/p25L) coexpressing hepatitis B surface antigen (HBsAg) and L-HDAg were used in this study. We compared the humoral response generated in mice using different plasmid DNA dosages and modes of immunization, including gene gun and *in vivo* electroporation (EP).

Results: Intramuscular injection with a high dose of plasmid DNA (10 mg/kg) produced strong antibodies to HBsAg earlier than the usual dose did, but did not augment the anti-HDV response. Gene gun DNA immunization could not provide a better humoral immune response to HDV. EP DNA immunization had a higher anti-HDV seroconversion rate of 80%, but the anti-HDV antibody responses were generally weak (titer ≤ 400:1).

Conclusion: The low humoral immunogenicity of DNA vaccine with L-HDAg cannot be ameliorated by different dosage, gene gun immunization, or *in vivo* EP intramuscular injection. DNA vaccine with a L-HDAg construct may not be a candidate HDV vaccine to generate anti-HDV humoral immunity. [*J Chin Med Assoc* 2006;69(1):7–13]

Key Words: DNA vaccine, gene gun, hepatitis D virus, *in vivo* electroporation

Introduction

Hepatitis D virus (HDV) is a circular single-stranded RNA virus that requires the presence of hepatitis B surface antigen (HBsAg) from hepatitis B virus (HBV) for assembly and transmission.[1,2] Approximately 350 million individuals are chronically infected with HBV worldwide.[3] Of these, nearly 8% are co-infected with HDV.[4] It is estimated that HDV infects 20 million people globally.[5] HDV superinfection is one of the major causes of fulminant hepatitis in Taiwan.[6] Superinfection with HDV often results in chronic hepatitis, and many cases progress to cirrhosis.[7–10] Furthermore, HDV superinfection also increases the risk of hepatocellular carcinoma (HCC) and the mortality in HBV-related cirrhotic patients.[11]

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Currently, there is still no effective treatment for chronic hepatitis D (CHD). Interferon is a licensed drug to treat CHD, but the relapse rate is high after discontinuation.[12] Lamivudine, a nucleotide analog, can inhibit HBV replication but shows no response in the treatment of CHD.[13] It is necessary to develop an alternative approach to prevent unfavorable outcomes in patients with HDV infection. DNA vaccine can induce a broad range of immune responses and is considered to be a promising method to prevent and treat persistent viral infections. The development of HDV DNA vaccine has a potential use for HBV carriers who are at risk of HDV superinfection or for CHD patients to serve as a therapeutic agent.

HDV has 2 forms of viral proteins, large and small hepatitis D antigens (HDAg). Large hepatitis D antigen (L-HDAg) has an additional 19 amino acids at the C-terminus of small hepatitis D antigen (S-HDAg) due to an RNA editing event.[14,15] Both antigens have their distinct functions. L-HDAg is responsible for HDV assembly and S-HDAg is responsible for viral replication.[2,16] Our previous study demonstrated that HDV DNA vaccine produces Th1 immune response to HDV, but L-HDAg-encoded DNA vaccine could only produce low titers of anti-HDV antibody.[17] In contrast, DNA vaccine expressing S-HDAg can generate stronger anti-HDV antibody titers.[18] The gene gun approach has been reported to increase antibody responses.[19] Another new mode of DNA vaccine immunization, electroporation (EP), can greatly increase the uptake of DNA into cells and is capable of overcoming the cell barrier to enhance the antibody responses.[20,21] In addition, the optimal dosage of DNA immunization is still uncertain. Therefore, it is of interest to determine whether the low humoral immunity of DNA vaccine with L-HDAg construct is due to inadequate dosage or can be ameliorated by other modes of immunization. Consequently, we conducted this study to compare the humoral responses generated in mice using different DNA dosages, gene gun immunization, and *in vivo* EP intramuscular injection.

Methods

Construction of expression vector

The L-HDAg gene was amplified by polymerase chain reaction (PCR) with pairs of primers (δx25: 5' GGCTCTAGAGTAAGAGTACTGAGG 3' and δS: 5' TCATGCATGCCGACCCGAAGAGGA 3') from plasmid TWD2667 (AF104263), which contained the HDV coding region (genotype I) in the PCR II vector, and cloned into the Xba I/Sph I sites in plasmid pCMV EBNA (Invitrogen, San Diego, CA, USA) to produce plasmid p25L.[17] A bicistronic plasmid, pTCAE,[17,22] was used to express HBV major S protein (*adw*) and L-HDAg (genotype I) in the same construct but under discrete CMV promoter control. The HBV major S gene was amplified by PCR from plasmid pEco63 (ATCC 31518; American Type Culture Collection, Rockville, MD, USA) with pairs of primers (5' ACCCTAGATCTAACATGGAGAACA 3' and 5' GCCGAAGCTTACTAGTTACCCAGAGACA 3') and cloned into the Bgl II/Hind III sites of pTCAE to generate plasmid pTCAE/S. The L-HDAg gene was amplified by pairs of primers (5' TCTTGTCGACATGAGCCGTTCCGAG 3' and 5' CGAGATCTTCACTGGGGTCGGCAACTC 3') and cloned into the Sal I/Bam HI sites of pTCAE/S to generate the bicistronic plasmid designated pS/p25L. The expression of HDAg or HBsAg by plasmid p25L or pS/p25L has been demonstrated by immunohistostaining in our previous work.[17] Plasmid pcDNA3 was used as a negative control. Plasmid DNA was purified from transformed *Escherichia coli* DH5α (Gibco BRL, Life Technologies, Gaithersburg, MD, USA) using a QIAGEN Giga plasmid purification kit (TAIGEN Bioscience Corporation, Taipei, Taiwan) and stored as pellets at –70°C. DNA was reconstituted in sterile saline at a concentration of 1 mg/mL for experimental use.

Animals

Female BALB/c mice were obtained from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). Mice were housed at the Laboratory Animal Facility, Taipei Veterans General Hospital, and were immunized at 6–8 weeks of age.

Immunization

Intramuscular immunization

For intramuscular DNA immunization, cardiotoxin (Sigma, St Louis, MO, USA) was given to each mouse 1 week before immunization.[17,18] Groups of mice were anesthetized and given intramuscular injections, into the bilateral quadriceps, at a total dose of 200 μg of plasmid DNA dissolved in 100 μL of sterilized normal saline. Groups of 5 mice were immunized as follows: group 1 with 200 μg of p25L; group 2 with 100 μg of p25L plus 100 μg of pcDNA3; group 3 with 200 μg of pS/25L; group 4 with 100 μg of pS/25L plus 100 μg of pcDNA3; and group 5 with 200 μg of pcDNA3. Each mouse was given booster doses 3 and 6 weeks after the first immunization.

Gene gun immunization

For gene gun DNA immunization, a hand-held, helium-driven Helios gene delivery system (Bio-Rad, Hercules, CA, USA) was used.[23] Plasmid DNA was precipitated onto gold particles with a 1.6-μm average diameter, as specified by the manufacturer. The inner surface of a Tefzel (Bio-Rad) tubing was coated with the DNA-gold particle preparation with a tube loader (Bio-Rad), and the tubing was cut into 0.5-inch segments for delivery of 0.5 mg of gold and 1 μg of plasmid DNA per shot. Groups of 5 mice were immunized with the gene gun as follows: group 6 with p25L; group 7 with pcDNA3. Each animal received a gene gun injection into the abdominal epidermis 3 times at 3-week intervals with a helium pressure setting of 500 lb/in^2. Another group of mice (group 8) was intramuscularly immunized with 100 μg of p25L to serve as a control. Group 8 mice were given cardiotoxin pretreatment 1 week prior to immunization and 2 booster doses 3 and 6 weeks after the first immunization.

Electroporation

In vivo EP intramuscular injection was performed as previously described with some modifications.[24,25] Mice were anesthetized with acepromazine maleate (Fermenta Animal Health Co., Kansas City, MO, USA). One hundred micrograms of plasmid DNA was injected into the bilateral quadricep muscles. Immediately after the injection, a pair of electrode needles was inserted into the muscle to a depth of 5 mm to encompass the DNA injection sites, and electric pulses were delivered using an electric pulse generator (Electro Square Porator ECM 830; BTX, San Diego, CA, USA). The shape of the pulse was a square wave. The electrodes consisted of a pair of gold-plated stainless steel needles 5 mm in length and 0.8 mm in diameter, with a fixed distance of 5 mm between them. Ten pulses of 900 V each were administered to each injection site at a rate of 1 pulse/second, with each pulse being 100 μs in duration. Groups of 5 mice were immunized as follows: group 9 with 100 μg of p25L and group 10 with 100 μg of pcDNA3. Each mouse was given booster doses 3 and 6 weeks after the first EP immunization. Another group of mice (group 11) was intramuscularly immunized with 100 μg of p25L without EP. This group of mice was given 2 booster doses 3 and 6 weeks after the first immunization.

Serum sampling

Serum samples were collected by tail bleeding before each immunization, and 9 and 12 weeks after the first immunization.

Enzyme-linked immunosorbent assay (ELISA) of anti-HBs and anti-HDV antibodies

The total anti-HBs antibodies (Ab) were quantitated using the AUSAB-EIA kit (Abbott Laboratories, North Chicago, IL, USA). Assays were performed as specified by the manufacturer. The amount of anti-HBs antibody was calculated from a standard curve generated by using the anti-HBs positive control provided in the AUSAB quantitation panel (Abbott Laboratories). Serum samples from groups of mice were analyzed for the presence of HDAg-specific antibodies according to our previous reports.[17,18] In brief, microtiter plates were coated with 100 μL (1 μg/mL) of a fusion protein containing maltose-binding protein (MBP) and L-HDAg (MBP-DL2577), as reported previously.[26] After blocking, 100 μL of serial dilutions of tested sera in triplicate were added to each well. Bound proteins were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) (Sigma). Color was generated by adding 0.1 M citric acid (pH 5) and phenyldiamine (Sigma). The absorbance at 490 nm was measured on an ELISA reader. The results were considered significant if the optical density (OD) value of the tested sera was higher than the mean OD value + 3 SD of the control sera.

Statistical analysis

To compare the results between groups, the Wilcoxon signed-rank test was used. To compare the results among groups, a Kruskal-Wallis 1-way analysis of variance (ANOVA) was used.[27] A p value < 0.05 was considered significant.

Results

Humoral responses to intramuscular immunizations

Plasmid pS/p25L could co-express L-HDAg and HBsAg. When anti-HBsAb was detected after DNA immunization, it was confirmed to be successful. Consequently, plasmid pS/p25L was adopted in this study to serve as a control. As shown in Figure 1, mice immunized with pS/p25L showed a strong anti-HBs response and a seroconversion rate of 100%. Clinically, a protective immunity against HBV infection is at a level of 10 mIU/mL of anti-HBs antibody based on World Health Organization reference values. The maximal anti-HBs antibody response after DNA immunization was achieved in week 9 (> 300 mIU/mL). Mice immunized with 200 μg of pS/25L could reach a high anti-HBs response beginning at week 6,

about 3 weeks earlier than those immunized with 100 μg of pS/25L. However, the anti-HBs responses of both groups of mice were nearly identical in weeks 9 and 12. No statistical difference was noted between these 2 groups ($p > 0.05$).

The anti-HDV humoral immune response to various plasmids at different dosages was assessed (Figure 2) and was shown to be weak after intramuscular immunization with L-HDAg DNA vaccine. Even though the anti-HBs titers were high in mice immunized with pS/p25L, titers for anti-HDV antibodies were low. The anti-HDV seroconversion rate was 60% in group 1 compared to group 4 mice. It is worth noting that doubling the dosage of plasmid DNA could not augment the anti-HDV response.

Humoral responses to gene gun immunization

Gene gun immunization is a route of intra-epidermal DNA transfection. As shown in Figure 3, gene gun DNA immunization with L-HDAg plasmid could not provide a better humoral immune response to HDV. The anti-HDV titers were even lower than in the intramuscular route, but without statistical significance. The anti-HDV seroconversion rate was 60% for both routes of immunization.

Humoral responses to EP DNA immunization

The anti-HDV antibody titers in mice immunized with p25L by EP DNA immunization were similar to those in mice immunized by intramuscular injection (Figure 4). The titers also peaked at 9 weeks. Generally, the anti-HDV titers were also low by EP immunization (titer < 400:1). However, EP DNA immunization had an anti-HDV seroconversion rate of 80%, which seemed better than intramuscular injection alone (60%).

Discussion

HDV DNA vaccine with L-HDAg can produce Th1 immune response to HDV but the anti-HDV titer is weak by this mode of immunization.[17] In this study, we doubled the plasmid DNA to 200 μg per injection for each mouse. This dosage is equivalent to 10 mg/kg for a mouse that weighs 20 g. At this dosage, mice immunized with pS/p25L could rapidly reach high anti-HBs antibody titers in 6 weeks. However, the anti-HDV response was still weak regardless of dosage change. All mice immunized with pS/p25L had seroconversion to anti-HBs-positive, which confirmed that the immunization technique was successful. According to our previous reports, the anti-HDV seroconversion rate was around 60% for mice immunized with L-HDAg DNA vaccine.[17,18] Comparable with these results, the anti-HDV seroconversion rate was 60% by intramuscular immunization, and was unaffected by the dosage increment.

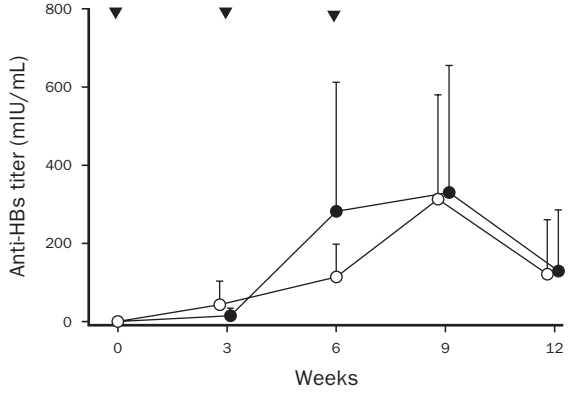

Figure 1. Kinetics of anti-HBs antibodies in mice immunized with 200 μg of plasmid pS/p25L (●) or 100 μg of pS/p25L plus 100 μg of pcDNA3 (○). All mice were given an intramuscular injection of 200 μg of plasmid DNA at 0, 3 and 6 weeks (▼). Sera were obtained at different time points and assayed for the presence of anti-HBs. Concentrations of anti-HBs antibodies were quantitated with a commercial kit, and values were determined relative to the standard reference preparation. Mean titers are expressed in milli-international units per milliliter (mIU/mL). The threshold for seroconversion was defined as ≥ 10 mIU/mL. The data are presented as mean ± SD for 5 animals per time point.

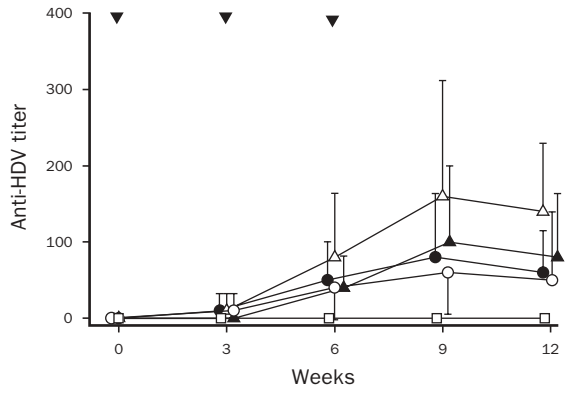

Figure 2. Kinetics of anti-HDV antibodies in mice immunized with 200 μg of plasmid pS/p25L (●), 100 μg of pS/p25L plus 100 μg of pcDNA3 (○), 200 μg of p25L (▲), 100 μg of p25L plus 100 μg of pcDNA3 (△), or 200 μg of pcDNA3 (□). Immunization at 0, 3 and 6 weeks (▼). After intramuscular injection, sera were assayed for the presence of anti-HDV. Titers of anti-HDV antibodies were assayed by ELISA and determined by a serial dilution of the sera. The results were defined as significant if the OD 490 value was greater than the mean OD + 3 SD of the control sera. The data were presented as mean ± SD for 5 animals per time point.

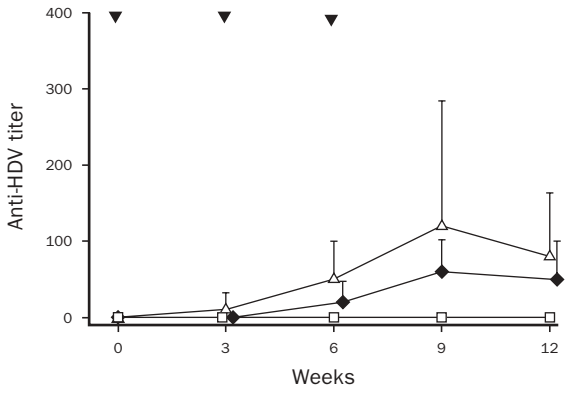

Figure 3. Kinetics of anti-HDV antibodies in mice immunized with 1 μg of plasmid p25L by gene gun (◆), 100 μg of p25L by the intramuscular route (△), or 1 μg of pcDNA3 by gene gun (□). Immunization at 0, 3 and 6 weeks (▼). After injection, sera were assayed for the presence of anti-HDV. The data are presented as mean ± SD for 5 animals per time point.

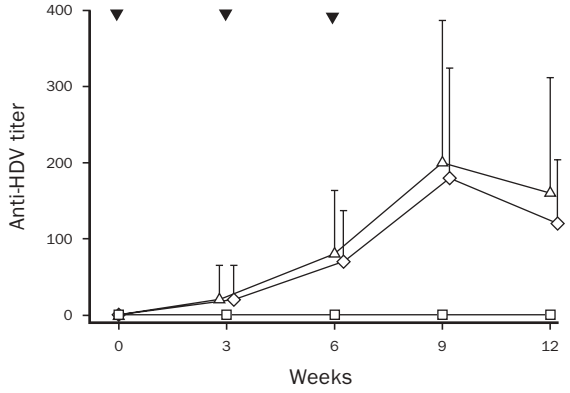

Figure 4. Kinetics of anti-HDV antibodies in mice immunized with 100 μg of plasmid p25L by EP (◇), 100 μg of p25L by the intramuscular route (△), or 100 μg of pcDNA3 by EP (□). Immunization at 0, 3 and 6 weeks (▼). After injection, sera were assayed for the presence of anti-HDV. The data are presented as mean ± SD for 5 animals per time point.

Gene gun immunization can propel gold beads coated with DNA into the epidermis. In a clinical trial of HBV DNA vaccine with a gene gun, all vaccinees immunized with plasmid encoding HBsAg seroconverted. Most importantly, some of them failed to respond to the licensed recombinant protein vaccine.[28,29] Although gene gun DNA immunization has been proven to generate an antibody response, the anti-HDV titers in mice after gene gun immunization with L-HDAg DNA vaccine are still low. The anti-HDV seroconversion rate was nearly the same in mice immunized by intramuscular injection or gene gun. The humoral immunogenicity of DNA vaccine with L-HDAg could not be improved by this mode of immunization.

In vivo EP intramuscular injection is a new delivery device that increases the uptake of DNA into cells and expression of encoded protein.[20,21] This approach makes holes in cells by simply delivering electric current *in vivo* and permits the injected DNA to enter the cells more efficiently. In this study, EP DNA immunization improved the anti-HDV seroconversion rate to 80%. Thus, EP substantially increased the DNA uptake after injection of HDV DNA vaccine. However, even under a more optimal condition of DNA transfection, the anti-HDV antibody titer was still low. Consequently, this low humoral immunogenicity of DNA vaccine with L-HDAg is not related to modes of immunization or the efficiency of DNA transfection. Consistent with our recent finding, the immunogenicity of DNA vaccines with large or small HDAg is different.[18] The low humoral immunogenicity of DNA vaccine with L-HDAg arises from the masking effect of the isoprenylated motif. This motif of L-HDAg can mask the B-cell epitope of HDAg at the location of amino acids 170–195 but does not interfere with the cellular immunity following DNA-based immunization.[18] This may explain why the DNA dosage and modes of immunization are not related to the antibody response of DNA vaccine with L-HDAg.

HDAg is an intracellular antigen. The weak humoral immunogenicity of L-HDAg DNA vaccine may be due to its intracellular location in nature. However, a recent study using expression vector to secrete HDAg showed no improvement in anti-HDV response.[30] In this study, we adopted BALB/c mice for study as this inbred mouse strain can produce an anti-HDV titer using S-HDAg DNA vaccine.[18] Consequently, the possibility that HDAg response might be under the control of a specific mouse immune response gene should be ignored.

HDV DNA vaccine has been proven to produce Th1 immune response, HDV-specific interferon-γ secreting CD8 T cells, and cytotoxic T-cell response.[17,18,30] Currently, there is no doubt that HDV DNA vaccine can generate cellular immunity. In a woodchuck model, HDV DNA vaccine seemed to be able to modulate the disease course of HDV superinfection but the protective ability was not sufficient.[31] In that experiment, no anti-HDV antibody was induced by HDV DNA vaccine before HDV challenge. Generally, both humoral and cellular immunity are necessary for a protective vaccine. Although the neutralizing ability of the anti-HDV antibodies is undetermined, a stronger anti-HDV humoral immune response in cooperation with cellular immune responses might be important

for a candidate HDV vaccine. In a human study, it was discovered that HDV variants with amino acid changes in B-cell epitopes usually emerged after a severe hepatitis attack in CDH patients.[32] This finding implies that the anti-HDV antibodies might have immune selection effects, which can deteriorate as a result of the low humoral response generated by the L-HDAg construct DNA vaccine.

In conclusion, the low humoral immunogenicity of DNA vaccine with L-HDAg construct cannot be improved by different dosages, gene gun immunization, or *in vivo* EP intramuscular injection. DNA vaccine with L-HDAg construct may not be a candidate HDV vaccine.

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