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DNA-based vaccination induces humoral and cellular immune responses against hepatitis B virus surface antigen in mice without activation of C-myc

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Abstract

AIM To develop a safe and effective DNA vaccine for inducing humoral and cellular immunological responses against hepatitis B virus surface antigen (HBsAg).

METHODS BALB/c mice were inoculated with NV-HB/s, a recombinant plasmid that had been inserted S gene of hepatitis B virus genome and could express HBsAg in eukaryotes. HBsAg expression was measured by ABC *immunohis*-tochemical assay, generation of anti-HBs by ELISA and cytotoxic T lymphocyt e (CTL), by MTT method, existence of vaccine DNA by Southern blot hybridization and activation of oncogene C-myc by *in situ* hybridization.

RESULTS With NV-HB/s vaccination by intramuscular injection, anti-HBs was initially positive 2 weeks after inoculation while all mice tested were HBsAg positive in the muscles. The titers and seroconversion rate of anti-HBs were steadily increasing as time went on and were dose-dependent. All the mice inoculated with 100ìg NV-HB/s were anti-Bs positive one month after inoculation, the titer was 1:1024 or more. The humoral immune response was similar induced by either intramuscular or intradermal injection. CTL activities were much stronger (45.26%) in NV-HB/s DNA immunized mice as compared with those (only 6%) in plasma-derived HBsAg vaccine immunized mice. Two months after inoculation, all muscle samples were positive by Southern-blot hybridization for NV-HB/s DNA detection, but decreased to 25% and all were undetectable by *in situ* hybridization after 6 months. No oncogene C-myc activation was found in the muscle of inoculation site.

CONCLUSION NV-HB/s could generate humoral and cellular immunological responses against HBsAg that had been safely expressed *in situ* by NV-HB/s vaccination.

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INTRODUCTION

Infection with hepatitis B virus (HBV) is the most important and most common cause of acute and chronic liver disease worldwide. Some of them eventually progress to cirrhosis or/and liver failure. Persistent HBV infection is associated with a high risk of primary hepatocellular carcinoma. Hepatitis B virus surface antigen (HBsAg) is the protein product of the S gene of the HBV genome and is the protective immunogen used for developing vaccine against HBV infection. The current commercial HBV vaccines which were divided into plasma-derived HBV vaccine and recombinant HBV vaccine by genetic techniques induce neutralizing antibody (anti-HBs) against HBV. Unfortunately, up to 5% of the adult population may not respond to the currently available HBV vaccines, so great efforts have been made to develop more successful vaccines to prevent and even treat chronic HBV infection[1]. The newest approach is the use of naked DNA vaccine, the so-called genetic immunization, which has been shown to be effective at generating protective immune responses against a wide variety of diseases[2-18]. This technique involves the transfer of a viral gene into muscle or skin cells of host by a plasmid vector with subsequent endogenous production and intracellular processing of the viral structural proteins into small antigenic peptide. Such peptides are expressed subsequently on the cell surface in the context of major

histocompatibility complex (MHC) molecules, and may be secreted from the cells to stimulate a humoral and T-helper cell immune response. After genetic immunization, both humoral and cell-mediated immune responses developed[19-24]. In this study, we evaluated both humoral and cellular immune responses against HBsAg, which were generated in a mice model by DNA-based immunization with naked DNA vaccine NV-HB/s.

MATERIALS AND MATHODS

Preparation of nucleic acid vaccine NV-HB/s

S sequences encoding surface antigen of HBV were inserted into eukaryotic expression vector pRc/CMV (Invitrogen, San Diego, CA) under the transcription control of the cytomegalovirus early promoter. The reconstructed plasmid was then amplified in *E. Coli* DH5α. After extraction and purification plasmid DNA was dissolved in PBS (pH 7.2) at the concentration of 1μg/L as the DNA vaccine NV-HB/s and stored at -70℃ until use for animal immunization.

DNA vaccine immunization in mice

Vaccine trials in mice were accomplished by the administration of NV-HB/s to 6-week-old BALB/c mice. Dosage of DNA vaccine inoculation was 100μg/100μL, 10μg/100μL, and 1μg/100μL. DNA vaccine was administrated by the following routes: ¢Ù intramuscular injection (I.M.) into both sides of tibialis anterior muscles, half dose for each side; ② intradermal injection (I.D.) at 1cm distal from the tail base; and ③ inoculated sites of tibialis anterior muscles were pretreated with the injection of bupivicaine (0.2 μg per site) 7 days before the vaccination by I.M. at the same sites. The control groups included: pRc/CMV (as negative control), PBS (as blank control); commercial plasma-derived HBV vaccine which was the purified HBsAg (as positive control).

Serum samples were collected 3 days, 1 and 2 weeks, 1, 2, 4 and 6 months after inoculation through retrobulbar puncture and stored under -70℃. Tibialis anterior muscle samples were collected from sacrificed mice 7 times with the same time schedule of the serum sample collection. Each muscle sample was divided into 2 pieces, one was stored at -70℃, the other was fixed in formalin and embedded with paraffin for making into slides.

For detection of CTL activity, another group of mice was inoculated I.M. with 100μg NV-HB/s (commercial plasma-derived HBV vaccine as control) and boosted one month later with the same dosage. After one week of boosting, the mice were sacrificed to collect spleen cells for detection of CTL activity.

Detection of HBsAg and anti-HBs

By ABC immunohistochemistry assay, all muscle tissue samples collected from the inoculation sites were tested for HBsAg expression; and by enzyme-linked immunoadsorbent assay (ELISA), all sera samples were detected for anti-HBs, which were immunologically induced by HBsAg.

Detection of CTL activity

Spleen cells were collected from the sacrificed mice, washed and suspended with HEPES solution at a concentration of 5×10^6 cells/mL as effect cells. SP2/0-HBs cells (myeloma cell line SP2/0 derived from BALB/c mice had been transfected with NV-HB/s DNA *in vitro*) were suspended with HEPES at a concentration of 5×10^4 cells/mL as target cells. The effect cells were pre-cultured with stimulating cells, which were SP2/0-HBs irradiated with 10000 rad, and mixed with target cells by the optimal ratio. After a 3h incuba tion at 37℃, 5% CO_2 and 95% relevant humidity, CTL activities were detected according to the manual described for MTT test kit of CTL activity detection (Boehringer Mannheim Company). In brief, the mixtures were spinned and the supernatant was collected, then LDH substrates were added to the supernatants. Half an hour later, O.D. value of the solution was measured. The percentage specific cytolysis was determined by the following formula: the specific cytolysis (%)= (experimental release-spontaneous release of effect cell-spontaneous release of target cells)/(maximum release of target cell-spontaneous release of target cells)×100%.

Existence of NV-HB/s plasmid in the muscles tissue

To find out how long the plasmid DNA could exist in the muscle tissue at the injection sites after inoculation, NV-HB/s was detected by *in situ* hybridization and Southern blot hybridization with Dig-pRc/CMV probe prepared in our laboratory with the protocol described by Zhao *et al*[25,26]. For *in situ* hybridization, the fixed muscle samples slides were hybridized with Dig-pRc/CMV probe and for Southern blot hybridization, the frozen muscle samples at -70℃ were thawed, homogenized, digested with protease, and extracted with phenol/CH-3CL, and the cell DNA recovered was precipitated with ethanol and dissolved in T.E. solution for Southern blot hybridization.

Pathological examination and detection for C-myc activation

Special attention was paid to the health condition of mice after inoculation. Routine pathological examination was made for all mice to know if there is any pathological change. The muscle slides were detected for C-myc mRNA by hybridization *in situ* with Dig-C-myc-cDNA probe with the protocol described by Zhao *et al*[26,27].

RESULTS

HBsAg expression and antibody induction

After 3 days of inoculation, no HBsAg was detected in the muscle samples. However, 1/3 mice after 1 week of inoculation and 3/3 mice after 2 weeks, were HBsAg positive in the muscles collected from the mice vaccinated with NV-HB/s (Table 1). On the other hand, there was no detectable HBsAg in all sera samples.

Anti-HBs were initially positive 2 weeks later after inoculation while all mice were HBsAg positive in the muscles. The titers and seroconversion rate of anti-HBs were steadily increasing as time goes on. All the mice inoculated with 100μg NV-HB/s were anti-HBs positive one month later after inoculation, the titer could be 1:1024 or more. Titers and seroconversion rate were dosage-dependent. For the mice inoculated with 1μg or 10μg NV-HB/s, the positive rate of anti-HBs wa s only 3/6 or 5/6 respectively even 2 months after inoculation, while their titers of anti-HBs were also lower than that of mice inoculated with 100μg NV-HB/s (P<0.05).

Humoral immune response induced with NV-HB/s vaccination were similar by either intramuscular or intradermal injection. The positive rates of anti-HBs were nearly the same although the titers induced by I.M. were higher than those by I.D. Pretreatment with bupivicaine before I.M. did not promote the humoral immune response in this experiment.

Table 1 HBsAg expression and anti-HBs induction in the mice inoculated with DNA vaccine against hepatitis B (NV-HB/s)

	Positive rate after inoculation						
	3d	1wk	2wks	1mo	2mos	4mos	6mos
HBsAg(%)	0 (0/4)	33.3 (1/3)	100 (3/3)	75 (3/4)	5 (3/4)	83.3 (5/6)	75 (6/8)
Anti-HBs(%)	0 (0/4)	0 (0/3)	66[a]±7 (2/3)	100 (4/4)	100 (4/4)	100 (6/6)	100 (8/8)

HBsAg was detected from mice muscle tissue samples of injection sites by ABC immunochemistry. Anti-HBs was detected from sera samples by ELISA. The numbers in parenthesis indicate the number of mice with positive detection results / the total number of mice detected.

NV-HB/s vaccination induces HBsAg-specific CTLs

In our data, CTL activities were much stronger in NV-HB/s immunized mice (45.26%) as compared with those in plasma-derived HBsAg vaccine immunized mice (only 6%) (P<0.01). Certainly there was no CTL activity detected from any negative control groups.

Persistence of NV-HB/s DNA in the muscles

Two months later after inoculation, all muscle samples were positive by Southern-blot hybridization for NV-HB/s DNA detection. However, 6 months after inoculation, the positive rate decreased to 25%, and all the muscle samples were negative by *in situ*úJ hybridization for NV-HB/s DNA detection. Considering that the DNA detection sensitivity by *in situ* hybridization is lower than by Southern-blot hybridization, the above-mentioned result implies that the amount of NV-HB/s existed in the muscle was very low and degenerated rapidly as time goes on.

Effect of NV-HB/s vaccination on oncogene C-myc

In mice sacrificed 3 days after injection, hyaline degeneration was observed, including swollen muscular fiber, disappearance of cross striation and red stain of myocytes plasma, which were slightly more apparent in mice inoculated with NV-HB/s or pRc/CMV than those inoculated with PBS or plasma-derived vaccines. In the former groups, some lymphocytes aggregated, and in one of them neutr ophile clustering was found. These pathological changes disappeared 1 week later in the PBS-injected mice and 4 weeks later in NV-HB/s or pRc/CMV inject ed mice. During the experiment the mice looked healthy, and no C-myc mRNA was detected in all the muscle sample collected from the mice inoculated.

DISCUSSION

In this study, we evaluated the humoral and cellular immune response induced in BALB/c mice by DNA-mediated immunization with NV-HB/s, a recombinant plasmid which had been inserted S gene and could express HBsAg in eukaryotes. The results showed that even after a single intramuscular injection of DNA, a detectable antibody response could be induced and sustained which resembles that of natural HBV infection in terms of the fine specificity. There was no significant difference between the humoral immune response induced with NV-HB/s by intramuscular injection and those by intradermal injection[28]. These data provided evidence that the envelope proteins encoded by the recombinant DNA has adopted a conformation similar to that of the proteins present during natural infection. This conclusion validates the use of DNA-based *in vivo* synthesis of the antigen for immunization purposes.

It is the important feature that DNA-based immunization is the *in situ* production of the expressed protein subsequent to the introduction of DNA carrying the protein coding sequences, mimicking in this respect a viral infection[29]. In our data, the muscle samples had positive HBsAg expression in 1 week for 1/3 mice, and in 2 weeks after NV-HB/s inoculation for all the mice detected by ABC immunohistochemistry assay. However, no HBsAg was detected from any sera sample, which suggested that the quantity of HBsAg protein expression was high enough to induce immune

response in mice. Such endogenous protein synthesis could allow presentation of antigens by class I molecules of the major histocompatibility complex (MHC), thus resulting in the induction of $CD8^+$ cytotoxic T lymphocytes (CTL). Therefore, the potential of DNA-mediated immunization in partially mimic viral infection promises the efficacy of live attenuated vaccines without the risk of inadvertent infection[30-31].

DNA-based immunization was shown to induce a broad range of immune responses, including neutralizing antibodies, CTL, T-cell proliferation, and protection against challenge with the various pathogens. In this study, direct injection of NV-HB/s DNA encoding for HBsAg into the muscle of mice could induce humoral and, more important, cell-mediated immune responses.

Compared with immunizations with soluble recombinant proteins or peptides (e.g. the commercial plasma-derived HB vaccine), the advantage of DNA-mediated immunization is to induce a more Th1-like immune response with the production of inflammatory $CD4^+$ T cell as well as cytotoxic T cell activity, presumably due to the intracellular processing of viral proteins into peptides and subsequent loading onto MHC class I molecules in transfected muscle cells and to be defined interactions of the complex with APCs. Immunization with soluble protein primarily leads to a humoral immune response due to processing through the MHC class II pathway. The disadvantage of the immunization with foreign peptides is that there is only a limited number of epitopes available for stimulation of the host immune response. In contrast, all naturally occurring B and T cell epitopes encoded for each protein by the DNA construct of interest are presumably preserved for recognition by TCRs and will consequently generate very broad humoral and cellular immune responses. In our data, NV-HB/s vaccination generated much stronger CTL activities (45.26%) than plasma-derived HBsAg vaccine inoculation could (6%) ($P<0.01$).

Some types of local APCs will take up and process antigens to induce MHC class I and II restricted T-cell responses in DNA-mediated immunization. For intramuscular inoculation, myoblasts and myotubes express MHC class I molecules but not MHC class II and other co-stimulatory molecules[32]. It was reported that pretreatment of the tissues with the anesthetic bupivacaine could dilate local vessels, thus enhancing DNA uptake by myocytes[33]. Our data did not prove that the use of bupivacaine could improve responses to HNV-HB/s vaccination[34]. As CTLs can recognize cells that are already infected, it might be desirable particularly in the prevention, and even treatment of chronic viral infection. Moreover, unlike antibody responses, which are usually type specific CTLs can crossreact against different viral epitopes, thus potentially affording greater protection against disparate viral strains. During active viral replication, HBV has a very high mutation rate[35]. In this approach, the vaccine escape mutants HBV strains could be conquered.

It is known that cytotoxic T lymphocyte (CTL) activity against HBV structural proteins is not detectable in peripheral blood lymphocytes derived from the individuals with persistent HBV infection. Some chronic HBV-infected individuals who had spontaneous clearance of HBV DNA from sera are often accompanied by increased $CD4^+$ T-helper responses and acute exacerbation of liver disease. So an attractive hypothesis for the development of persistent viral infection is that HBV-specific CTLs are unable to clear virus from the liver because of substantially decreased introhepatic levels or qualitative changes in CTLs activity[36,37]. On the other hand, the observation of spontaneous HBV clearance in some individuals indicated that the suboptimal cellular immune response may be reversible. Therefore, strategies designed to boost the HBV-specific immune response or to alter the balance between the cytopathic and the regulatory component of the response may be able to terminate persistent infection. There is strong evidence that the efficacy of the CTL, response to HBV structural proteins may be crucial for eradication of persistent viral infection. It has been shown that the adoptive transfer of HBsAg-specific CTLs into HBV transgenic mice was associated with HBV clearance from the liver by antiviral effects of secreted IFN-F and tumor necrosis factor α derived fro m sensitized cells, without killing hepatocytes[38,39].

In this study, we presented evidence that DNA vaccine inoculation is capable of eliciting Ag-specific immune responses in both effector pathways of the immune system: the humoral and cellular immune responses. We have shown that NV-HB/s vaccination is able to induce humoral and CTL activity in mice using this approach. Our data may be beneficial for possible antiviral therapy of chronic HBV infection. Thus, NV-HB/s could be promising candidates as antiviral agents for persistent viral infection of the liver by inducing a strong cellular immune response after intramuscular immunization. However, it was noteworthy that the generation of such protective immune responses in humans remains to be established.

Finally, the safety of DNA vaccine remains theoretical concerns, for example, if the foreign DNA may integrate into the host genome with the possibility of disrupting normal genes and malignant transformation[33]. Our study showed no proof of oncogene C-myc activation with specific immune response induced by NV-HB/s inoculation. The mice looked healthy except a brief trauma reaction at the inoculation site caused by injection.

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