**HBV DNA vaccine with adjuvant cytokines induced specific immune responses against HBV infection**

De-Wei Du, Zhan-Sheng Jia, Guang-Yu Li, Yong-Ying Zhou

**METHODS:** The pCR3.1-S plasmid and the eukaryotic expression vectors expressing murine IL-2 (pDOR-IL-2) or IL-12 (pWRG3169) were injected into mice subcutaneously. The immune responses to pCR3.1-S and the adjuvant effect of the cytokines plasmid were studied. Meanwhile the effect of pCR3.1-S on anti-translated subcutaneous tumor of P815 mastocytoma cells stably expressing HBsAg (P815-HBV-S) was also studied. Anti-HBs in serum was detected by enzyme-linked immunosorbent assay (ELISA) and HBsAg specific cytotoxic T lymphocytes (CTLs) activity was measured by 51Cr release assay. After three weeks of DNA immunization, the cells of P815-HBV-S were inoculated into mice subcutaneously and the tumor growth was measured every five days. The survival rate and living periods of mice were also calculated.

**RESULTS:** After 8 wk DNA immunization, the A 450 nm values of sera in mice immunized with pCR3.1, pCR3.1-S and pCR3.1-S codelivered with IL-2 or IL-12 plasmids were 0.03±0.01, 1.24±0.10, 1.98±0.17 and 1.67±0.12 respectively. Data in mice codelivered pCR3.1-S with IL-2 or IL-12 plasmids were significantly higher than that of mice injected pCR3.1 or pCR3.1-S only. The HBsAg specific CTL activities in mice coimmunized to mice with pCR3.1-S and their effects as adjuvants for immune responses were also studied. Meanwhile, we established the HBV-infectious animal model through the inoculating of the P815-HBV-S and observed the treatment and preventive effect of pCR3.1-S to HBV infection in vivo.

**MATERIALS AND METHODS**

**Plasmids, cell lines and mice**

Plasmid expressing hepatitis B virus surface antigen (pCR3.1-S) was constructed by Prof. Yao ZHQ (in this Department). Plasmids expressing murine IL-2 (pDOR-IL-2) and IL-12 (pRW1369) were generous gift from Dr. Feng ZHH (in this Department). P815 mastocytoma cells were generous gift from Dr. Zhao (Department of Pathology, the Fourth Military Medical University). Female Balb/c (H-2d) mice were obtained from the Center for Experimental Animals of the Fourth Military Medical University and used at the age of 5-8 weeks.

**Transfection and expression of pCR3.1-S in P815**

P815 cells were maintained in RPMI 1640 (Sigma) with 10 mL L-1 fetal bovine serum in a six-well tissue culture plate at 37 °C in 5 % CO2 humidified atmosphere and then transfected with the pCR3.1-S or the pCR3.1 alone by using lipofectamine (GIBCO). For each transfection 20 µg of plasmid and 15 µL of lipofectamine in 0.2 mL of serum free medium were mixed in tube for 30 minutes. After 0.8 mL of serum-free medium were added to the tube, the DNA-lipofectamine complexes were overlaid onto the cells. After incubated 12 hours, the cells were washed two times with the complete culture medium and then the CTL activities. DNA vaccine may be useful for both prophylaxis and treatment of HBV infection.

**INTRODUCTION**

Many animal models of infectious diseases have been reported[1-3] which shows DNA vaccine induced broad range of protective immunities, including antibodies. CD8+ CTL, CD4+Th cells against challenge with the pathogens, such as plasmodium[4], influenza virus[5] simplex virus[6] and HIV-1[7]. Application of this genetic vaccination approach has been extended to the treatment of cancers[8,9] as well as allergic diseases[10,11] and autoimmune disease[12]. Because DNA vaccines can induce weak and short-lived immune responses in large out-bred animal[13], seeking for an effective way to promote the immune responses of DNA immunization is an urgent case. Relying on the knowledge above, we constructed a recombination vector expressing HBV S protein, pCR3.1-S, to study the possibility of DNA vaccine in controlling and preventing the HBV infection. In addition, the eukaryotic expression vectors expressing murine IL-2 or IL-12 were codelivered to mice with pCR3.1-S and their effects as adjuvants for immune responses were also studied. Meanwhile, we established the HBV-infectious animal model through the inoculating of the P815-HBV-S and observed the treatment and preventive effect of pCR3.1-S to HBV infection in vivo.
the medium was replaced with 2 mL of the complete culture medium. After another 24–48-hour incubation, the cells were transferred from the culture medium, which was then replaced by medium containing 100 mg L−1 G418 (Promega). Two weeks later, the G418-resistant clones were selected and the expression of HBsAg was detected by using indirect immunofluorescence (IIF). The HBsAg expressed cells were designated as P815-HBV-S and used as the target cells for CTL assay.

**DNA immunization in mice**

Four groups of mice were used, each consisting of 5 mice which were immunized with one of the following regiments in 100 μL of sterile saline: (1) 100 μg of pCR3.1-S; (2) mixture of 100 μg of pCR3.1-S and 100 μg of pDOR-IL-2 (IL-2); (3) mixture of 100 μg of pCR3.1-S and 100 μg of pRW1369 (IL-12); (4) 100 μg of pCR3.1 vector. The mice in the last group served as negative control. All injections were done intramuscularly into the lift thigh quadriceps muscle of mice at 0, 2, 4, 6 and 8 weeks.

**HBsAg-specific antibody assay**

Sera samples were collected by tail bleeding at different times, beginning at 1 wk after immunization, and the presence of HBsAg-specific antibody was analyzed by ELISA. The ELISA kits for the HBsAg-specific antibody detection were purchased from Huamei Co. and performed according to the manufacturer’s instructions.

**CTL assay**

Spleen cells of mice were segregated 8 weeks after immunization and the CTL activities were measured by 51Cr releases assay. Na2CrO4 was purchased from Dubang Co. Target cells (1×10⁴) were labeled with 3.7MBq radiolabeled sodium chromate. The assays were performed in triplicate with 1×10⁶ targets/well at various effector cell/target cell (E:T) ratios of 100:1. Results were expressed according to the formula: % specific lysis = (experimental release-spontaneous release/maximum release-spontaneous release). Experimental release represents the mean count per-minute released by target cells in the presence of effector cells. Maximum release represents the radioactivity released after lysis of target cells with 50 μL of L1 Triton X-100. Spontaneous release represents the radioactivity present in medium derived from target cells alone.

**Blocking of CTL response by monoclonal antibodies**

At the effector cell/target cell ratio of 100:1, CTL assays were performed in the presence of 10 μL of anti-CD4+ or anti-CD8+ monoclonal antibody added to the spleen cells in 96-well plates. As a control an unrelated antibody was added to the spleen cells. The monoclonal antibodies to mouse CD4+ or CD8+ cell were purchased from Sigma Chemical Co.

**DNA vaccine against subcutaneous translated tumor**

Four groups of mice were used, each consisting of 5 mice immunized with one of the following regimens in 100 μL of sterile saline: (1) 100 μg of pCR3.1-S; (2) mixture of 100 μg of pCR3.1-S and 100 μg of pDOR-IL-2 (IL-2); (3) mixture of 100 μg of pCR3.1-S and 100 μg of pRW1369 (IL-12); (4) 100 μg of pCR3.1 vector. One group of mice without immunization. Three weeks after DNA immunization, cells of P815-HBV-S were inoculated into mice by subcutaneous injection in abdomen. The growing tumors were measured every five days with a calipers using average diameter. The survival period of mice was observed and the survival rate of mice was calculated.

**Statistical analysis**

Data were reported as means and were analyzed by professional statistical computer software SPSS. Significance was set at P<0.05.

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**RESULTS**

**Codelivery of cytokines gene augmented the titer of antibody induced by pCR3.1-S**

The pCR3.1-S showed a strong antigenticity in humoral immunity and the anti-HBsAg could be detected in sera of mice after pCR3.1-S vaccination. The serum titres of anti-HBsAg in mice increased with the times of immunization in a period of time. The titres of anti-HBsAg in sera of mice were significantly promoted by genes expressed murine IL-2 or IL-12, especially by IL-2 gene (Figure 1).

![Figure 1](image-url)

**Figure 1** Serum anti-HBsAg level of Balb/c mice. 

**Cytokines gene effected on the CTL activity induced by pCR3.1-S**

The HBsAg specific CTL activities were developed in the mice after pCR3.1-S immunization. The CTL activities were augmented by coimmunized with IL-2 or IL-12 gene. The mice immunized with pCR3.1 alone did not elicited detectable HBsAg specific CTL activities. IL-12 was more effective than IL-2 in promoting the HBsAg specific CTL activities. The CTL activity was blocked by anti-CD8+ monoclonal antibody but not by anti-CD4+ monoclonal antibody or unrelated antibody (Table 1). Taken together, these results indicated that the CTL activity induced by pCR3.1-S in vivo was executed by cells expressing CD8+/CD4+ surface phenotype and the CTL activity could be enhanced or suppressed depending on the cytokines gene expressed.

| Group                  | Untreated  | Unrelated | Anti-CD4+ | Anti-CD8+ |
|------------------------|------------|-----------|-----------|-----------|
| pCR3.1-S               | 10.1±2.1   | 10.7±1.9  | 9.7±1.2   | 3.2±0.8   |
| pCR3.1-S+IL-2          | 50.5±6.4   | 49.7±6.1  | 48.3±5.9  | 10.6±4.4  |
| pCR3.1-S+IL-12         | 61.9±7.1   | 62.0±6.8  | 56.2±7.5  | 13.5±1.9  |
| pCR3.1-S+IL-12         | 73.3±8.8   | 69.9±7.6  | 75.6±9.1  | 16.9±2.3  |

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**pCR3.1-S+IL-12 vs pCR3.1-S+IL-2

DNA vaccine inhibits the formation of subcutaneous translating tumor derived from P815-HBV-S**

After inoculated with P815-HBV-S, all five mice with or without pCR3.1 (100 %) formed the tumor. The rate of tumor formation was 20 % (1/5) in mice immunized with pCR3.1-S and there was no tumor formed in mice coimmunized with pCR3.1-S and IL-12. The survival rate of mice immunized with pCR3.1-S alone or coimmunized with IL-12 increased significantly (Figure 2) and the tumor growth was evidently slower than that of mice immunized with or without pCR3.1 (Figure 3).
The level of antibody may be related to its ability to induce the proliferation and differentiation of B cells and the antibody level of the HBsAg specific antibody and the latter mainly relates to the specific killing effect of CTLs to P815-HBV-S cells induced by DNA vaccine. Furthermore, these effects of pCR3.1-S can be enhanced obviously by IL-12 gene as shown in the experiment. The therapeutic potential of DNA-based immunization for the chronic HBV carrier states has also been demonstrated in a transgenic mouse model[37]. This mode of immunization has been shown to successfully eliminate HBsAg in circulation.

These features of DNA-based immunization make it an attractive strategy for prophylactic and therapeutic vaccination against extra- and intracellular pathogens. Recently, DNA vaccines induced the specific humoral[38] and cellular[39-41] immune responses were observed in human experiments. The results of these experiments suggest that DNA vaccine might be a potential therapy for chronic HBV infection.

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Figure 2 Survival rate in different group. P <0.05 vs pCR3.1

Figure 3 Growth curve of subcutaneous translating tumor in different group of mice. P <0.01 vs pCR3.1; P <0.05 vs pCR3.1-S

DISSCUSSION
HBV infection is very common in China[16-23]. It is estimated that approximately 130 million people in the world is infected by hepatitis B virus (HBV). These people are at risk of developing chronic hepatitis leading to liver cirrhosis and hepatocellular carcinoma. Up to now, vaccination is a main way in prevention[24-31].

It has been suggested that the MHC class II and I restricted T cell responses to the virus are relatively weak during chronic HBV infection[29] and there are no specific therapies to cope with it. DNA vaccine contains the gene for an antigenic portion of a pathogen, such as the core or the envelope protein, usually under the transcriptional control of a viral promoter[26,30]. In DNA-based vaccination, immunogenic proteins are expressed in transfected cells of the vaccine recipients in their native conformation with correct post-translational modifications from antigen-encoding expression plasmid DNA in vivo. This ensures the integrity of antibody-defined epitopes and supports the generation of protective antibody. DNA vaccination is furthermore an exceptionally potent strategy to stimulate CD8+ cytotoxic T lymphocyte (CTL) responses because antigenic peptides are efficiently generated by endogenous processing of intracellular protein antigen[34].

The results of our experiment indicate that the plasmids expressing either IL-2 or IL-12 can enhance the specific humoral and cellular immune responses against HBV infection elicited by pCR3.1-S in mice. The former mainly enhances the level of the HBsAg specific antibody and the latter mainly enhances the HBsAg specific CTL activity.

IL-2 can enhance the immune responses through promoting proliferation and differentiation of B cells and the antibody development. The proliferation and activation of many kinds of T-cells and the production of various cytokins can also be promoted and stimulated by IL-2. The effect of IL-2 enhancing the level of antibody may be related to its ability to induce the increase of the Th1 cells but not Th2 cells. This can increase IgG2a type of antibody and lead to the increase of the total level of the antibody subsequently[32-34]. IL-12 is so far the most potent cytokine with the widest scope of modulation of immune responses. The immune responses can be modulated by IL-12 promoting the production of Th1 type T cells and secretion of other cytokines, stimulating the polarization and proliferation of T cells and through promoting the maturity of CTL cells and LAK cells. All of these can enhance the ability of the host to kill and eliminate pathogens. The adjuvant efficacies of cytokines were also observed by others[25,32,36].

The situations in vivo are different from that in vitro after all. In order to search the preventive and therapeutic effects of HBV DNA vaccine to the HBV infection in vivo, we serve the mouse injected with P815-HBV-S cells subcutaneously for an animal model of HBV infection. The preventive and therapeutic effects of pCR3.1-S on HBV infectious animal model were investigated by observing the inhibiting effects of pCR3.1-S on the neoplasia of P815-HBV-S cells inoculated by subcutaneous injection. The result indicates that the pCR3.1-S can reduce the formative rate of the subcutaneous translating tumor significantly, inhibit the growth of tumor, prolong the living periods and promote the survival rate of mice injected with the P815-HBV-S cells. Maybe all of these relate to the specific killing effect of CTLs to P815-HBV-S cells induced by DNA vaccine. Furthermore, these effects of pCR3.1-S can be enhanced obviously by IL-12 gene as shown in the experiment. The therapeutic potential of DNA-based immunization for the chronic HBV carrier states has also been demonstrated in a transgenic mouse model[37]. This mode of immunization has been shown to successfully eliminate HBsAg in circulation.

These features of DNA-based immunization make it an attractive strategy for prophylactic and therapeutic vaccination against extra- and intracellular pathogens. Recently, DNA vaccines induced the specific humoral[38] and cellular[39-41] immune responses were observed in human experiments. The results of these experiments suggest that DNA vaccine might be a potential therapy for chronic HBV infection.
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