Journal of Zhejiang University SCIENCE
ISSN 1009-3095
http://www.zju.edu.cn/jzus
E-mail: jzus@zju.edu.cn

Enhancing cellular immune response to HBV M DNA vaccine in mice by codelivery of interleukin-18 recombinant

CHEN Jian-zhong (陈建忠)†1, ZHU Hai-hong (朱海红)[2], LIU Ke-zhou (刘克洲)[2], CHEN Zhi (陈 智)[2]

([1]Institute of Immunology, School of Medicine, Zhejiang University, Hangzhou 310031, China)

([2]Institute of Infectious Diseases, First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310003, China)

†E-mail: chenjzh@mail.hz.zj.cn

Received May 10, 2003; revision accepted Sept. 1, 2003

Abstract: Objective: To investigate the effect of interleukin-18 (IL-18) on immune response induced by plasmid encoding hepatitis B virus middle protein antigen and to explore new strategies for prophylactic and therapeutic HBV DNA vaccines. Methods: BALB/c mice were immunized with pCMV-M alone or co-immunized with pcDNA3-18 and pCMV-M and then their sera were collected for analysing anti-HBsAg antibody by ELISA; splenocytes were isolated for detecting specific CTL response and cytokine assay *in vitro*. Results: The anti-HBs antibody level of mice co-immunized with pcDNA3-18 and pCMV-M was slightly higher than that of mice immunized with pCMV-M alone, but there was not significantly different (P>0.05). Compared with mice injected with pCMV-M, the specific CTL cytotoxity activity of mice immunized with pcDNA3-18 and pCMV-M was significantly enhanced (P<0.05) and the level of IFN-γ in supernatant of splenocytes cultured with HBsAg *in vitro* was significantly elevated (P<0.05) while the level of IL-4 had no significant difference (P>0.05). Conclusion: The plasmid encoding IL-18 together with HBV M gene DNA vaccines may enhance specific TH1 cells and CTL cellular immune response induced in mice, so that IL-18 is a promising immune adjuvant.

Key words: Interleukin-18, Hepatitis B virus, DNA vaccines, Immune response
Document code: A **CLC number:** R392

INTRODUCTION

Chronic infection with HBV affects more than 250 million people worldwide. There are more than 120 million chronic HBV carriers in China; approximately 10 percent of them remain in state of chronic hepatitis and have a high risk of development of cirrhosis and hepatocellular carcinoma. But there is no effective method to control chronic HBV infection at present. Recent data indicated that immunotherapeutic strategies stimulating both cellular and humoral immune responses to HBV antigens are essential for curing chronic HBV infection (Chisari and Ferrari, 1995). In this regard, DNA-based vaccination appears as a particularly appropriate approach for chronic hepatitis B therapy, since viral proteins are expressed in the cell after transient *in vivo* transfection with plasmid DNA and are subjected to the same post-translational modification as during viral infection leading to extremely potent stimulation of humoral and cellular responses (Tang *et al.*, 1992; Babiuk *et al.*, 2002). But there still exists a distance before application of DNA-based immunization in clinic. To meet the target many optimization methods are being studied. Among them co-immunization with cytokines may enhance immune response via attracting APC to the local site and enhancing their ability to process and present antigen to T or B cells, or by continuously activating T or B cells. Injection of a plasmid en-

coding cytokine with DNA vaccines is more effective than injection of recombinant protein as cytokines expressed in local environment may enhance the interaction of immune cells in local environment.

IL-18, originally identified as IFN-γ-inducing factor (Nakanishi *et al.*, 2001; Biet *et al.*, 2002), is a cytokine that plays an important role in development of Th1 cell and cellular immune response such as enhancing cytotoxity activity of CTL, NK and Th1, inducing the production of IFN-γ, IL-2 and GM-CSF. To investigate the effect of interleukin-18 (IL-18) pCMV-M on immune response induced by plasmid encoding hepatitis B virus M antigen, we co-immunized mice with pcDNA3-18 and pCMV-M and evaluate the cellular and humoral immunities in the immunized mice.

MATERIALS AND METHODS

Plasmids, cell lines and mice

Plasmid encoding for HBV MHBs pCMV-M was kindly offered by Dr. Davis. Plasmid encoding for mouse IL-18 pcDNA3-18 was constructed by ours (Chen *et al.*, 2002). Plasmid pcDNA3 was perchased from Invitrogen (V79020). The mastocytoma cell line P815 (H-2d) was sustained by our institute. Female Balb/c (H-2d) mice, 6−8 weeks, 16−18 g were purchased from the Shanghai experimental animal center.

Reagents

Large plasmid purification kit was purchased from the Shanghai Huashun Biological Engineering Corporation. CytoTox96^{R} Non-Radioactive Cytotoxicty Assay was purchased from Promega; anti-HBs ELISA kit was purchased from Aldevron. Mouse IFN-γ, IL-4 ELISA kit were purchase from Endogen.

Genetic immunization

The BALB/c mice were divided into four groups with each containing ten mice. Mice from various groups were immunized as follows: co-immunized group injected with 100 μg pcDNA3-18 and 100 μg pCMV-M; pCMV-M group injected with 100 μg pCMV-M and 100 μg pcDNA3; control group injected with 200 μg pcDNA3; blank group injected with 200 μl normal saline. Each mouse was immunized three times by intramuscular injection into two hind legs at different sites for interval of three weeks. Two weeks after last immunization, the mice were killed and their sera were collected for analyzing anti-HBsAg antibody by ELISA and splenocytes were isolated for specific CTL response and cytokine induction assay.

Serum anti-HBs antibody assays

Anti-HBs antibodies were measured using a commercial ELISA kit.

Cytokine assays

In vivo primed spleen cells were co-cultured with recombinant proteins HBs for 48 hours. IL-4 and IFN-γ levels in culture supernatant were measured by commercial kits.

Cytotoxicity assay

Splenocytes from immunized mice were suspended in complete RPMI-1640 containing 10% FCS and 50 μmol/L 2-mercaptoethanol; the cells were then analyzed for cytotoxic activity 5 days after in vitro stimulation. Cytotoxic effector lymphocyte populations were harvested after 5 days of incubation. LDH-release assay was performed in a 96-well round bottom plate using P815/S cells as target cell. CTL assays were performed at lymphocyte effector: target (E:T) ratios of 20:1. The results were expressed according to the formula:

$$\% \text{ specific analysis} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})}$$

Statistical analysis

To compare the results between the different groups, we used *t* to compare the difference between the groups.

RESULTS

Serum anti-HBs antibody assays

To investigate whether the humoral responses to pCMV-M vaccine can be affected by simultaneous expression of IL-18 gene, two weeks after the last injection, the serum from each mouse was obtained for analysis of anti-HBs responses. As shown in Table 1, the anti-HBs level of the mice immunized with pcDNA3-18 and pCMV-M (46.34 ± 14.78 mIU/ml) was higher than that of mice immunized with pCMV-M (41.76 ± 5.73 mIU/ml), but was not significantly different (*P*>0.05). The anti-HBs level of control and blank group were both under 10 mIU/ml.

Table 1 Anti-HBs induced in mice immunized with HBV DNA vaccine ($\bar{x}$±*s*)

Group	*N*	mIU/ml
Co-immunized	10	46.34±14.78*
pCMV-M	10	41.76±15.73
Control	10	<10
Blank	10	<10

*Compared with pCMV-M, *P*>0.05

Cytokine assay

It is known that the subsets of TH cells can be distinguished by the pattern of cytokine co-expression. TH1 cells produce IFN-*γ* and IL-2, and TH2 cells produce IL-4, IL-5, and IL-10. To study the effect of IL-18 co-expression on the development of TH cells induced by pCMV-M vaccination. IFN-*γ* and IL-4 released from splenocytes stimulated with HBsAg *in vitro* were assayed. Splenocytes isolated from mice immunized with pcDNA3-18 and pCMV-M significantly increased in IFN-*γ* production compared with mice immunized with pCMV-M only (*P*<0.05). There was no significant change in the production of IL-4 of these two groups (*P*>0.05). These results suggested that IL-18 may enhance the cellular immune response by induction of TH1 type cytokines. See Table 2.

Table 2 Cytokines produced by splenocytes stimulated by HBsAg (pg/ml) ($\bar{x}$±*s*)

Group	IFN-*γ*	IL-4
Co-immunized	289.59±74.36*	55.76±14.88#
pCMV-M	201.49±57.41	58.21±12.16
Control	<50.00	<20.00
Blank	<50.00	<20.00

*Compared with pCMV-M, *P*<0.05; #Compared with pCMV-M, *P*>0.05

CTL assay

It is well known that cellular immunity is highly dependent on the types of cytokines produced by TH cells. Thus we tested whether the CTL activity in mice induced by pCMV-M was influenced by co-expression of IL-18 (Table 3). CTL activity against P815/S cells expressing the HBV S epitope was assayed by LDH release assay. CTL activity in mice immunized with pcDNA3-18 and pCMV-M was (51.36±8.42)% at E:T ratio of 20:1 while in mice immunized with pCMV-M, it was (38.76±7.46)%, indicating that the CTL activity was significantly enhanced (*P*<0.05). Animals immunized with plasmid pcDNA3 and normal saline had no CTL activity (<5%). Taken together, the data showed that CTL activity was enhanced in mice immunized with pcDNA3-18 and pCMV-M.

Table 3 CTL assay of immunized mice (%) ($\bar{x}$±*s*)

Group	E:T 20:1
co-immunized	51.36±8.42 *
pCMV-M	38.76±7.46
Control	<5.00
Blank	<5.00

*Compared with pCMV-M, *P*<0.05

DISCUSSION

Nucleic acid vaccine or named DNA vaccine is a novel vaccine developed recently (Tang *et al.*, 1992; Babiuk *et al.*, 2002). It can induce humoral immune response and more effective cellular immune response, showing it is relatively more feasible for prophylactic and therapeutic vaccination against chronic virus infection such as HBV, HCV, HIV infection (Beckebaum *et al.*, 2002). HBV DNA vaccines have made great advances in recent years (Wolff *et al.*, 1990; 1992; Davis *et al.*, 1996; Chow *et al.*, 1997; 1998; Geissler *et al.*, 1998). Results following injection of plasmid DNA encoding HBsAg, HBcAg and HBeAg of HBV in animal models indicated HBV DNA vaccines can induce humoral and cellular immune response. Moreover, HBsAg-encoding plasmid DNA can induce immune response in some strains of mice which have no

response to recombinant HBsAg protein (Geissler *et al.*, 1997). Thus HBV DNA vaccines may be superior to recombinant HBsAg for prophylactic vaccination especially in cases of non- or hypo-response, or for induction of a humoral response in infants born to chronic carrier mothers. HBV DNA vaccines also can induce anti-HBs antibodies and specific CTL responses in HBV transgenic mice which are tolerant to HBV (Mancini *et al.*, 1996; Oka *et al.*, 2001). This demonstrated that HBV DNA vaccines can be used as therapeutic vaccines for patients with chronic hepatitis type B.

Co-immunization with plasmid encoding cytokine with DNA vaccines had shown that cytokines may enhance immune response. In mice, IL-2 may enhance the induction of anti-HBs to 100 times by co-immunization with HBsAg DNA vaccine and the proliferation activity of spenosytes was also enhanced (Chow *et al.*, 1997). Co-immunized IL-4 and GM-CSF with HBsAg DNA vaccine also can enhance the cellular and humoral immunity in different level (Chow *et al.*, 1998) .To study the feasibility of IL-18 as immune adjuvant, we co-immunized with vector encoding mouse IL-18 and pCMV-M and studied how immune response may be modulated by simultaneous intramuscular injection of them and is its underlying mechanism. The plasmid encoding IL-18 together with pCMV-M significantly enhanced the specific CTL response and significantly elevated the production of IFN-γ of splenocytes induced by HBsAg. The humoral immune response induced in mice had no change. The result obtained here is consistent to former results using plasmid encoding HBsAg (Chen *et al.*, 2002). Therefore IL-18 can enhance the specific CTL activity and TH1 response in immunized mice.

It was reported recently that plasmid vectors containing an unmethylated CpG dinucleotide motif can elicit much stronger humoral and cellular responses to the encoded Ag than vectors that do not contain this sequence (Krieg and Davis, 2001). The adjuvant effect of the cytokine vectors could be attributed to the presence of the CpG motif in the coding sequences of cytokines instead of functioning through their biologic effects. Our studies provide evidence that rules out this possibility, since co-injection of the control plasmid pcDNA3, which was used to construct the cytokine vectors, did not increase immune responses to the HBV DNA vaccine.

DNA vaccines are attractive because of their ability to induce T cell responses, including CD8+ MHC class I-restricted CTL. Several studies have emphasized a critical role for the cellular immune response to HBV antigens in resolution of an established infection. Acutely infected individuals who successfully clear HBV develop vigorous, multi-specific CD4+ and CD8+ T cell responses against the virus. In contrast, chronically infected patients who do not clear the virus develop limited or undetectable T cell responses (Rehermann *et al.*, 1995; 1996; Jung *et al.*, 1999). The pivotal role of CD8+CTL in the clearance of HBV infection is supported by the observation that adoptive transfer of HBV-specific CTL into mice transgenic for the HBV genome results in the suppression of HBV gene expression (Mancini *et al.*, 1996). After recognizing their viral target antigen on hepatocytes, CTL release cytokines, especially IFN-γ and TNF-α. This cytokine environment supports the degradation of viral RNA and nucleocapsid particles intracellularly (Guidotti *et al.*, 1999). CD4+ helper T lymphocytes may also contribute to the clearance of HBV infection by secreting cytokines that inhibit viral replication (Franco *et al.*, 1997). In addition T helper cell responses may probably play a role in supporting the induction and proliferation of HBV-specific CTL (Livingston *et al.*, 1999) and B cells (Leroux-Roels *et al.*, 1994). These results suggested that vaccine induction or augmentation of HBV-specific CD4+ and CD8+ T cell responses may be an effective strategy for treatment of chronic HBV infection. IL-18 can induce TH1 cellular response and enhance the CTL response in mice immunized by DNA vaccine, indicating IL-18 and HBV DNA vaccine co-immunization may play an important role in treatment of chronic hepatitis B infection. The result obtained in this study may also provide an immune strategy and specific basis for HBV prophylactic and therapeutic vaccines and indicated that IL-18 is a promising adjuvant. We are now investigating the immunotherapeutic effect in HBV transgenic mice by co-immunization of IL-18 and HBV DNA vaccine.

References

Babiuk, L.A., Babiul, S.L., Baca-Estrada, M.E., 2002. Novel vaccine strategies. *Adv Virus Res*, **58**:29-80.

Beckebaum, S., Cicinnati, V.R., Gerken, G., 2002. DNA-based immunotherapy: potential for treatment of chronic viral hepatitis? *Rev Med Virol*, **12**(5):297-319.

Biet, F., Locht, C., Kremer, L., 2002. Immunoregulatory functions of interleukin 18 and its role in defense against bacterial pathogens. *J Mol Med*, **80**(3):147-162.

Chen, J.Z., Zhu, H.H., Liu, K.Z., Chen, Z., 2002. Immuneresponse induced in mice by codelivery of IL-18 recombinant and HBV S gene DNA vaccine. *Chin J Infect Dis*, **20**(3):156-158 (in Chinese).

Chisari, F.V., Ferrari, C., 1995. Hepatitis B virus immunopathogenesis. *Annu Rev Immunol*, **13**:29-60.

Chow, Y.H., Huang, W.L., Chi, W.K., Chu, Y.D., Tao, M.H., 1997. Improvement of hepatitis B virus DNA vaccines by plasmids coexpressing hepatitis B surface antigen and interleukin-2. *J Virol*, **71**(1):169-178.

Chow, Y.H., Chiang, B.L., Lee, Y.L., Chi, W.K., Lin, W.C., Chen, Y.T., Tao, M.H., 1998. Development of Th1 and Th2 populations and the nature of immune response to hepatitis B virus DNA vaccines can be modulated by codelivery of various cytokine genes. *J Immunol*, **160**(3):1320-1329.

Davis, H.L., Mancini, M., Michel, M.L., Whalen, R.G., 1996. DNA-mediated immunization to hepatitis B surface antigen: longevity of primary response and effect of boost. *Vaccine*, **14**(9):910-915.

Franco, A., Guidotti, L.G., Hobbs, M.V., Pasquetto, V., Chisari, F.V., 1997. Pathogenic effector function of CD4-positive T helper 1 cells in hepatitis B virus transgenic mice. *J Immunol*, **159**(4):2001-2008.

Geissler, M., Tokushige, K., Chante, C.C., Zurawski, V.R. Jr.,Wands, J.R., 1997. Cellular and humoral immune response to hepatitis B virus structural proteins in mice after DNA-based immunization. *Gastroenterology*, **112**(4):1307-1320.

Geissler, M., Schirmbeck, R., Reimann, J., Blum, H.E., Wands, J.R., 1998. Cytokine and hepatitis B virus DNA co-immunizations enhance cellular and humoral immune responses to the middle and but not the large hepatitis B virus surface antigen in mice. *Hepatology*, **28**(1):202-210.

Guidotti, L.G., Rochford, R., Chung, J., Shapiro, M., Purcell, R., Chisari, F.V., 1999. Viral clearance without destruction of infected cells during acute HBV infection. *Science,* **284**(5415):825-829.

Jung, M.C., Hartmann, B., Gerlach, J.T., Diepolder, H., Gruber, R., Schraut, W., Gruner, N., Zachoval, R., Hoffmann, R., Santantonio, T., Wachtler, M., Pape, G. R., 1999. Virus-specific lymphokine production differs quantitatively but not qualitatively in acute and chronic hepatitis B infection. *Virology*, **261**(2):165-172.

Krieg, A.M., Davis, H.L., 2001. Enhancing vaccines with immune stimulatory CpG DNA. *Curr Opin Mol Ther*, **3**(1):15-24.

Leroux-Roels, G., Van, Hecke, E., Michielsen, W., Voet, P., Hauser, P., Petre, J., 1994. Correlation between in vivo humoral and in vitro cellular immune responses following immunization with hepatitis B surface antigen (HBsAg) vaccines. *Vaccine*, **12**(9):812-818.

Livingston, B.D., Alexander, J., Crimi, C., Oseroff, C., Celis, E., Daly, K., Guidotti, L.G., Chisari, F.V., Fikes, J., Chesnut, R.W., Sette, A., 1999. Altered helper T lymphocyte function associated with chronic hepatitis B virus infection and its role in response to therapeutic vaccination in humans. *J. Immunol*, **162**(5):3088-3095.

Mancini, M., Hadchouel, M., Davis, H.L., Whalen, R.G., Tiollais, P., Michel, M.L., 1996. DNA-mediated immunization in a transgenic mouse model of the hepatitis B surface antigen chronic carrier state. *Proc Natl Acad Sci USA,* **93**(22):12496-12501.

Nakanishi, K., Yoshimoto, T., Tsutsui, H., Okamura, H., 2001. Interleukin-18 regulates both Th1 and Th2 responses. *Annu Rev Immunol*, **19**:423-474.

Oka, Y., Akbar, S.M., Horiike, N., Joko, K., Onji, M., 2001. Mechanism and therapeutic potential of DNA-based immunization against the envelope proteins of hepatitis B virus in normal and transgenic mice. *Immunology,* **103**(1):90-97.

Rehermann, B., Fowler, P., Sidney, J., Person, J., Redeker, A., Brown, M., Moss, B., Sette, A., Chisari, F.V., 1995. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. *J Exp Med*, **181**(3):1047-1058.

Rehermann, B., Lau, D., Hoofnagle, J.H., Chisari, F.V., 1996. Cytotoxic T lymphocyte responsiveness after resolution of chronic hepatitis B virus infection. *J Clin Invest*, **97**(7):1655-1665.

Tang, D.C., DeVit, M., Johnston, S.A., 1992. Genetic immunization is a simple method for eliciting an immune response. *Nature*, **356**(6365):152-154.

Wolff, J.A., Malone, R.W., Williams, P., Chong, W., Acsadi, G., Jani, A., Felgner, P.L., 1990. Direct gene transfer into mouse muscle in vivo. *Science*, **247**(4949 Pt 1): 1465-1468 .

Wolff, J.A., Ludtke, J.J., Acsadi, G., Williams, P., Jani, A., 1992. Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum Mol Genet*, **1**(6):363-369.