ENHANCEMENT OF IMMUNE RESPONSES TO THE HEPATITIS B VIRUS CORE PROTEIN THROUGH DNA VACCINES WITH A DNA FRAGMENT ENCODING HUMAN IL-1β 163–171 PEPTIDE

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Summary. – DNA vaccines have been widely used as effective means of eradicating a variety of viruses, parasites, bacteria as well as means of alleviating allergic and autoimmune diseases and tumors. As interleukin 1 (IL-1) plays an essential role in augmenting both cellular and humoral immune responses to foreign antigens, it may represent a good candidate for an adjuvant to DNA vaccines. Since the inflammatory activity of IL-1 may have a restricted application to DNA vaccines, we explored the possibility of augmenting immune response without unwanted inflammatory effect using IL-1β 163–171 peptide, which is essential for IL-1 receptor 1 binding. A DNA fragment encoding the human IL-1β 163–171 peptide of concern was fused to the Hepatitis B virus (HBV) core DNA vaccine, and injected into mice to analyze its immune responses. Compared with the control mice which received hepatitis B virus core antigen (HBcAg) alone, significant increase in not only the HBcAg-specific antibody response but also in T cell proliferation was observed in mice which received IL-1β 163–171-HBcAg. These results suggest that the DNA fragment encoding the IL-1β polypeptide of aa 163–171 might represent a good candidate for an adjuvant of DNA vaccines.

Key words: interleukin 1β; hepatitis B virus core antigen; DNA vaccine

Introduction

Direct injection of plasmids expressing an antigen under the control of eukaryotic promoter into muscles has been shown to be an effective method of inducing immune response to a variety of viral (Wang *et al*., 1993; Xiang *et al*., 1994; Manickan *et al*., 1995; Geissler *et al*., 1998), parasitic and bacterial (Lozes *et al*., 1997; Lowrie *et al*., 1997) antigens. In addition, it has also shown efficacy of treatment of allergic diseases, autoimmunity and model tumors (Ciernik *et al*., 1996; Weber *et al*., 1998; Stevenson *et al*., 1995). DNA vaccines result in intracellular processing of antigenic peptides, secreting expressed proteins from muscle cells, and their presentation to the immune system by antigen presenting cells (APCs) (Tang *et al*., 1992). Cytokines play an important role in affecting antigen presentation to MHC class I and II molecules on APCs.

IL-1 amplifies both the cellular and humoral immune responses to foreign antigens (Lin *et al*., 1995), but its inflammatory activity restricts its application as an adjuvant *in vivo* (Vetri and Smith, 1996; Verschraegen *et al*., 1996). Human IL-1β peptide of the sequence VQGEESNDK (aa 163–171), which is crucial for binding to the IL-1 receptor 1, was shown previously to be effective both in the T cell-independent and T cell-dependent immune response by enhancing both primary and secondary responses

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Abbreviations: APCs = antigen presenting cells; BSA = bovine serumalbumin; ELISA = enzyme-linked immunosorbent assay; HBV = Hepatitis B virus; HBcAg = HBV core antigen; HRP = horseradish peroxidase; IL-1 = interleukin 1; PBS = phosphate-buffered saline; RBC = red blood cell; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; SI = stimulation index

(Tagliabue and Boraschi, 1993; Beckers *et al*., 1993; Antoni *et al*., 1986).

In this paper, the DNA fragment encoding the IL-1β 163–171 peptide was studied for its potential to elicit a significantly enhanced immunogenicity of DNA vaccines. The HBcAg was chosen as a model. Our results indicate that the IL-1β 163–171 peptide could enhance both the cellular and humoral immune responses to HBcAg by fusing the DNA fragment encoding the peptide of concern with HBcAg cDNA. This IL-1β 163–171 peptide might also have a general application as an adjuvant to DNA vaccines.

Materials and Methods

Construction of recombinant plasmids. The HBcAg gene was amplified from the plasmid pBI 1301-HBcAg constructed by us earlier by PCRusing the sense primer HBcAg-F (5'-GCGGCGGATCCATGGACATTGACCCGTAT-3') with a *Bam*HI site and the antisense primer HBcAg-R (5'-GCCGCGCGGCCGCCTAACATTGAGATTCCCGAGA-3') with a *Not*I site (restriction sites are underlined). The PCR product was digested with *Bam*HI and *Not*I and inserted into the eukaryotic expression vector pcDNA3 (Invitrogen) under the control of an early enhancer and promoter of cytomegalovirus and a bovine growth hormone terminator. The resulting recombinant plasmid was pcDNA3-HBcAg.

HBcAg gene was recloned into the prokaryotic expression vector pET-28a (Novagen). The resulting recombinant plasmid pET-28a-HBcAg served for prokaryotic expression of HBcAg. The expression of HBcAg in *Escherichia coli* strain BL21(DE3)pLysS (Novagen) was induced with IPTG (Sigma). HBcAg was checked by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE).

The DNA fragment encoding the IL-1β 163–171 peptide was prepared by annealing of complementary oligonucleotides IL-1β-F (5'-GATCCATGGTGCAGGGCGAGGAGAGCAACGACAAGG-3') and IL-1β-R (3'-GTACCACGTCCCGCTCCTCTCGTTGCTGTTCCCTAG-5') (BioAsia, P.R. China). The annealing was performed in a volume of 20 µl containing 50 mmol/l IL-1β-F and 50 µmol/l IL-1β-R oligonucleotides. After denaturation at 94°C for 5 mins, the mixture was cooled slowly to room temperature. The annealing product represented the DNA fragment encoding the IL-1β 163–171 peptide with cohesive *Bam*HI termini at both ends. It was inserted into the *Bam*HI site occurring at the 3'-end of the HBcAg gene in the recombinant plasmid pcDNA3-HBcAg so that upon expression a fused protein [HBcAg]-[IL-1β 163–171] was produced.

Clones of new recombinant plasmids were sequenced using the dideoxy chain termination method. The plasmids were prepared by standard alkaline lysis followed by double CsCl density gradient centrifugation (Sambrool *et al*., 1989). The A_{260}/A_{280} values of plasmid purificates were about 1.9. They were stored at -20°C until used.

HBcAg purification. The bacteria were resuspended in 10 mmol/l Tris-Cl, sonicatied and centrifuged at 10,000 x g for 15 mins at 4°C. The protein was purified from the supernatant using a Ni^{2+}-NTA-Sepharose 6B column according to the manufacturer's instructions (Hilden, Germany).

Transfection of mammalian cells. Expression of HBcAg from the plasmid pcDNA3-HBcAg was carried out by transfection of the 293 cells (Invitrogen) by the calcium phosphate-DNA precipitation method in a standard way (Sambrool *et al.,* 1989).

Western blot analysis was employed for assaying the expressed HBcAg. Extracts from transfected 293 cells were electrophoresed in 12% SDS gels and blotted onto a nylon membrane (Amersham Pharmacia, England). The blot was blocked with PBS containing 10% BSA and 0.05% Tween-20. After 1 hr of incubation, the blot was washed three times with PBS and incubated with a mouse HBcAg antibody for 1 hr. After washing three times, a 1/1000 dilution of a goat anti-mouse horseradish peroxidase (HRP)-conjugated antibody (Huamei, China) was added and the mixture was incubated for 1 hr. The blot was washed three times before adding DAB (Sambrook *et al*., 1989).

Immunization of mice. Male BALB/c mice were bred in the facilities of this Institute. Groups of anesthetized mice (six mice in each group) were injected intramuscularly in the left hind thigh muscles with 100 µg of plasmid DNA in 0.9% NaCl. The mice were boosted 2 weeks later. Animals of the group 1 were injected with plasmid pcDNA3 (negative control). The group 2 was given the plasmid pcDNA3-HBcAg; while the group 3 the plasmid pcDNA3-IL1β-HBcAg. The sera were collected at indicated time intervals.

Measurement of antibody response. Sera from vaccinated mice were assayed for the antibody response using a competitive enzyme-linked immunosorbent assay (ELISA) kit (Kehua, China) and an ELISA plate reader (Biorad, USA). In brief, if the sample contained an anti-HBcAg antibody that reacted with HBcAg, the antibody would inhibit binding of the HRP-labeled anti-HBcAg antibody and no color reaction would occur. If the sample did not contain an antibody that reacted with HBcAg, the HRP-labeled anti-HBcAg antibody would bind and a color reaction would occur.

T cell proliferation assay. Spleens from the vaccinated mice were removed and digested with trypsinase K for 2 hrs. Red blood cells (RBC) were removed by incubation in 0.83% NH_4Cl and 0.17 mol/l Tris pH 7.4 for 10 mins at 37°C. T cells (2 x 10^6/ml) were grown in 96-well plates and then incubated with purified HBcAg. Splenocytes were incubated at 37°C in 5% CO_2 for 3 days. Cultures were incubated with 50 µg MTT (Sigma) for 6 hrs before the addition of acidified isopropanol and were analyzed at 570/630 nm in an ELISA plate reader. The results were expressed as a stimulation index (SI, SI = $A_{570/630\,nm}$ with antigen /$A_{570/630\,nm}$ without antigen), which was regarded positive if SI ≤ 2.

Results

Expression of HBcAg in E. coli

The HBcAg cDNA was inserted into the plasmid pET-28a downstream of the 6×His tag to facilitate its protein purification. The "empty"plasmid pET-28a served as a negative control. *E. coli* strain BL21(DE3)pLysS was transfected with the above plasmids. The protein expression

was induced by IPTG. In contrast to the negative control, a protein of 21 K was specifically expressed from the pET-28a-HBcAg plasmid as revealed by SDS-PAGE and Coomassie Brilliant Blue staining (data not shown). The protein was purified up to 95% purity using a Ni^{2+}-NTA-Sepharose 6B column.

Expression of IL-1β-HBcAg in mammalian cells

The HBcAg gene digested with corresponding restriction endonucleases and the DNA fragment encoding the IL-1β 163–171 peptide, were cloned into the pcDNA3 plasmid, resulting in the pcDNA3-IL1β-HBcAg plasmid. Digestion with restriction endonucleases, PCR and sequence analysis were used to verify correct insertion and sequence fidelity of the recombinant plasmid.

293 cells were transfected with the recombinant plasmid and the "empty" plasmid served as the negative control. The cell lysates were subjected to Western blot analysis. A 21 K protein was specifically detected by HBcAg antiserum from the lysate of cells transfected with pcDNA3-IL1β-HBcAg plasmid. The empty plasmid pcDNA3 (negative control) gave a negative result (Fig. 1).

Fig. 1
Western blot analysis of expression of HBcAg in 293 cells
pcDNA3 (lane 1); pcDNA3-IL1β-HBcAg (lane 2).

Immunization of mice with the DNA vaccine producing HBcAg and the effect of the IL-1β 163–171 peptide

To test the possibility that the IL-1β 163–171 peptide might boost the immune responses of DNA vaccines in general, three groups of anesthetized mice (six mice in each group) were injected intramuscularly in the left hind thigh muscles with either the empty plasmid pcDNA3 (negative control), pcDNA3-HBcAg or pcDNA3-IL1β-HBcAg. The injections were repeated two weeks later to boost the immune responses, and the sera from the vaccinated mice were assayed for the antibody responses by competitive ELISA.

After the primary injection, no blood samples collected from any of the immunized mice groups showed a detectable level of immune response. At day 10 after the second injection, the sera from three out of six mice injected with pcDNA3-HBcAg were positive, while all of the six mice injected with pcDNA3-IL1β-HBcAg were positive. The mean A of the pcDNA3-IL1β-HBcAg group was significantly lower than that of the pcDNA3-HBcAg group (Fig. 2). These results suggested that the IL-1β 163–171 peptide increased this particular antibody response and might increase the antibody responses to DNA vaccines in general.

T cell proliferation response to the DNA vaccine producing HBcAg and the effect of the IL-1β 167–171 peptide

To determine whether T cell proliferation response to the DNA vaccine producing HBcAg may be boosted by IL-1β 163–171 peptide, splenocytes from vaccinated mice were examined for proliferation. Although the mice injected with plasmids pcDNA3-IL-1β-HBcAg or pcDNA3-HBcAg elicited significant proliferation responses over a wide range of HBcAg concentration (5–40 μg), pcDNA3-IL1β-HBcAg increased T cell proliferation index two-three-fold compared with plasmid pcDNA3-HBcAg (Table 1). No specific stimulation with the plasmid pcDNA3 (negative control) was observed. Thus, the IL-1β 163–171 peptide could not only increase the humoral immune response but also the cellular

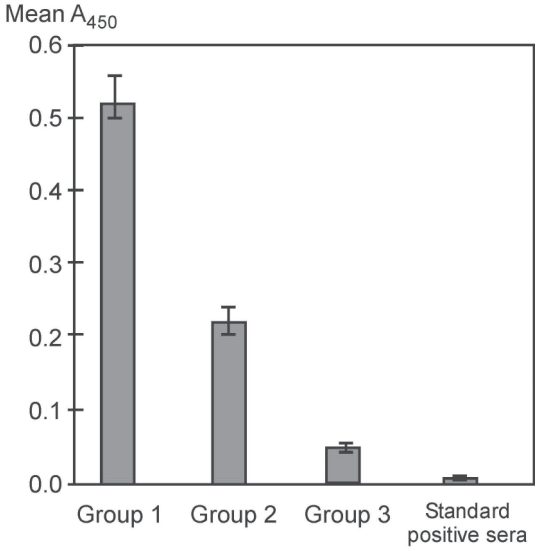

Fig. 2
HBcAg antibodies in the immunized mouse sera 10 days after the second plasmid injection
Competitive ELISA. pcDNA3 (group 1); pcDNA3-HBcAg (group 2); pcDNA3-IL1β-HBcAg (group 3).

Table 1. Proliferation of murine splenocytes induced by HBcAg

Plasmids	Stimulation index (SI)				
	HBcAg (μg)				Con A
	40	20	10	5	
pcDNA3	2.11 ± 0.23	1.04 ± 0.25	0.87 ± 0.18	0.84 ± 1.12	10.33 ± 0.35
pcDNA3-HBcAg	3.71 ± 0.41	3.19 ± 0.61	2.74 ± 0.37	2.52 ± 0.22	9.42 ± 0.21
pcDNA3-IL1β-HBcAg	9.77 ± 0.35	8.22 ± 0.41	8.37 ± 0.31	6.78 ± 0.22	11.02 ± 1.37

Results are expressed as mean SI from triplicate wells ± SE. SI = the mean $A_{570/630\ nm}$ of the culture stimulated with HBcAg/the mean $A_{570/630\ nm}$ of the culture not stimulated with HBcAg.

immune response when used as an adjuvant to this particular DNA vaccine.

Discussion

A few adjuvants to DNA vaccines have been so far described (Kim *et al*., 2001; Nobiron *et al*., 2001; Wang *et al*., 2000). However, none of them have been shown to be highly effective, and at the same time they all have side effects in humans. It is now widely believed that IL-1 plays an important role in B and T cell activations. Purified IL-1 was shown to enhance secondary antibody response of protein antigens both *in vitro* and *in vivo* (Lin *et al*., 1995; Staats *et al*., 1999). But its potent pyrogenic and inflammatory effects (such as leukocyte increase and activation, induction of acute phase reactants, serum ion alteration, temperature increase, etc.) restrict its application as an adjuvant (Veltri and Smith, 1996; Verschraegen *et al*., 1996). Several short peptides of murine and human IL-1 have been synthesized to determine the minimal structure responsible for their biological activities (Antoni *et al*., 1986). Among those peptides, a nonapeptide from human IL-1β (VQGEESNDK, aa 163–171) was shown to maintain the capacity to activate B and T cells without any inflammatory responses (Antoni *et al*., 1986; Boraschi and Tagliabue, 1999; Tagliabue and Boraschi, 1993). Excessive amount (100 mg of the peptide per kg of body weight) of the IL-1β 163–171 peptide had to be used to elicit a significant enhancement of antibody response (Rao and Nayak, 1990); however, it limited its applicability as an adjuvant. DNA vaccines have provided a promising approach to overcome this problem.

In our study, the *in vivo* immunostimulatory activities of the IL-1β 163–171 peptide were tested on the immune responses to HBcAg DNA vaccine. No sera collected from all the immunized groups showed a detectable level of immune responses after the primary injection. After the secondary injection, HBcAg gene fused with the DNA fragment encoding the IL-1β 163–171 peptide caused fourfold increase of HBcAg antibody compared to HBcAg alone. However, when the IL-1β 163–171 peptide was used as an adjuvant for a protein vaccine, a much greater increase was observed (Rao and Nayak, 1990). This discrepancy might be well explained as follows: (i) compared with protein vaccines, DNA vaccines induce comparatively low immune response; (ii) there is a species-specific sequence variation between murine and human IL-1β 163–171 peptides.

IL-1 can act as a direct B cell growth and differentiation factor (Dinarello, 1992, 1996) to enhance antibody production. On the other hand, the adjuvant activity of this nonapeptide could be the result of induction of IL-2 production and of IL-2 receptor expression on T cells. The IL-1β 163–171 peptide as well as IL-1 have been shown to be potent enhancers of IL-2 production *in vitro* (Tagliabue and Boraschi, 1993; Nencioni *et al*., 1987).

A lot of work needs to be done to verify the generality of immunostimulation effects of IL-1β 163–171 peptide. Nevertheless, our data provided an evidence that the IL-1β 163–171 peptide might represent a good candidate for an adjuvant *in vivo*.

References

Antoni G, Presentini R, Perin F, Tagliabue A, Ghiara P, Censini S, Volpini G, Villa L, Boraschi D (1986): A short synthetic peptide fragment of human interleukin 1 with immunostimulatory but not inflammatory activity. *J. Immunol. 137*, 3201–3204.

Beckers W, Villa L, Gonfloni S, Castagnoli L, Newton SM, Cesareni G, Ghiara P (1993): Increasing the immunogenicity of protein antigens through the genetic insertion of VQGEESNDK sequence of human IL-1β into their sequence. *J. Immunol*. **151**,1757–1764.

Boraschi D and Tagliabue A (1999): Interleukin-1 and interleukin-1 fragments as vaccine adjuvants. *Methods* **19**, 108–113.

Ciernik IF, Berzofsky JA, Carbone DP (1996): Induction of cytotoxic T lymphocytes and antitumor immunity with DNA vaccines expressing single T cell epitopes. *J. Immunol*. **156**, 2369–2375.

Dinarello CA (1992): The biology of interleukin-1. *Chem. Immunol*. **51**, 1–32.

Dinarello CA (1996):Biologic basis for interleukin-1 in disease. *Blood* **87**, 2095–2147.

Geissler M, Tokushige K, Wakita T, Zurawski VR Jr, Wands JR (1998): Differential cellular and humoral immune responses to HCV core and HBV envelope proteins after genetic immunizations using chimeric constructs. *Vaccine* **16**, 857–867.

Kim JJ, Yang JS, Manson KH, Weiner DB (2001): Modulation of antigen-specific cellular immuneresponses to DNA vaccination in rhesus macaques through the use of IL-2, IFN-γ, or IL-4 gene adjuvants. *Vaccine* **19**, 2497–2505.

Lin R, Tarr PE, Jones TC (1995): Present status of the use of cytokines as adjuvants with vaccines to protect against infectious diseases. *Clin. Infect. Dis*. **21**,1439–1449.

Lowrie DB, Silva CL, Colston MJ, Ragno S, Tascon RE (1997): Protection against tuberculosis by a plasmid DNA vaccine. *Vaccine* **15**, 834–838.

Lozes E, Huygen K, Content J, Denis O, Montgomery DL, Yawman AM, Vandenbussche P, Van Vooren JP, Drowart A, Ulmer JB, Liu MA (1997): Immunogenicity and efficacy of a tuberculosis DNA vaccine encoding the components of the secreted antigen 85 complex. *Vaccine* **15**, 830–833.

Manickan E, Rouse RJ, Yu Z, Wire WS, Rouse BT (1995): Genetic immunization against herpes simplex virus. Protection is mediated by CD4+ T lymphocytes. *J. Immunol*. **155**, 259–265.

Nencioni L, Villa L, Tagliabue A, Antoni G, Presentini R, Perin F, Silvestri S, Boraschi D (1987): In vivo immunostimulating activity of the 163–171 peptide of human IL-1 beta. *J. Immunol*. **139**, 800–804.

Nobiron I, Thompson I, Brownlie J, Collins ME (2001): Cytikine adjuvancy of BVDV DNA vaccine enhances both humoral and cellular immune responses in mice. *Vaccine* **19**, 4226–4235.

Rao KVS, Nayak AR (1990): Enhanced immunogenicity of a sequence derived from hepatitis B virus surface antigen in a composite peptide that includes the immunostimulatory region from human interlaukin 1. *Proc. Natl. Acad. Sci. USA* **87**, 5519–5522.

Sambrool J, Fritsch F, Maniatis T (1989): *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory Press.

Staats HF, Ennis FA Jr (1999): IL-1 is effective adjuvant for mucosal and systemic immune responses when coadministered with protein immunogens. *J. Immunol.* **162**, 6141–6147.

Stevenson FK, Zhu D, King CA, Ashworth LJ, Kumar S, Hawkins RE (1995): Idiotypic DNA vaccines against B-cell lymphoma. *Immunol. Rev.* **145**, 211–228.

Tagliabue A, Boraschi D (1993): Cytokines as vaccine adjuvants: Interleukin 1 and its synthetic peptide 163–171. *Vaccine* **11**(5), 594–595.

Tang DC, DeVit M, Johnston SA (1992): Genetic immunization is a simple method for eliciting an immune response. *Nature* **356**, 152–154.

Xiang ZQ, Spitalnik S, Tran M, Wunner WH, Cheng J, Ertl HCJ (1994): Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus. *Virology* **199**, 132–140.

Veltri S, Smith JW 2nd (1996): Interleukin1 trials in cancer patients: a review of the toxicity, antitumor and hematopoietic effects. *Stem Cells* **14**, 164–176.

Verschraegen, CF, Kudelka AP, Termrungruanglert W, de Leon CG, Edwards CL, Freedman RS, Kavanagh JJ, Vadhan-Raj S (1996): Effects of interleukin-1 alpha on ovarian carcinoma in patients with recurrent disease. *Eur. J. Cancer* **32**A, 1609–1611.

Wang B, Ugen KE, Srikantan V, Agadjanyan MG, Dang K, Refaeli Y, Sato AI, Boyer J, Williams WV, Weiner DB (1993): Gene inoculation generates immune responses against human immunodeficiency virus type I. *Proc. Natl. Acad. Sci. USA* **90**, 4156–4160.

Wang S. Liu X, Fisher K, Smith JG, Chen F, Tobery TW, Ulmer JB, Evans RK, Caufield MJ (2000): Enhanced type immune response to a hepatitis B DNA vaccine by formulation with calcium- or aluminium-phosphate. *Vaccine* **18**, 1227–1235.

Weber LW, Bowne WB, Wolchok JD, Srinivasan R, Qin J, Moroi Y, Clynes R, Song P, Lewis JJ, Houghton AN (1998): Tumor immunity and autoimmunity induced by immunization with homologous DNA. *J. Clin. Invest.* **102**, 1258–1264.