Biotechnology and Bioprocess Engineering **2005**, 10: 29-33

Construction of CpG Motif-enriched DNA Vaccine Plasmids for Enhanced Early Immune Response

Young Seoub Park[1], Seung Ha Hwang[2], and Cha-Yong Choi[1,2]*

[1] Interdisciplinary Program for Biochemical Engineering and Biotechnology, Seoul National University, Seoul 151-742, Korea
[2] School of Chemical Engineering, Seoul National University, Seoul 151-742, Korea

Abstract A DNA vaccine methodology using eukaryote expression vectors to produce immunizing proteins in the vaccinated hosts is a novel approach to the development of vaccine and immuno-therapeutics, and it has achieved considerable success over several infectious diseases and various cancers. To further enhance its efficiency, attempts were made to develop novel plasmid vectors containing multiple immunostimulatory CpG motifs, for rapid and strong immune response. First, a 2.9 kb compact plasmid vector (pVAC), containing CMV promoter, polycloning site, BGH poly(A) terminator, ampicillin resistance gene and pBR322 origin was constructed. A pVAC-hEPO was also constructed, which contained a human erythropoietin gene, for evaluating the transfection efficiency of naked plasmid DNA both *in vitro* and *in vivo*. To examine the adjuvant effect of multi-CpG motifs on naked plasmid DNA, 22 and 44 enriched and unmethylated CpG motifs were introduced into pVAC to generate pVAC-ISS1 and pVAC-ISS2, respectively. 100 μg of pSecTagB, pVAC, pVAC-ISS1 or pVAC-ISS2 were each injected intramuscularly into the tibilias anterior muscle of Balb/c mice. The level of interleukin-6 induced in the mice injected with pVAC-ISS1 and pVAC-ISS2 were significantly elevated after 12 hours, which were almost 2 and 2.5 times higher than that in the mice injected with pSecTagB, respectively. These results suggest that DNA vaccine plasmids with enriched CpG motifs can induce rapid secretion of interleukin-6 by lymphocytes. In conclusion, these vectors can contribute to the development of adjuvant-free DNA vaccinations against infectious diseases and various cancers.

Keywords: DNA vaccine, CpG motifs, erythropoietin, interleukin-6, immunostimulatory sequence

INTRODUCTION

DNA vaccines use eukaryote expression vectors to produce immunizing proteins in the vaccinated host, which represents a new and powerful approach for the generation of needed vaccines [1-3]. There has been considerable interest in enhancing the activity of naked plasmid DNA vaccine for treating various infectious diseases and cancers [4,5].

Bacterial DNA contains unmethylated CpG motifs that trigger B and NK cells, and secrete a variety of cytokines such as IL-12, IL-6 and TNF-α [1,2]. It has been reported that synthetic oligonucleotides and plasmid DNA containing CpG motifs boost early immune responses and support the induction of Th1 responses *in vivo* [4,6,7]. Interleukin-6 (IL-6) is a 26 kDa cytokine that is synthesized and secreted by mononuclear phagocytes, vascular endothelial and other cells in response to interleukin-1. IL-6 is an important pro-inflammatory cytokine due to its ability to regulate fever, immune function, and acute-phase responses [6]. For this reason, IL-6 was measured to evaluate its adjuvant effects of plasmid DNA vaccines.

In this study, it was demonstrated that novel plasmid vectors for the induction of early immune responses can result in an improvement in therapeutic gene therapy based on plasmid DNA. Firstly, a 2.9 kb compact plasmid vector (pVAC) was constructed by retaining only the essential elements for a DNA vaccine vector, derived from a mammalian expression vector. Secondly, a human erythropoietin (hEPO) gene was cloned into the pVAC in order to test the transfection efficiency of naked plasmid DNA both *in vitro* and *in vivo*. hEPO is the essential hormone which is made and secreted in the kidney and stimulates the differentiation and propagation of reticulocytes [8,9]. Finally, pVAC-ISS1 and pVAC-ISS2 which contain repeating unmethlyated CpG motif genes were cloned. The plasmids were administered to Balb/c mice and the induced levels of IL-6 measured by ELISA.

***Corresponding author**
Tel: +82-2-880-7071 Fax: +82-2-888-7295
e-mail: choicy@snu.ac.kr

METERIALS AND METHODS

Plasmid Preparation and Purification

The restriction endonucleases, T4 DNA ligase, and vent DNA polymerase, were purchased from New England Biolabs (Beverly, MA, USA). The pSecTag B was purchased from Invitrogen (Carlsbad, CA, USA) and the pUC19 was purchased from Novagen (Darmstadt, Germany). All the oligonucleotides used in the PCR reaction were synthesized at Cosmogentech (Seoul, Korea). The plasmids were amplified in DH-5α cells at 37°C, purified by Qiagen EndoFree Giga Kit (Qiagen; Germantown, MD, USA) and diluted in sterile and endo-free phosphate buffered saline (Promega; Seoul, Korea).

Oligodeoxynucleotides

Phosphodiester oligodeoxynucleotides (ODN) which contain enriched CpG motifs were synthesized with a 5′ phosphorylated end by Bioneer (Daejeon, Korea). The ODN used herein were: 5′ PGATCCAAAAGACGTTGAC GTTAAAGACGTTAAQCGTCAAA 3′ (41-mer), 5′ PCC CGCCCGCCCCCCCCAACGTTAACGTTGACGTCGAC GTCGCGGACGTTCCA 3′ (54-mer), 5′ PGATCTGGAA CGTCCGCGACGTCGACGT CAACGTTAACGTT 3′ (41-mer), and 5′ PGGGGGGGGCGGGGCGGGTTTT GACGTTAACGTCTTTAACGTCAACGTCTTTT 3′ (53-mer). All ODNs were hybridized with each other by boiling for 10 minutes then cooled slowly to room temperature. Hybridized double stranded DNA was inserted into a *Bgl*II restriction site in the pVAC. A CpG motif is 5′-purine-purine-cytosine-guanosine-pyrimidine-pyrimidine-3′, which a stimulator of primate leukocytes [6,7]. The hybridized ODNs contain 22 CpG motifs.

Plasmid Vectors

First, a 2.9 kb compact plasmid vector (pVAC) derived from pSecTagB and pUC19 (Novagen), was constructed. A CMV-IE promoter, an Ig κ–chain leader sequence, a multiple cloning site, a BGH poly A terminator were derived from the pSecTag B. And an ampicillin resistance gene and a pBR322 origin were derived from the pUC19. To construct pVAC, 1,201 base pair fragment obtained by PCR of pSecTagB with sense and antisense primers. The primers were 5′ TTTTTTAATAAT(*Ssp*I)GG ATCC(*Bam*HI) CGATGTACGGGCCAGAT 3′ and 5′ TTTTTTGATTC (*Tfi*I)AGATCT(*Bgl*II)TCCCCAGCATGCCTGCT 3′, respectively. Restriction sites of *Bam*HI and *Bgl*II were introduced to the fragment for the further cloning strategy. On digestion of the pUC19 with *Tfi*I (sticky end) and *Ssp*I (blunt end), the second fragment containing ampicillin resistance gene and the pBR322 origin was obtained. The ligation was carried out with these two fragments.

To construct the pVAC-ISS1, four phosphorylated oligodeoxynucleotides (5′ PGATCCAAAAGACGTTGACGT TAAAGACGTTAAQCGTCAAA 3′, 5′ PCCCGCCCGCC CCCCCCAACGTTAACGTTGACGTCGACGTCGCGGA CGTTCCA 3′, 5′ PGATCTGGAACGTCCGCGACGTCG ACGTCAACGTTAACGTT 3′, and 5′ PGGGGGGGGC GGGGCGGGTTTTGACGTTAACGTCTTTAACGTC AACGTCTTTT 3′) were hybridized with each other, and then ligated into pVAC which was digested with a *Bgl*II restriction endonuclease. Since the *Bam*HI and *Bgl*II share the same recognition sequence, ligating the *Bam*HI- and *Bgl*II-digested DNAs resulted in a plasmid DNA unrecognizable any further with these enzymes. The pVAC-ISS2 was constructed by inserting the same hybridized ODN into the unique *Bgl*II site of pVAC-ISS1 (Fig.1). The pVAC-hEPO and pSecTagB-hEPO plasmids were constructed by inserting the human erythropoietin (hEPO) gene into the *Hind*III and *Xba*I recognition sites of pVAC and pSecTagB, respectively (Fig.1).

Animals and Cell Lines

The 6-week-old female Balb/c mice were obtained from the Seoul National University Laboratory Animal Center and maintained under specific pathogen-free and standard conditions with a 12-h light-dark cycle. NIH3T3 (mouse fibroblast) and COS-7 (monkey kidney) cell lines were purchased from the Korean Cell Line Bank (KCLB) (Seoul, Korea). All culture media and fetal bovine serum were obtained from GIBCO/BRL (Carlsbad, CA, USA). All cells were cultured at 37°C in a 5% CO_2 humidified incubator and maintained in DMEM supplemented with 10% heat inactivated FBS (v/v), sodium bicarbonate (3.7 g/L), penicillin (100 U/mL), and streptomycin (100 μg/mL).

Transfection of Plasmids

The NIH3T3 mouse fibroblast and COS-7 African green monkey kidney cell lines were plated at a concentration of 2×10^5 cells per well in a six-well plate under normal growth conditions (37°C and 5% CO_2). The cells were each transiently transfected with 2 μg of the pVAC-EPO, pSecTagB-EPO or pSecTagB. After incubation for 24 h at 37°C, the culture supernatants were collected and assayed to detect the expression level of the hEPO gene.

Intramuscular DNA Injection

Balb/c mice were injected i.m. with 100 μg of purified plasmids (1 μg/μL, diluted in PBS) into the tibilias anterior muscle. Blood was collected three times by retro-orbital venipuncture at weekly intervals. The hematocrit level, which is the percent of whole blood that is comprised of red blood cells, was determined by measuring the cellular portion of the total amount of blood after centrifuging the whole blood in heparinized capillary tubes [10]. The cellular portion is almost entirely red blood cells because the portion of white blood cells is very small. All animal procedures were performed at the Seoul National University Laboratory Animal Center and conducted in conformity with national and international laws and policies.

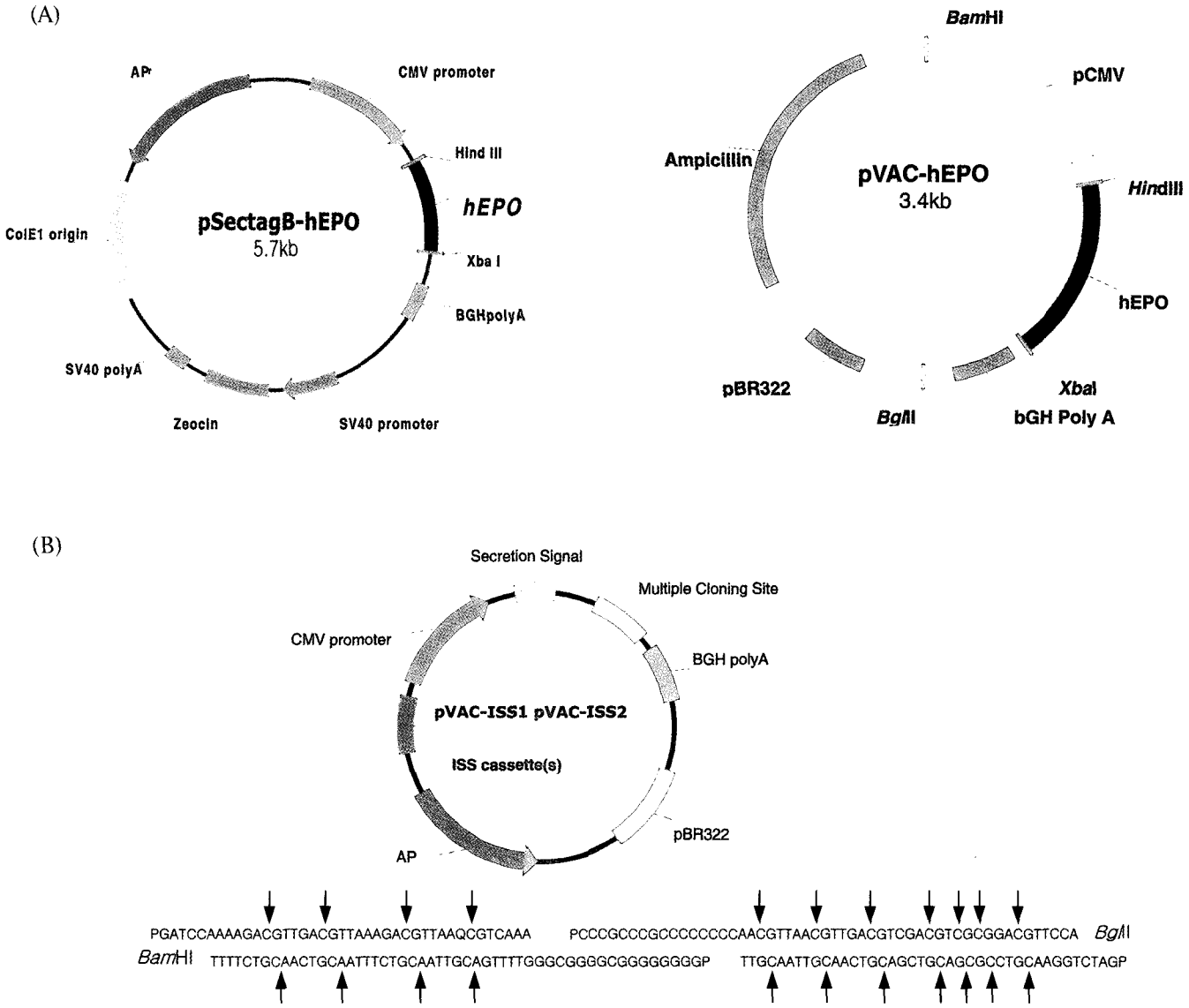

Fig. 1. (A) Gene maps of hEPO-containing plasmid vectors and (B) schematic illustration for construction of pVAC-ISS1 and pVAC-ISS2. All vectors contain a CMV promoter, BGH poly A terminator, ampicillin resistance gene and ColE1 origin. The pVAC contains multiple cloning site and unique restriction sites of *Bam*HI and *Bgl*II for further cloning strategies. The pVAC-hEPO-1 (3.4 kb) is the smallest plasmid vector in size among hEPO expression vectors. A single cassette of ISS has 22 enriched and unmethylated CpG motifs. pVAC-ISS1 and pVAC-ISS2 have single and double ISS cassette(s), respectively. Arrows mean each CpG motif.

Measurement of hEPO and IL-6

Culture supernatants and mice blood samples were collected at the indicated times and the levels of EPO and interleukin-6 measured using ELISA kits (Roche, Basel, Switzerland; Pharmingen, San Diego, CA, USA, respectively). Recombinant hEPO (Roche) mouse interleukin-6 and human serum were used as standard and control, respectively.

RESULTS AND DISCUSSION

To examine the transfection efficiency, pVAC, pVAC-hEPO, and pSecTagB-hEPO were transfected into COS-7 and NIH3T3 cells, and a competitive enzyme-linked immunosorbent assay (ELISA) was performed to detect expressed and secreted hEPO levels. The hEPO expression level of transfected pVAC-hEPO was found to be significantly elevated: two times higher than that of the transfected pSecTagB-hEPO in the case of COS-7 and four times higher than in the case of NIH3T3 (Fig. 2). The 6-week-old female Balb/c mice were injected with 100 μg of the pVAC, pVAC-hEPO, and pSecTagB-hEPO into the tibilias anterior muscle and with PBS as a negative control. The same muscles were treated with cardiotoxin before injection of the plasmids to induce muscle degeneration and regeneration as previously described

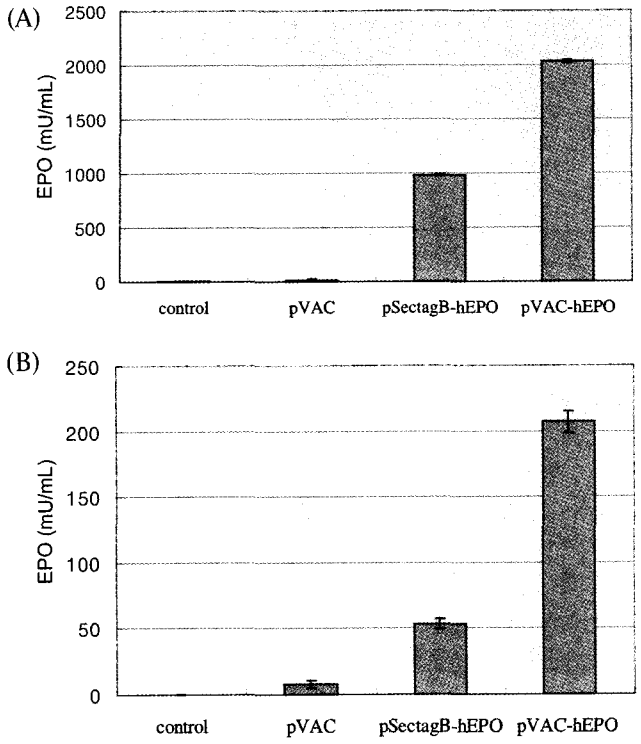

Fig. 2. The hEPO expression levels of plasmid vectors transfected into COS-7 and NIH3T3 cells. (A) COS-7. (B) NIH3T3. Cell lines were transfected with 2 μg of all plasmids and the culture supernatants were collected after 24 hours. The hEPO levels were measured by EPO ELISA. Data are presented as the mean (+/-) SD values of the hEPO levels.

[11]. The administration of pVAC-hEPO led to a rise in a mean hematocrit level from 47% to 58% (Fig. 3). The hEPO expression in the blood sample increased approximately two-fold with the pVAC-hEPO compared with that of the pSecTagB-hEPO. These results show that pVAC-hEPO, the smallest of the constructed vectors, was the most effective vector for hEPO expression both *in vitro* and *in vivo* and an enhancement of EPO producing ability of pVAC-hEPO could guarantee an increased efficiency of direct plasmid delivery into the skeletal muscles *in vivo*. Therefore, this result reassures the previous finding that the size of plasmid vectors is one of the important factors related to the transfection efficiency of various DNA vaccines based on naked plasmid DNA [12]. To determine the adjuvant effect of enriched CpG motifs on pVAC-ISS1 and pVAC-ISS2, 100 μg of pSecTagB, pVAC, pVAC-ISS1 or pVAC-ISS2 were each injected i.m. into the tibilias anterior muscle of Balb/c mice. The pVAC-ISS1 has a single ISS cassette, which contains 22 repeating CpG motifs and the pVAC-ISS2 has double ISS cassettes, which contain 44 repeating CpG motifs. The levels of interleukin-6 induction in the mice injected with pVAC-ISS1 and pVAC-ISS2 was significantly elevated within 12 h, which were almost 2 and 2.5 times higher, respectively, than those injected with pSecTagB (Fig. 4). These results suggest that DNA vaccine plasmids with

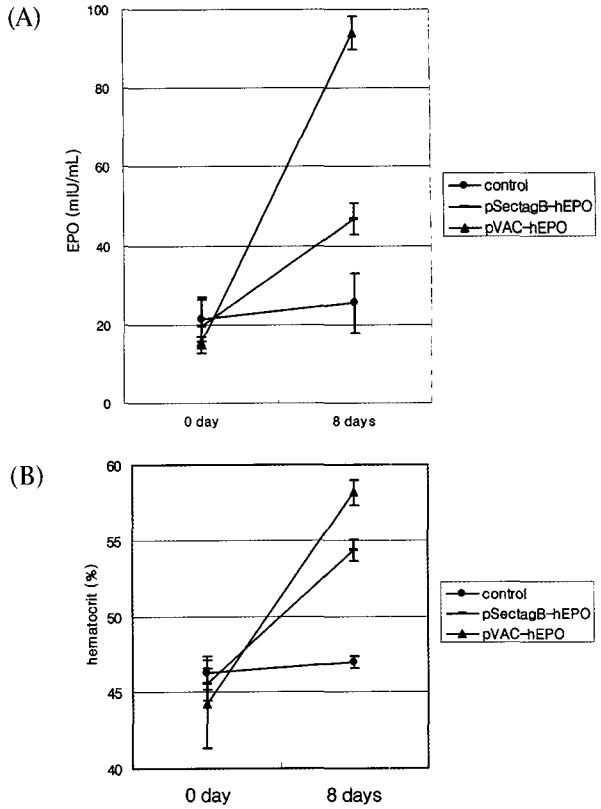

Fig. 3. The serum hEPO and hematocrit levels after intramuscular injection with the constructed vectors in Balb/c mice. Groups of five mice were injected i.m. with 100 μg of plasmids into the tibilias anterior muscle. After 8 days, blood samples were collected and hematocrit and serum hEPO levels were detected. (A) Serum EPO levels. (B) Hematocrit levels. Data represent the mean (+/-) SD of the hematocrit and serum EPO. The experiment was repeated at least three times with similar results.

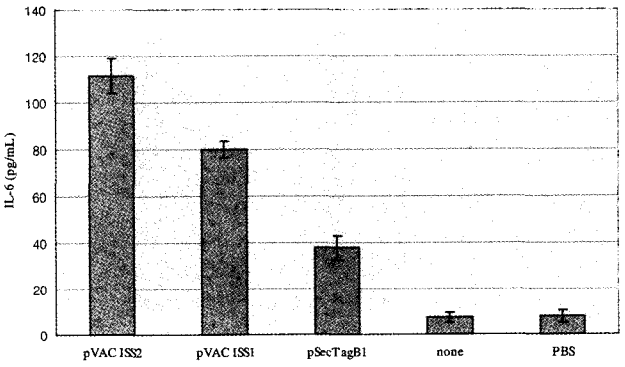

Fig. 4. The serum IL-6 levels after intramuscular injection with pSecTagB, pVAC-ISS1 or pVAC-ISS2 in Balb/c mice. Groups of eight mice were injected i.m. with 100 μg of plasmids into the tibilias anterior muscle. After 12 hours, blood samples were collected by retro-orbital venipuncture and IL-6 levels were detected using IL-6 ELISA. Data represent the mean (+/-) SD of IL-6 levels. The experiments were carried out at least twice with similar results.

enriched CpG motifs can induce the rapid secretion of interleukin-6 by lymphocytes and small plasmid vector containing enriched CpG motifs are more efficacious as DNA vaccines.

CpG DNA could rapidly induce early immune responses by activating transcriptional factor such as NFκB and activated protein-1. These transcriptional factors are regulated via reactive oxygen species (ROS), which activate cytokine synthesis [7,13,14]. There have been reports revealing that repeating sequence of CpG motif in a DNA vaccine could enhance the induction of Th1-type responses *in vivo* but the optimal number and sites of CpG motif cloned to DNA vaccine is yet to be determined [15,16].

The potential of a DNA vaccine plasmid include the direct and easy expression of the encoded protein into the vaccinated host and also the induction of early immune responses *via* CpG motif in the DNA vaccine itself. Therefore, a cloning strategy for a DNA vaccine based on a plasmid vector should be considered for the development a more efficacious vaccine. Herein, a powerful cloning method has been provided using compact vector of pVAC and ISS cassette(s) with a view to easily and simply amplifying CpG motifs and constructing effective DNA vaccine against various targets.

There have been studies concerning developing methods for efficient DNA vaccines such as co-administration of hyaluronidase, plasmid transfer into skeletal muscle mediated by electric pulses *in vivo* and liposome-mediated DNA transfer [17-22]. An increase of activity by using plasmid DNA provides a synergistic enhancement to these methods of DNA vaccination.

REFERENCES

[1] Hele, T., C. Maripat, R. Mark, and R. Eyal (1998) Gene vaccination: Plasmid DNA is more than just a blueprint. *Immunol. Today* 19: 89-97.

[2] Harriet, L. and C. A. T. Celia (1997) DNA vaccines. *Semin. Immunol.* 9: 271-283.

[3] Lindsay, W., R. Fernando, Z. Jie, and E. H. Daniel (1999) DNA immunization: Mechanistic studies. *Vaccine* 17: 1612-1619.

[4] Yoshtsugu, K., X. Ke-Qin, O. Takaki, H. Kenji, O. Tomohiro, S. Kaori, O. Tomomi, H. Yuka, J. Nao, N. Masatoshi, K. Dennis, and O. Kenji (2002) Adjuvant effect of multi-CpG motifs on an HIV-1 DNA vaccine. *Vaccine* 20: 2857-2865.

[5] Shin, S., T. Fumihiko, X. Ke-Qin, I. Norihisa, and O. Kenji (2003) Technical issues in construction of nucleic acid vaccines. *Methods* 31: 199-206.

[6] Yi, A. K., D. M. Klinman, T. L. Martin, S. Matson, and A. Krieg (1996) Rapid immune activation by CpG motifs in bacterial DNA: Systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. *J. Immunol.* 157: 5394-5402.

[7] Krieg, A. M., A. K. Yi, S. Matson, T. J Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374: 546-549.

[8] Benjamin, L. E. and B. Franklin (1999) Regulation of the erythropoietin gene. *Blood* 94: 1864-1877.

[9] Kendall, R. G. (2001) Erythropoietin. *Clin. Lab. Haematol.* 23: 71-80.

[10] Klinman, D. M., J. Conover, J. M. Leiden, A. S. Rosenberg, and J. M. Sechler(1999) Safe and effective regulation of hematocrit by gene gun administration of an erythropoietin-encoding DNA plasmid. *Hum. Gene Ther.* 10: 659-665.

[11] Duchen, L. W., B. J. Excell, R. Patel, and B. Smith (1974) Changes in motor end-plates resulting from muscle fiber necrosis and regeneration: A light and electron microscopic study of the effects of the depolarizing fraction (cardiotoxin) of *Dendroaspis jamesoni* venom. *J. Neurol. Sci.* 21: 391-417.

[12] Herrera, A. B., E. G. Rodriguez, T. Hernandez, B. Sandez, and C. A. Duarte (2000) A family of compact plasmid vectors for DNA immunization in humans. *Biochem Biophys. Res. Commun.* 279: 548-551.

[13] Nicole, A., R. Doria, and L. H. Nancy (2003) DNA vaccine strategies: Candidates for immune modulation and immunization regimens. *Methods* 31: 207-216.

[14] Ronald, K. S. (2000) The role of CpG motifs in immunostimulation and gene therapy. *Adv. Drug Deliv. Rev.* 44: 119-134.

[15] Klinman, D. M. and A. K. Yi (1996) CpG motifs present in bacterial DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon γ. *Proc. Natl. Acad. Sci. USA* 93: 2879-2883.

[16] Klinman, D. M., G. Yamshchikov, and Y. Ishgatsubo (1997) Contribution of CpG motifs to the immunogenicity of DNA vaccines. *J. Immunol.* 158: 3635-3639.

[17] Tripathy, S. K., E. C. Svensson, H. B. Black, E. Goldwasser, M. Margalith, P. M. Hobart, and J. M. Leiden (1996) Long-term expression of erythropoietin in the systemic circulation of mice after intramuscular injection of a plasmid DNA vector. *Proc. Natl. Acad. Sci. USA* 93: 10876-10880.

[18] Mennuni, C., F. Calvaruso, I. Zampaglione, G. Rizzuto, D. Rinaudo, E. Dammassa, G. Ciliberto, E. Fattori, and N. La Monica (2002) Hyaluronidase increases electrogene transfer efficiency in skeletal muscle. *Hum. Gene Ther.* 13: 355-365.

[19] Mir, L. M., M. F. Bureau, J. Gehl, R. Rangara, D. Rouy, J. M. Caillaud, P. Delaere, D. Branellec, B. Schwartz, and D. Scherman (1999) High-efficiency gene transfer into skeletal muscle mediated by electric pulses. *Proc. Natl. Acad. Sci. USA* 96: 4262-4267.

[20] Y. Perrie, P. M. Frederik, and G. Gregoriadis (2001) Liposome-mediated DNA vaccination: The effect of vesicle composition. *Vaccine* 19: 3301-3310.

[21] Lee, H. S., J. H. Jeong, J. H. Lee, and T. G. Park (2001) Enhancing transfection efficiency using polyethylene glycol grafted polyethylenimine and fusogenic peptide. *Biotechnol. Bioprocess Eng.* 6: 269-273.

[22] Kim, Y. J., T. W. Kim, H. S. Chung, I. C. Kwon, and S. Y. Jeong (2001) Counterion effects on transfection activity of cationic lipid emulsion. *Biotechnol. Bioprocess Eng.* 6: 279-283.

[Received September 13, 2004; accepted February 21, 2005]