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Cloning, Protein Expression and Immunogenicity of HBs-Murine IL-18 Fusion DNA Vaccine

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SUMMARY Hepatitis B is a global serious disease caused by hepatitis B virus (HBV). There is no known cure for hepatitis B. The best way to deal with the disease is by preventing with hepatitis B vaccine. However, the current protein-based vaccines made up of recombinant hepatitis B surface antigen (HB_SAg) are ineffective in chronic HBV carriers and a significant number of the vaccinees do not mount the protective immune response. Novel DNA-based immunization may overcome the deficits of the protein-based immunization and may provide more effective prophylactic and therapeutic outcomes. In this study, we constructed a recombinant plasmid carrying gene encoding the HBV surface antigen (*HBs*) linked to DNA segment encoding full-length murine interleukin-18, i.e. pcDNA-HBs-IL-18. Immunogenicity of the DNA construct was carried out in BALB/c mice in comparison with mock, i.e. pcDNA3.1+ and vaccines comprised of pRc/CMV-HBs and pRc/CMV-HBs plus pcDNA-IL-18. All vaccinated mice revealed significant serum anti-HBs IgG response after two intramuscular injections of the vaccines at 28 day interval as compared to the level of mock. Co-administration of pRc/CMV-HBs and pcDNA-IL-18 elicited arbitrarily higher levels of anti-HBs IgG than the levels in mice immunized with pRc/CMV-HBs alone and mice that received pcDNA-HBs-IL-18 although not statistically different. Further experiments are needed to investigate the sub-isotypes of the IgG antibody, the kinetics of cytokine and the cell-mediated immune response. For this communication, the prototype HBs-IL-18 DNA vaccine was successfully constructed and the gene encoding murine IL-18 was successfully cloned. The latter can be co-injected with the antigen coding DNA or used as a fusion partner to the DNA for priming the immune response. The recombinant HBs and full-length IL-18 proteins have potential for other research purposes. They may be used also as standard proteins in the protein quantification assay.

Hepatitis B is a worldwide serious disease caused by hepatitis B virus (HBV). There is no known cure for hepatitis B. The best way to deal with the disease is by prevention using hepatitis B vaccine. However, the current protein-based vaccines made up of recombinant hepatitis B virus surface antigen (HB_SAg) are ineffective in chronic HBV carriers and a significant number of the vaccinees do not elicit the protective immune response to the vaccines.[1] Novel DNA-based immunization may overcome the deficits of the protein-based immunization and may provide more effective prophylactic and therapeutic outcomes. Hepatitis B DNA vaccine mimics virus infection in that the antigen is produced in its native conformation and can evoke cellular, *i.e.* cytotoxic lymphocytes, and humoral immune responses.[2-5] There have been studies on immunogenicity and efficacy of hepatitis B DNA vaccines.[6-7] In a phase I study of HBV DNA vaccination administered *via* a gene gun, it was found that low dose of

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the vaccine without adjuvant did not induce priming immune response.[8] Thus, several approaches have been used to enhance the immune response of vaccines, including the use of T-cell epitope-based immunization[9] and co-injection of the vaccine with adjuvants such as cytokines.[10,11]

Vaccine adjuvanticity of a number of cytokines has been studied.[12] The cytokines were used in the form of either a protein-cytokine mixture or a fusion DNA. In the latter, the cytokine is produced *de novo* and most exert the immunological effect over a short distance and a short time span at low concentrations. Cytokines may be produced by various cell populations; nevertheless, the major producers are the helper T cells (Th) and the macrophages.[13]

Interleukin-18 (IL-18) is a proinflammatory cytokine with potent interferon-γ (IFN-γ) inducing activity.[14] It is synthesized as a biologically inactive precursor molecule lacking a signal peptide. The protein requires activation to molecularly mature by intracellular cysteine protease cleavage.[14,15] In general, IL-18 is produced by T cells. Other cells that can produce IL-18 after being stimulated include monocytes, macrophages and keratinocytes. IL-18 acts on Th1 cells, natural killer (NK) cells and dendritic cells to produce IFN-γ in the presence of IL-12. Thus, IL-18 might play an important role in viral clearance. Studies have shown that IL-18 could contribute in the protection of mice from experimental bacterial infections through induction of IFN-γ.[15] IL-18 could enhance the Th1 immune responses in a mouse tumor model.[16]

In this study, a mammalian expression recombinant plasmid carrying genes encoding HBsAg and murine IL-18 was prepared and used as a vaccine to immunize BALB/c mice. Immunogenicity of the DNA construct was investigated.

MATERIALS AND METHODS

Preparation of plasmids carrying genes encoding HBsAg

Hepatitis B virus (HBV) was obtained from an HBV chronically infected Thai male. The virus genomic DNA was extracted and PCR amplified by oligonucleotide primers designed from the HBs nucleotide sequence of wild type HBV (GenBank access number X98077). A *Hin*dIII restriction site and a Kozak's sequence (ACCATGG) was included at the 5' of the forward primer for optimal expression in the mammalian cells. For constructing fusion DNA, the reverse primer contained the final 21 nucleotides of coding region without the stop codon but with a 12 nucleotide linker and *Xho*I restriction site. For constructing a DNA encoding only recombinant HBs protein, a similar reverse primer containing a stop codon (without the nucleotide linker) was used. PCR condition was: first DNA denaturation at 94°C for 4 minutes and 30 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute and extension at 72°C for 1 minute. The final cycle was followed by a 10 minutes extension step at 72°C. The amplified product containing the open reading frame of 678 bp of HBs gene was verified by DNA sequencing and cloned into a pGem-Teasy cloning vector (Promega, USA) as instructed by the manufacturer. The plasmids carrying the HBs gene were designated pG-HBs.

Preparation of plasmids carrying gene encoding full-length murine IL-8

Spleen cells of a BALB/c mouse were isolated and incubated with phytohemaglutinin (PHA) for 1 hour. Total mRNA was extracted from the activated cells using TRIzol® reagent (Invitrogen, New Zealand). Single stranded cDNA was synthesized using the reverse-transcription system (RevertAid™ H Minus First Stand cDNA Synthesis Kit, Fermentas, USA). The full length IL-18 gene was amplified by PCR using two couples of primers designed from the published sequence of *Mus musculus* (GenBank, accession number NP032386.1). For pcDNA-HBs-IL-18 fusion DNA preparation, the forward primer contained *Xho*I restriction site and a start codon (ATG). The reverse primer contained a stop codon and *Xba*I restriction site. For only IL-18 protein expression, the forward primer included *Eco*RI restriction site, a Kozak's sequence and a start codon (ATG). The PCR condition was similar to that of HBs gene amplification except the annealing temperature was changed from 58°C to 55°C. The PCR products were cloned into pGEM-Teasy vector. The recombinant plasmids carrying the verified IL-18 gene sequence were designated pG-IL-18.

pcDNA-HBs-IL-18 fusion DNA

To prepare the HBs-IL-18 fusion DNA, the murine IL-18 gene fragments were cut from the pG-IL-18 using *Xho*I and *Xba*I endonucleases. The cut fragments were purified and ligated into pcDNA3.1(+) expression vector which was previously cleaved with the same restriction enzymes. The pcDNA3.1(+) plasmids carrying the murine IL-18 gene were designated pcDNA-IL-18. Fragments of HBs gene were cut from the pG-HBs by using *Hin*dIII and *Xho*I restriction enzymes and inserted into pcDNA3.1(+) plasmids and pcDNA-IL-18 which were individually pre-cut with the *Hin*dIII and *Xho*I enzymes. The recombinant plasmids carrying HBs gene and the pcDNA-IL-18 with HBs gene insert were designated pcDNA-HBs and pcDNA-HBs-IL-18 fusion DNA, respectively. Individual recombinant plasmids, *i.e.* pcDNA-IL-8, pcDNA-HBs and pcDNA-HBs-IL-18 fusion DNA were introduced into *E.coli (*DH5α) and the respective transformed *E. coli* clones were selected by using ampicillin agar plates.

Verification of plasmid DNA

For plasmid DNA verification, the recombinant plasmids extracted from selected *E. coli* clones were extracted and purified using Qiagen Miniprep (Qiagen, Germany). The DNA sequences were analyzed with chain terminator method using BigDye™ terminator-cycle V. 3.1 sequencing kit and ABI Prism DNA Sequencer (ABI PRISM™, Foster City, CU, USA). To confirm that all of the gene inserts were in frame, the amino acids were deduced from the DNA sequences and protein alignments were done with the sequences of the database using the All-in-One- Seq Analyzer Version 1.35.[17]

Expression of HBs protein in prokaryotic cells

To investigate the antigenicity of the recombinant HBs protein, the HBs gene segment was cut from plasmid pcDNA-HBs with *BamH*I and *Xho*I enzymes and ligated into a prokaryotic expression vector, *i.e.* pET-32a(+) which was pre-cut with the same restriction enzymes. The ligated plasmids were introduced into gene storage JM109 *E. coli* and also into a prokaryotic expression host, *i.e.* BL21 (DE3 *E. coli*). Small scale protein expression was performed by culturing a selected transformant to mid-log phase and recombinant protein expression was induced by adding IPTG to the final concentration of 1 mM. The culture was incubated at 37°C with continuous aeration for 3 hours. The cells were harvested, washed twice with normal saline solution and resuspended in 1 ml of a lysis buffer. The cells were disrupted using Labsonic®P (Sartorious, Germany) (0.5 second per cycle and 50% amplitude for 10 minutes) in an ice bath. After the cells debris was removed by centrifugation at 4°C, the supernatant (*E. coli* lysate) was subjected to a sandwich ELISA for determining the binding activity of the HBs protein with the anti-HBs-antibody

HBs protein expression in mammalian cells

Chinese hamster ovary (CHO-K1) cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics in an 8-well tissue culture plate. The cells at log-phase culture were aliquoted, *i.e.* 1×10^4 cells per well. The cells in individual wells were transfected with 2 μg of pcDNA-HBs, pcDNA-HBs-IL-18, and blank pcDNA3.1(+) using Polyfect® (Qiagen, Germany) as directed by the manufacturer. The plate was incubated in a 5% CO_2 incubator at 37°C for 16 hours. The spent culture medium was changed with fresh medium and the plate was further incubated for 48 hours. The cells from individual wells were harvested and disrupted in 200 μl of lysis buffer (50 mM Tris HCl, 1 mM EDTA, and 100 mM NaCl) in an ice bath. The cell debris was removed by centrifugation and HBV antigen in the cell lysate was detected by sandwich ELISA.

Sandwich ELISA for determining antigenicity of the recombinant HBs

Antigenicity of the recombinant HBs protein prepared from the prokaryotic and the mammalian expression systems were investigated by using a commercial kit, *i.e.* Murex HBsAg version 3 (Abbott Laboratories, USA). Wells in the ELISA plate were appropriately coated with monoclonal anti-HBs provided in the kit. Seventy-five microliters of positive and negative controls, also provided in the kit, and HBs samples (cell lysates) were individually and appropriately added into the monoclonal antibody coated wells. The plate was incubated at 37°C for 1

hour. After washing with washing buffer (PBS containing 0.05% Tween-20 [PBST]) to removed unbound material, 50 µl of polyclonal anti-HBs-HRP was added into each well and incubated for 30 minutes. The wells were extensively washed with the PBST and 100 µl of substrate solution (TMB +0.015% H_2O_2) was added to each well and mixed. The plate was incubated at 37°C for 30 minutes; the enzymatic reaction was stopped by adding 50 µl of 1 N H_2SO_4 into each well. Optical density (OD) of the content in each well was measured at absorbance 405 nm (A_{450nm}) against blank (well to which PBS was added instead of the protein sample).

Protein analysis by SDS-PAGE and Western blotting

Transformed BL21 (DE3) *E. coli* harboring recombinant pET32a-HBs was grown in 500 ml LB medium and the expression of recombinant HBs protein was induced by adding IPTG to the concentration of 1 mM. After three hours of IPTG induction, bacterial cells were harvested by centrifugation. Cell pellet (1.5 mg) was disrupted in 1 ml of the lysis buffer containing protease inhibitors in an ice bath. After centrifugation to remove cell debris, recombinant HBs protein in the preparation was purified by loading the *E. coli* lysate onto a Ni/iminoacetic acid column (ProPure®, Nunc, Denmark) which was pre-equilibrated with a binding buffer (50 mM sodium phosphate, pH 7.4, 300 mM NaCl, 10 mM imidazole). The column was washed with the same buffer containing 30 mM imidazole until the O.D of the eluate was zero at A_{280nm}. The HBs protein was eluted out using a buffer containing 300 mM imidazole. The eluate was dialysed against PBS, pH 7.4, at 4°C overnight and lyophilized. The dried protein was reconstituted with 200 µl of distilled water, boiled in equal volume of sample buffer for 4 minutes; then 10 and 20 µl of the sample were subjected to SDS-PAGE and Coomassie Brilliant Blue R-250 staining or SDS-PAGE and Western blotting.

Western blotting was performed by transferring the SDS-PAGE separated-components onto a nitrocellulose membrane (NC). After blocking the unoccupied sites on the NC with 3% bovine serum albumin (BSA) and the excess blocking reagent was removed by washing, the NC was incubated with anti-HBs-HRP (Murex HBsAg version 3, Abbott Laboratories, USA) for 1 hour. After washing with PBST, the membrane was equilibrated in 1/15 M PBS pH 7.6 for 10 minutes. The antigen (HBs)-antibody reactive bands on the NC were revealed by adding substrate (10 mg of DCIP, 5 µl of 30% H_2O_2 in 5 ml of 1/15 M PBS pH 7.6). The membrane was washed with distilled water. In the case of detection of histidine-tagged protein, the HRP labeled anti-histidine was used instead of the anti-HBs-HRP conjugate.

Mouse immunization

BALB/c mice aged 6 weeks old were purchased from The National Laboratory Animal Center, Mahidol University, Salaya, Nakhon Pathom province, Thailand. They were divided into four groups of six mice. Each mouse received intramuscular injection with 50 µg of plasmid DNA mixed with 50 µg of aluminum phosphate ($AlPO_4$). Mice of the first groups received blank pcDNA3.1+ and served as negative control. Mice of the second group received pRc/CMV-HBs. Mice of the third group were injected with pcDNA-HBs-IL-18 fusion DNA. Mice of the fourth group received pcDNA-IL-18 plasmid mixed with pRc/CMV-HBs.[18] Immunizations were repeated on day 28. Mouse blood samples were collected on day 42. The sera were tested for IgG anti-HBs.

Detection of humoral immune response elicited by the DNA vaccines

Hepatitis B antibody expressed in mIU/ml in each mouse serum sample was detected by double antigen sandwich ELISA using a commercial test kit (Monolisa anti-HBs, Version 3.0, Bio-Rad, France). One hundred microliters of mouse serum was placed into well of an ELISA plate. The plate was incubated at 37°C for 60 minutes. The unbound protein was removed and all wells were washed with PBST. One hundred microliters of biotin-HBsAg-streptavidin peroxidase conjugate mixture was added into each well. The plate was incubated at 37°C for 60 minutes, washed and each well was added with 100 µl TMB substrate containing 0.015% H_2O_2. Enzymatic reaction was allowed to take place in the dark for 30 minutes at ambient temperature. The reaction was stopped by adding 50 µl of 1 N H_2SO_4 to each well. The optical density of the content in each well was

read at A_{450nm}. Values of anti-HBs were expressed as mean ± S.D.

Statistical analysis

The Mann-Whitney *U* test was used to compare mean values of the IgG antibody to HBs protein between groups of mice. A $p < 0.05$ was considered significantly different.

RESULTS

Fig. 1 shows the schematic diagrams of recombinant DNA constructs for mammalian expression vector. The transcriptional control sequences were: cytomegalovirus (CMV) early promoter/enhancer sequence and poly A, bovine growth hormone (BGH) terminator. In the fusion DNA sequence, the IL-18 DNA (Fig. 1a) and HBs DNA (Fig. 1b) were linked *via* a nucleotide linker sequence encoding Gly-Gly-Gly-Ser (Fig. 1c).

Full-length IL-18 and HBs genes were successfully linked, amplified and cloned. Their nucleotide sequences were analyzed using an online available Basic Local Alignment Search Tool (BLAST).[21] This program compares nucleotide and protein sequences to those in database and calculates the statistical significance of matches. A DNA sequence was deduced to amino acid sequence using online available software, All-in-One Seq Analyzer Version 1.35.[17] Fig. 2 shows the nucleotide sequence of pcDNA-HBs-IL-18 extracted from a selected transformed *E. coli* clone harboring the recombinant plasmids. The DNA sequence alignment indicated that the inserted sequences of the HBs and murine IL-18 genes were complete and their orientations were correct.

Fig. 3 shows the deduced amino acid sequences of the recombinant HBs and murine IL-18. The recombinant HBs contained 225 amino acids of HBV surface protein with high homology to many of the recently identified HBV surface proteins. Protein-protein BLAST with online available tool revealed that our recombinant HBs protein had 99% identity to the HBs surface protein of accession number ABC00824, which was reported by Mendy *et al*.[22] The sequence was derived from HBV subtype *ayw4* isolated from an HBsAg-positive child.

Full length 579 bp nucleotide fragment of murine IL-18 gene was successfully cloned. The fragment contained the entire open reading frame encoding 192 amino acids. The nucleotide and deduced amino acid sequences BLAST algorithm of IL-18 showed that our IL-18 DNA sequence had 98% homology to that of the reported sequence (accession number NP032386.1).

Recombinant HBs expressed by transformed BL21(DE3) *E. coli* (clones no. 1-5) harboring pET32a-HBs and by CHO-K1 cells transfected with pcDNA-HBs and pcDNA-HBs-IL-18 could react readily with the specific monoclonal antibody against HBsAg provided in the commercial test kit (Fig. 4).

Despite the high signal of ELISA results showing reactivity of the recombinant HBs proteins

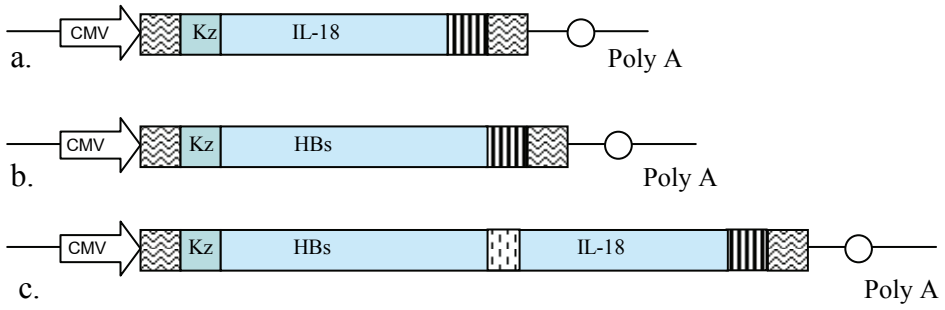

Fig. 1 Schematic diagrams of the expression vectors encoding murine IL-18 protein (a), HBV small surface protein (HBs) (b), and pcDNA-HBs-IL-18 fusion DNA (c).

expressed from prokaryotic and mammalian cells to the HBsAg specific monoclonal antibody, the presence of the recombinant HBs protein, *i.e.* a his-tagged-HBs protein at expected molecular mass (*Mr*) ~45 could not be seen after SDS-PAGE and Coomassie Brilliant Blue R-250 staining (Fig. 5). Instead, a thick band of bacterial protein containing histidine was predominant at the lower *Mr*. However, when the nitrocellulose membrane blotted with the SDS-PAGE separated-cell lysate was probed with polyclonal antibody to HBs protein, a band at *Mr*~45 was seen (Fig. 6) indicating that the recombinant HBs protein could be successfully expressed and the protein contained the HBs antigenic epitope.

Fig. 7 shows the mean anti-HBs IgG values in all experimental groups. The mean anti-HBs IgG value of mice in the pcDNA3.1+ group was 1.4 mIU/ml and those from the groups injected with pRc/CMV-HBs, pRc/CMV-HBs plus pcDNA-IL-18, and pcDNA-HBs-IL-18 fusion DNA were 3.8, 4.2 and 2.5 mIU/ml, respectively. The mean anti-HBs IgG level of mice immunized with Rc/CMV-HBs plus pcDNA-IL-18 was significantly higher than that of the pcDNA3.1+ group (p <0.05) but was not significantly higher than those of mice immunized with pRc/CMV-HBs and pcDNA-HBs-IL-18 fusion DNA (p > 0.05).

Name:pcDNA3.1(+)-HBs-IL18 (psIL18c1)

AAGCTTACCATGGCATGGAGAGCATCGCATCAGGATTCCTAGGACCCCTT
CTCGTGTTACAGGCGGGGTTTTTCTTGTTGACAAGAATCCTCACAATACCG
CAGAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGAACTACC
GTGTGTCTTGGCCAAAATTCGCAGTCCCCAACCTCCAATCACTCACCAACC
TCTTGTCCTCCAACTTGTCCTGGTTATCGCTGGATGTGTCTGCGGCGTTTT
ATCATCTTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCTT
CTGGACTATCAAGGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCCTCA
ACAACCAGCACGGGACCATGCCGGACCTGCATGACTACTGCTCAAGGAACC
TCTATGTATCCCTCCTGTTGCTGTACCAAACCTTCGGACGGAAATTGCACC
TGTATTCCCATCCCATCATCCTGGGCTTTCGGAAAATTCCTATGGGAGTGG
GCCTCAGCCCGTTTCTCCTGGCTCAGTTTACTAGTGCCATTTGTTCAGTGG
TTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGTTATATGGATGATGTGG
TATTGGGGGCCAAGTCTGTACAGCATCTTGAGTCCCTTTTTACCGCTGTTA
CCAATTTTCTTTTGTCTTTGGGTATACGGCGGTGGATCCCTCGAGATGGCT
GCCATGTCAGAAGACTCTTGCGTCAACTTCAAGGAAATGATGTTTATTGAC
AACACGCTTTACTTTATACCTGAAGAAAATGGAGACCTGGAATCAGACAAC
TTTGGCCGACTTCACTGTACAACCGCAGTAATACGGAATATAAATGACCAA
GTTCTCTTCGTTGACAAAAGACAGCCTGTGTTCGAGGATATGACTGATATT
GATCAAAGTGCCAGTGAACCCCAGACCAGACTGATAATATACATGTACAAA
GACAGTGAAGTAAGAGGACTGGCTGTGACCCTCTCTGTGAAGGATAGTAAA
ATGTCTGCCCTCTCCTGTAAGAACAAGATCATTTCCTTTGAGGAAATGGAT
CCACCTGAAAATATTGATGATATACAAAGTGATCTCATATTCTTTCAGAAA
CGTGTTCCAGGACACAACAAGATGGAGTTTGAATCTTCACTGTATGAAGGA
CACTTTCTTGCTTGCCAAAAGGAAGATGATGCTTCCAAACTCATTCTGAAA
AAAAAGGATGAAAATGGGGATAAATCTGTAATGTTCACTCTCACTAACTTA
CATCAAAGTTAGTCTAGA…

AAGCTT : restriction site *Hind*III;

TCTAGA : restriction site *Xba*I;

ACCATGG : Kozak sequence;

ATG : start codon;

GGCGGTGGATCCCTCGAG : linker

Fig. 2 DNA sequences encoding HBs and murine IL-18 from a selected transformed *E. coli* clone harboring recombinant pcDNA-HBs-IL-18 plasmids.

DISCUSSION

Currently, DNA vaccines have received much attention in chronic viral infections as the immunogenic components represent natural viral molecules in stimulating the host immunity, especially the cytotoxic lymphocytes and Th1 cytokines that activate other effector cells. Usually DNA vaccine elicits both humoral and cell mediated immune responses in different degrees due to the pathways from which the host cells manufacture and present the viral epitopes. In most cases, the immunogenicity of DNA vaccines remains relatively low in large animals and non-primates compared to mice.[11,23] Several approaches have been used to improve the cell mediated immune response of DNA vaccines. One of which is the co-delivery of cytokine protein or cytokine expression plasmids which acts as the vaccine adjuvants.[16,24] Studies in a variety of animal models demonstrated that the cytokines could mediate immunomodulatory effects by altering the magnitude and direction of the host immune responses and could improve the vaccine efficacy.[11,25]

In this study, we report the production of plasmid DNA construct carrying gene encoding hepatitis B surface protein linked with a gene encoding full-length murine IL-18. The IL-18 gene was expected to drive the host immune response protein towards the Th1 and cytotoxic lymphocyte responses. Our recombinant HBs proteins expressed from both prokaryotic and mammalian cells were re-

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HBs Protein-protein BLAST:
Identities = 223/225 99%), Positives = 223/225 99%), Gaps = 0/225(0%)
to gb/ABC00824.1 (GenBank)

pSIL18c1   : MESIASGFLG PLLVLQAGFF XLTRILTIPQ SLDSWWTSLN FLGGTTVCLG 50
ABC00824.1 : MESITSGFLG PLLVLQAGFF LLTRILTIPQ SLDSWWTSLN FLGGTTVCLG 50

pSIL18c1   : QNSQSPTSNH SPTSCPPTCP GYRWMCLRRF IIFLFILLLC LIFLLVLLDY 100
ABC00824.1 : QNSQSPTSNH SPTSCPPTCP GYRWMCLRRF IIFLFILLLC LIFLLVLLDY 100

pSIL18c1   : QGMLPVCPLI PGSSTTSTGP CRTCMTTAQG TSMYPSCCCT KPSDGNCTCI 150
ABC00824.1 : QGMLPVCPLI PGSSTTSTGP CRTCMTTAQG TSMYPSCCCT KPSDGNCTCI 150

pSIL18c1   : PIPSSWAFGK FLWEWASARF SWLSLLVPFV QWFVGLSPTV WLSVIWMMWY 200
ABC00824.1 : PIPSSWAFGK FLWEWASARF SWLSLLVPFV QWFVGLSPTV WLSVIWMMWY 200

pSIL18c1   : WGPSLYSILS PFLPLLPIFF CLWVY  225
ABC00824.1 : WGPSLYSILS PFLPLLPIFF CLWVY  225


Murine IL-18 Protein-protein BLAST:
Identities = 190/192 (98%), Positives = 190/192 (98%), Gaps = 0/192
(0%) to refNP_032386.1 (GenBank)

pSIL18c1   :  MAAMSEDSCV NFKEMMFIDN TLYFIPEENG DLESDNFGRL HCTTAVIRNI 50
NP032386.1 :  MAAMSEDSCV NFKEMMFIDN TLYFIPEENG DLESDNFGRL HCTTAVIRNI 50

pSIL18c1   :  NDQVLFVDKR QPVFEDMTDI DQSASEPQTR LIIYMYKDSE VRGLAVTLSV 100
NP032386.1 :  NDQVLFVDKR QPVFEDMTDI DQSASEPQTR LIIYMYKDSE VRGLAVTLSV 100

pSIL18c1   :  KDSKMSALSC KNKIISFEEM DPPENIDDIQ SDLIFFQKRV PGHNKMEFES 150
NP032386.1 :  KDSKMSTLSC KNKIISFEEM DPPENIDDIQ SDLIFFQKRV PGHNKMEFES 150

pSIL18c1   :  SLYEGHFLAC QKEDDASKLI LKKKDENGDK SVMFTLTNLH QS 192
NP032386.1 :  SLYEGHFLAC QKEDDAFKLI LKKKDENGDK SVMFTLTNLH QS 192
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Fig. 3 Amino acid sequences of our HBs and murine IL-18 deduced from gene sequences of a selected transformed *E. coli* clone carrying recombinant pcDNA-HBs-IL-18 and the protein alignment with those in the database.

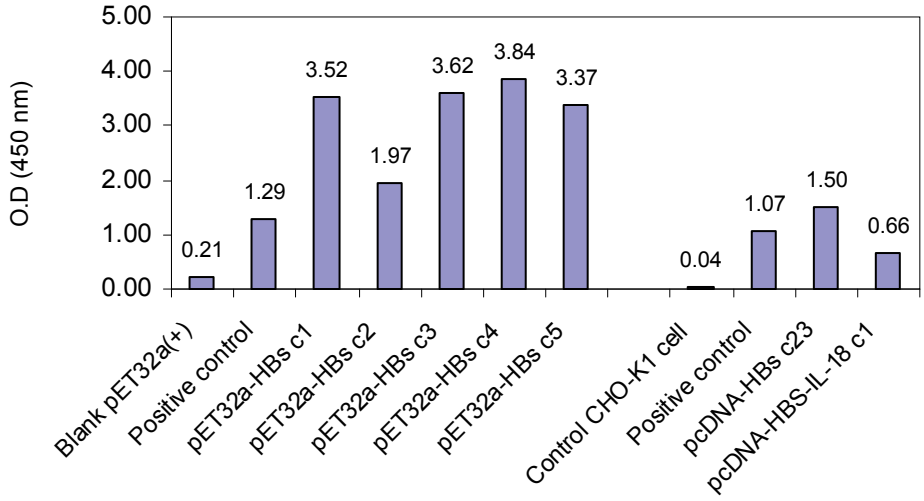

Fig. 4 ELISA results for determining the reactivity of recombinant HBs proteins expressed from *E. coli* BL21(DE3) clones 1-5 harboring pET32a-HBs plasmids and from CHO-K1 clone no. 23 transfected with pcDNA-HBs and clone no. 1 transfected with pcDNA-HBs-IL-18.

active to a monoclonal antibody to HBsAg in the commercial test kit which was designed for detection of hepatitis B viruses subtypes *ay* and *ad*, implying similarity of our HBs protein to the HBsAg of those subtypes.

The intramuscular injection of the DNA based cytokine expression plasmids should provide both direct and cross-presentation of the antigen to the immune cells. It was found that the specific IgG levels of mice injected with pRc/CMV-HBs plus pcDNA-IL-18 appeared higher than those injected with pRc/CMV-HBs alone or pcDNA-HBs-IL-18 fusion DNA, although was not statistically different. Unfortunately, a group of mice immunized with our pcDNA-HBs mixed with pcDNA-IL-18 was not included for comparison with the group injected with pRc/CMV-HBs plus pcDNA-IL-18. As most DNA vaccines are expected to elicit the host immunity that could control the intracellular pathogens such as viruses. In this study only total specific IgG levels were detected and the subisotypes of the IgG were not analyzed due to limitation of the mouse serum samples. Thus, further investigations are needed not only to more define the humoral immune response but also to investigate the kinetics of the cytokine profiles after the immunization and the cell-mediated immune response. For this communication, the conclusions that could be drawn are: 1) the prototype HBs-IL-18 DNA vaccine was successfully constructed; and 2) the gene encoding murine IL-18 was successfully cloned. The gene can be co-injected with the antigen coding DNA or as a fusion partner

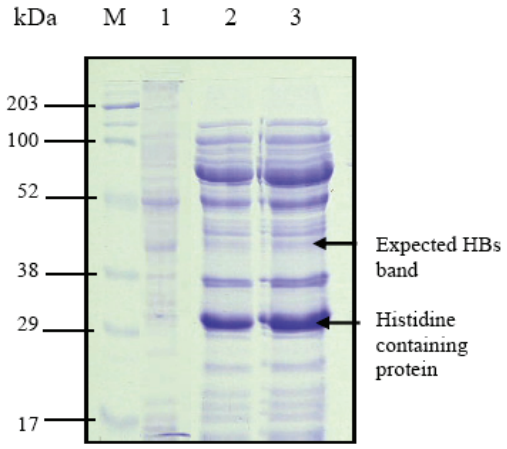

Fig. 5 Proteins in the lysate of a transformed BL21(DE3) *E. coli* harboring recombinant pET32a-HBs grown under IPTG induction. Lane M, pre-stained protein ladder; lane 1, crude *E. coli* lysate; lanes 2 and 3, loaded with 10 μl and 20 μl, respectively, of the 300 mM imidazole eluate from Ni/iminoacetic acid column

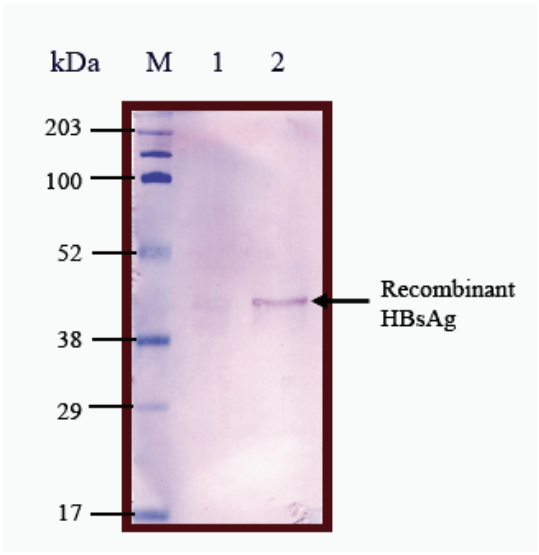

Fig. 6 Western blot patterns of the recombinant HbS protein probed with polyclonal antibody to HBsAg. Lane M, pre-stained protein ladder; lane 1, total proteins in lysate of *E. coli* harboring BL21-pET32a-HBs plasmids; lane 2, proteins in lysate of *E. coli* harboring BL21-pET32a-HBs plasmids that bound to NI/iminoacetic acid column.

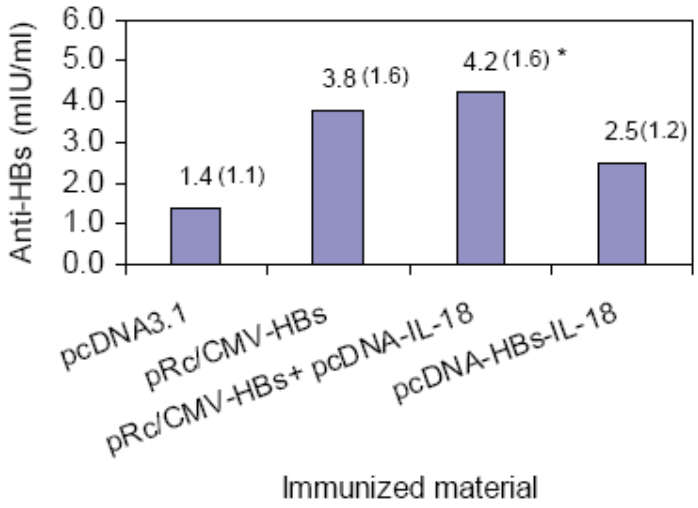

Fig. 7 Immune responses in BALB/c mice intramuscularly immunized with plasmid DNAs on days 0 and 28. The blood samples were collected on day 42. The results are expressed in mean of anti-HBs IgG in mIU/ml. The number in the brackets indicates the S.D. of the anti-HBs. IgG; * , indicates P <0.05, as compared to the levels of mice in the group immunized with blank pcDNA3.1+ plasmids.

to the DNA with rather promising potential as a genetic adjuvant for priming the immune response. The recombinant HBs and full-length IL-18 proteins have potential for other research purposes. They may be used also as standard proteins in a relevant quantification assay.

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