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Modulation of Immune Response Induced by Co-Administration of DNA Vaccine Encoding HBV Surface Antigen and HCV Envelope Antigen in BALB/c Mice

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Plasmid DNA vaccines encoding the hepatitis B virus (HBV) surface and hepatitis C virus (HCV) envelope antigens, respectively, were constructed, and attempt were made to find the possibility of a divalent vaccine against HBV and HCV. The expression of each plasmid in Cos-1 cells was confirmed using immunocytochemistry. To measure the induced immune response by these plasmids *in vivo*, female BALB/c mice were immunized intramuscularly with 100 μg of either both or just one of the plasmids. Anti-HBV and HCV-specific antibodies and related cytokines were evaluated to investigate the generation of both humoral and cellular immune responses. As a result, specific anti-HBV and anti-HCV serum antibodies from mice immunized with these plasmids were observed using immunoblot. The levels of IL-2 and RANTES showing a Th_1 immune response were significantly increased, but there was no change in the level of IL-4 (Th_2 immune response) in any of the immunized groups. Compared with each plasmid DNA vaccine, the combined vaccine elicited similar immune responses in both humoral and cell-mediated immunities. These results suggest that the combined DNA vaccine can induce not only comparable immunity experimentally without antigenic interference, but also humoral and Th_1 dominant cellular immune responses. Therefore, they could serve as candidates for a simultaneous bivalent vaccine against HBV and HCV infections.

Key words: DNA vaccine, HCV, HBV, IL-2, IL-4, RANTES

INTRODUCTION

Chronic hepatitis B and C infections represent major public health concerns due to their propensity to progress towards liver cirrhosis and cancer (Wright and Lau, 1993). Especially, chronic liver disease has been a major cause of mortality in Korea, and approximately 12 and 68% of hepatocellular carcinomas are attributable to HCV and HBV in Korea, respectively (Suh and Jeong, 2006).

However, the currently treatment modalities available for hepatitis B virus (HBV) or hepatitis C virus (HCV) infections are mostly interferon-α therapy, and the results of treatment are disappointing in chronically infected patients (Fried and Hoofnagle, 1995). Also, there are effective vaccines for HBV, but the development of an HCV vaccine remains at an early stage (Krahn *et al.*, 2005).

Several genetic antiviral strategies are currently being investigated through experimental studies, which have suggested alternative treatment strategies.

A DNA vaccine is considered as a novel and potentially powerful approach to prevent and treat various diseases, such as infectious diseases and cancer (Liu *et al.*, 2004).

Because DNA vaccines efficiently induce not only a humoral immune response, but also cytotoxic T lymphocytes (CTL), a new therapy against hepatitis B or C has been suggested using an animal model (Beckebaum *et al.*, 2002).

There has been a trend to develop combined vaccines because combined vaccination against multiple infectious

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diseases has various advantages, such as fewer inoculation, as well as the compliance and convenience for vaccine delivery compared with single antigen vaccines (Decker, 2001). The strategy of a combined vaccine has been applied in the development of a DNA vaccine for the treatment for both HBV and HCV (Musacchio *et al.*, 2001; Jin *et al.*, 2002).

In this study, attempts were made to investigate the possibility of a combined vaccine for both HBV and HCV, and the immune response against HBV and HCV induced by DNA immunization with a mixture of HBV and HCV plasmid constructs was evaluated. For this assessment, plasmid DNA vaccines for the HBV surface antigen (S+ preS2) and HCV envelope antigen (E2) were constructed and the type of immune responses induced by the plasmid DNA vaccines in combination and alone investigated.

MATERIALS AND METHODS

Plasmid DNA vaccine construction for immunization

The plasmid cloning the HBV-adr subtype sequence, pHBV315, was kindly provided by Dr. G.H. Kim., College of Pharmacy, Ehwa Woman's University, Korea. The coding region of the HBV surface antigen, the preS2+S sequence (845 bp), was amplified by PCR. The following primers were used for generation of the PCR fragment from HBV cDNA: forward primer (5'-gtt aag ctt atg cag tgg aac tcc acc ac-3') and reverse primer (5'-gca gaa ttc aat gta tac cca aag aca gaa g-3'). The plasmid containing Core, E1 and E2 coding sequences of HCV subtype 1b, pMT2 HCV st(N), was provided by Dr. Y. C. Sung, Pohang University of Science and Technology, Korea. The HCV E2 (1071 bp) gene of type 1b was amplified by PCR, using forward (5' gtt aag ctt atg agc acc cgc gtg aca gga gga) and reverse (5' gca gaa ttc gag gag cat cat cca caa gca) primers. The amplified products were digested with *Hind III* (Takara) and *EcoRI*(Takara), and then inserted into the pCDNA3.1/V5-HisA vector (Invitrogen) to generate pCDHBS and pCDHCE2. The pCDNA3.1/V5-HisA vector is a mammalian expression vector, containing the CMV and SV 40 promoters and an ampicillin resistance gene, for selection.

Preparation of HBV and HCV antigens

Recombinant HBV proteins were kindly provided by LG Life Science Corporation.

The HCV antigen was prepared as follows. The He HCV envelope antigen E2 (1071bp) gene was amplified by PCR, and then inserted into *Kpn I / Hind III* sites in the pQE-80L vector (Qiagen) to generate pQE/E2. The pQE vector is an N-terminal hexahistidine fusion expression vector from Qiagen. *E. coli* strain JM 109, which was used as the cloning and expression host.

The expression of the E2 protein in *E. coli* strain was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG; Gibco BRL) to LB media, at a final concentration of 1 mM. Purification of recombinant hexahistidine-tagged proteins, under denaturing condition, was performed on Ni^{2+}-nitrilotriacetate (Ni-NTA) resin (Qiagen) according to the manufacturer's instruction. The denatured extract was allowed to bind to a Ni-NTA agarose affinity column. The resin was then washed, with the resin bound proteins eluted with pH 4.5-50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole and 0.05% Tween 20. The eluted fractions were concentrated by trichloroacetic acid (TCA; Sigma) precipitation, and then washed three times with 100% iced acetone (Sigma).

The purified HBV and HCV E2 proteins were analyzed using SDS-PAGE, and then used for western blotting to detect HCV E2 antibodies.

In vitro antigens expression and immunofluorescence assay

Cos-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies), supplemented 10% fetal bovine serum (FBS; Gibco. Co.), at 37°C in a humidified 5% CO_2 incubator. Cells were seeded on 8-chamber slides (Falcon), at 7×10^3 cells per chamber well, for 1-2 days prior to transfection, and cultured to approximately 60-80% confluence. In order to ensure the plasmid DNA constructs were intact and functional, the expressions of pCDHBS and pCDHCE2 in Cos-1 cells were detected by immunofluoroscent staining. Transfection of Cos-1 cells with pCDHBS or pCDHCE2 was mediated by Lipofect-AMINE (Invitrogen), according to the manufacture's instruction. After transfection, Cos-1 cells were fixed with 70% ice cold ethanol and treated with rabbit anti-His polyclonal (1:1000 dilution; Medical & Biological Laboratories Co.) and fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG antibodies (Vector Laboratories, Inc.), with the cells then stained with 4, 6-diamidino-2-phenylindole (DAPI; Sigma) to identify the nuclei of the cells. To determine the gene expression, samples were observed under a fluorescence microscope.

Animals and immunization schedule

Female BALB/c mice were purchased from Orient Co. (Seoul, Korea). All animal experiment procedures were performed in accordance with program, which has been accredited by the association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The injection schedule and amount of total DNA were decided following preliminary experiments (data not shown). Five female mice per group, at 5-6 weeks old, were injected intramuscularly into their anterior tibialis muscle, with 100

μg of pCDHBS and/or pCDHCE2. Mice received 100 μg of plasmid DNA in a volume of 100 μL *via* three intramuscular injections, at two-week intervals. (Fig. 3A).

Western blot analysis for serum antibody detection

Sera were collected from the retroorbital venous plexus at selected time points, and used to monitor for the presence of specific HBV surface antigen (preS2 and S) and HCV envelope antigen (HCE2) by western blot analysis. HBS protein and purified HCV E2 protein from *E. coli* were loaded on a 10~12% SDS-PAGE gel (Invitrogen). After transfer to a polyvinylidene fluoride membrane (Invitrogen), the membrane was cut into strips, according to the immunization groups, and blocked with 3% BSA (Sigma). Each strip was incubated with sera from each group (1:100 dilution in 2.5% skim milk), and hybridized with HRP-conjugated anti-mouse IgG (Santa Cruz Biotechnology) at a 1:1000 dilution. After rinsing, the strips were developed with an ECL chemiluminescent detection kit (Amersham).

Assay for antigen specific splenocyte proliferation and the quantification of cytokines

Immunized mice were sacrificed 1 week after the last immunization, and their spleens were removed. Mononuclear cells from the spleen were harvested using the ficoll gradient method (Amersham Biosciences), and washed with fresh RPMI 1640 media (Gibco BRL) supplemented with 10% FBS. The cell viability was determined using the trypan blue exclusion method. The isolated cell suspensions were plated onto 24 well culture plates at a density of 10^7 cells per well. pCDHCE2 specific peptide SGPSQKIQLV (10 μg/mL) and HBS protein (20 μg/mL) were added to each well to stimulate the immune response. The cells were incubated at 37°C in a 5% CO_2 atmosphere for 3 days. Supernatants from each well were collected and analyzed for cytokine IL-2, IL-4 and RANTES (Regulated upon Activation, Normal T-cell Expressed and Secreted), using an ELISA kit (R&D Systems), according to the manufacture's instruction.

Statistics

All values were expressed as the mean ± standard deviation. The statistical significance was tested using a One way analysis of variance (ANOVA) test with $p<0.05$.

RESULTS

Cloning and expression of recombinant HBV and HCV antigen plasmids

For verification, the plasmid constructs were digested with *Hind III* and *EcoR I,* and the size of the inserts (HBS and HCE2) were checked by gel electrophoresis (Fig. 1). The HBV surface antigen fragment (845 bp) and HCV envelope antigen fragment (1071 bp) were confirmed with the *Hind III* and *EcoR I* restriction enzymes. Plasmid sequences were confirmed by sequencing analysis (data not shown). The pQE_E2 plasmid, expressing E2 antigen protein in *E. coli,* was also confirmed in the same manner (restriction enzyme site; *Kpn I and Hind III*). The purified E2 protein (39 kDa) was confirmed by N-terminal amino acid sequence (M R G S H H) analysis (data not shown).

In vitro expression of antigens

To confirm that HBV surface preS2/S and HCV envelope E2 proteins were expressed in the Cos-1 cells by transfection of pCDHBS and pCDHCE2, immunofluorescence staining was carried out using the anti-His specific antibody. Many cells were observed to express the specific protein by fluorescence detection under a microscope (Fig. 2).

The humoral immune response

To find if the HBS and HCE2 specific serum antibodies were induced by recombinant plasmid DNA vaccine immunization, western blot analysis was performed with the sera of immunized mice as sources of the primary antibodies (Fig. 3). The mice immunized with only the pCDNA 3.1 vector control showed no antibody response. Whereas, the specific HBV surface and/or HCV envelope antibodies were detected in the immunized mice group, using pCDHBS and pCDHCE2, respectively.

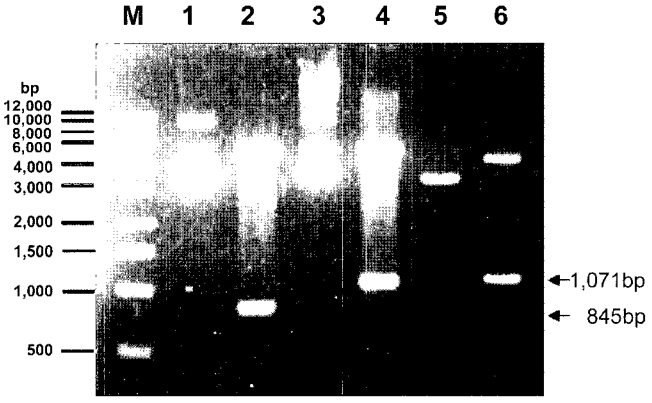

Fig. 1. Gel electrophoresis of cloned pCDHBS, pCDHCE2 and pQE_E2. Cloning of HBV pres2/preS and HCV E2 antigens into a pDNA3.1/V5-HisA vector. Lanes M, size marker (Perfect marker, Novagen); Lanes 1 and 2, supercoiled plasmid containing HBV preS2/S and its plasmid and the insert fragment (845 bp) of pCDHBS (6.4 kbp) digested with *Hind* III and *EcoR* I; Lanes 3 and 4, pCDHCE2 (6.6 kbp) supercoiled plasmid and its enzyme cutting plasmid and the insert (1071 bp) with *Hind* III and *EcoR* I; Lanes 5 and 6, supercoiled expression plasmid of pQE_E2 (5.7 kbp) and the digestion fraction with *Kpn* I and *Hind* III.

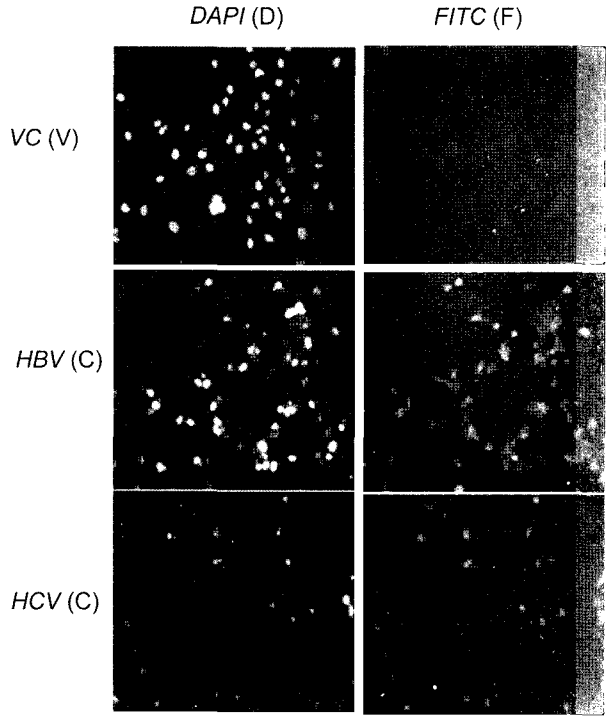

Fig. 2. *In vitro* expression of antigen proteins. Representative fields, using fluorescence microscopy at 100x magnification, are shown. D, nuclear staining of pCDHBS and pCDHCE2 DNA vaccines or pCDNA vector only-transfected Cos-1 cell, with DAPI staining; F, HBS and E2 protein visualized by staining with the FITC conjugated antibody. VC; pCDNA vector.

The cellular immune response

We also investigated if the DNA vaccine could induce a cellular immune response. IL-2, the representative cytokine of the Th_1 immune response, and RANTES, one of the CC chemokine, were evaluated with their respective ELISA. IL-2 was significantly induced by injection of the combined HBS and HCE2 plasmid DNA vaccines, as well as by immunization with HBS or HCE2 plasmid DNA vaccines (Fig. 4). RANTES, a small chemotactic protein that mediates many immunological and inflammatory responses, was also significantly induced in the group immunized by the combination of HBS and HCE2 plasmid DNA vaccines compared with those immunized with the single HBS or HCE2 plasmid DNA vaccines (Fig. 5). The combined HBS and HCE2 plasmid DNA vaccine induced stronger immune responses than the single HBS and/or HCE2 plasmid DNA vaccines, presumably because RANTES was rapidly up-regulated in response to a variety of stimuli. There was only a tendency for the expression level of RANTES to be increased in the HCV group. The secretion of IL-4, a marker representative of the Th_2 response, was also evaluated in splenocytes. Although the IL-4 level in the separate positive control group was not evaluated, there was no change in IL-4

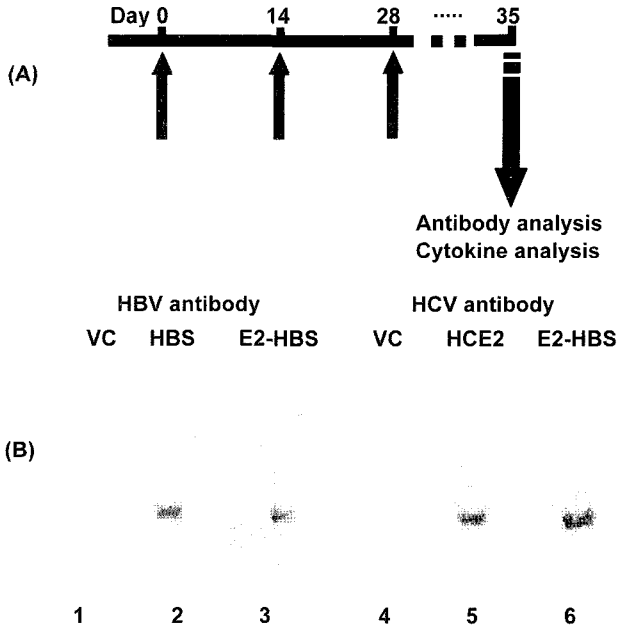

Fig. 3. Immunization schedule of animals, and the detection of the serum antibody by western blot analysis. After the first immunization, the mice were given a second immunization, and boost after 2 and 4 weeks, respectively. Serum samples were collected from the retro orbital venous plexus, at selected time points, and monitored for the presence of specific HBV surface antigen protein (preS2 and S) and HCV envelope antigen protein (HCE2) by western blot analyses. (A) : Immunization schedule (B): western blot analysis for detection of the serum antibody. Lanes 1 and 4(VC), pCDNA plasmid vector immunization; Lane 2, pCDHBS DNA vaccine immunization; Lane 5, pCDHCE2 DNA vaccine immunization; Lanes 3 and 6, the mixed DNA vaccines immunization.

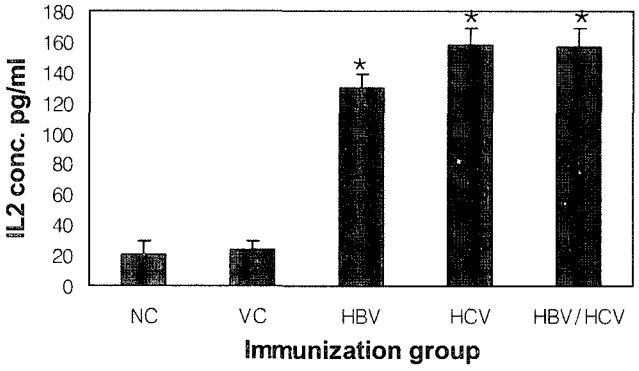

Fig. 4. HBV and HCV specific IL-2 production in mouse splenocytes. Each group consisted of five BALB/c mice, which were immunized with 100 μg of pCDHBS (HBC) and pCDHCE2(HCV) or pCDNA3.1(VC) plasmid vaccines at weeks 0, 2 and 4, respectively. At one week after the boost injection, the spleen cells from immunized mice were co-cultured in the presence of protein and T cell specific peptide. IL-2 secretions in the supernatants were assessed by ELISA. The asterisk indicates a statistically significant difference ($P < 0.05$) between data from the control (pCDNA3.1/His-A vector only) and the experimental (pCDHBS and pCDHCE2 or the mixture) samples. NC; Normal control.

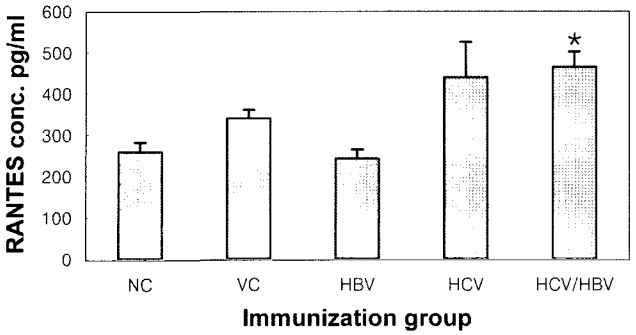

Fig. 5. HBV and HCV specific RANTES production in mouse splenocytes. Each group consisted of five BALB/c mice, which were immunized with 100 μg of pCDHBS(HBV) and pCDHCE2(HCV) or pCDNA3.1 plasmid(VC) vaccines at weeks 0, 2 and 4, respectively. One week after the boost injection, the spleen cells from immunized mice were co-cultured in the presence of protein and T cell specific peptide. RANTES secretions in the supernatants were assessed by ELISA. NC; Normal control.

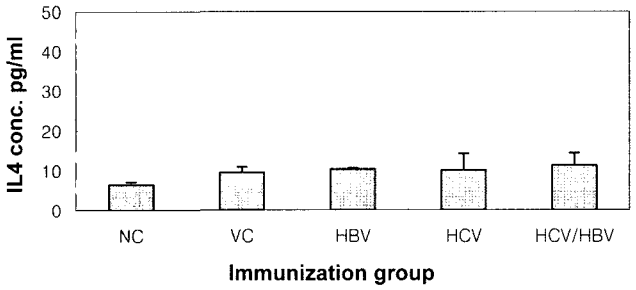

Fig. 6. HBV and HCV specific IL-4 production in mouse splenocytes. Each group consisted of five BALB/c mice, which were immunized with 100 μg of pCDHBS (HBV) and pCDHCE2(HCV) or pCDNA3.1 plasmid(VC) vaccines at weeks 0, 2 and 4, respectively. One week after the boost injection, spleen cells from immunized mice were co-cultured in the presence of protein and T cell specific peptide. IL-4 secretions in the supernatants were assessed by ELISA. NC; Normal control.

level in negative control or any of the DNA vaccine treated groups (Fig. 6).

DISCUSSION

The envelope of the hepatitis B virus contains three different proteins: the large, middle and small hepatitis B surface antigens (HBsAg). Several groups have shown that the gene of HBsAg is highly immunogenic in mice and stimulates a specific immune response (Hourvitz *et al.*, 1996; Yap *et al.*, 1992).

The HCV envelope proteins, E1 and E2, are considerably important, because antibodies against conserved epitopes of envelope proteins can be found in more than 90% of HCV infected patients. In addition, antibodies directed at the hypervariable region 1 (HVR1), near the N-terminus of E2 proteins, have been shown to inhibit viral attachment to susceptible cells, suggesting these antibodies might have a neutralizing capability for HCV infection (Lagging *et al.*, 1995; Major *et al.*, 1995; Nakano *et al.*, 1997; Tedeschi *et al.*, 1997).

Therefore, plasmid DNA vaccines for the HBV surface antigen (S+preS2) and HCV envelope antigen (E2) were constructed and the humoral or cellular immune responses induced by the plasmid DNA vaccines, either in combination or alone, investigated.

There have been many studies on the development of multivalent HBV and HCV DNA vaccine, using the fusion construct or mixed plasmids encoding HBV and HCV (Geissler *et al.*, 1998; Jin *et al.*, 2002; Musacchio *et al.*, 2001).

In this study, the immunization of mice with pCDHBS (HBV) and pCDHCE2 (HCV) DNA vaccines induce the respective antigen specific antibodies. In addition, the combined DNA vaccine similarly induced the production of anti-HBV and anti-HCV antibodies. Musacchio et al. reported that by mixing HBV core and surface antigen-encoded plasmids, antibody responses and T cell proliferation can be effectively raised, similarly to the independent administration of both constructs.

Likewise, in this study, the combined DNA vaccine was demonstrated to induce a comparable immune response, without antigenic interference.

The level of cytokines released by T cells reflects the direction and magnitude of the T cell immune response. Typical cytokines of the Th_1 response include IL-2 and IFN-γ, and characteristics of the Th_2 response include IL-4 and IL-10 (Romagnani, 2000). The cytokine IL-2 and IL-4 induced by DNA vaccination, as representative cytokines of the Th_1 subset that mediates the cell mediated immunity, and the Th_2 subset that modulates antibody production were evaluated using ELISA (Chong *et al.*, 2003). With regard to the increased level of IL-2 expression, there was statistically significant difference between the groups stimulating the protein and peptide, but in the case level of IL-4 expression, there was no difference between any of the DNA vaccine immunized groups compared with the control. This result implied that immunization with the DNA vaccines dominantly induced the Th_1 cell immune response (Yang *et al.*, 2001; Ljungberg *et al.*, 2002).

Interestingly, compared with single antigen targeting DNA vaccines (pCDHBS and pCDHCE2 groups), the combined plasmid DNA vaccine was also observed at a similar level to the Th_1 cell mediated immunity.

RANTES can chemoattract unstimulated CD4+ memory T cells and stimulated CD4+ and CD8+ T cells with naive and memory phenotypes (Pinto *et al.*, 2003). Th_1 and Th_2 cells were found to have distinct CC-chemokine secretion profiles. Th_1 cells preferentially secrete a number of CC-

chemokines, which have selective recruitment properties, including MIP-1α and RANTES (Song *et al.*, 2003). Thus, the secretion of RANTES was also evaluated by ELISA as further evidence of a Th_1 type immune activation due to DNA vaccine immunization. Although there was no significant change in the levels of RANTES expression in the HBV group, there was a tendency for the level of RANTES expression in the HCV group to increase. Also, the level of RANTES expression was significantly increased in the combined plasmid DNA vaccine group, due to the stimulation effect of RANTES secretion by the HCV DNA vaccine. However, the exact reason for only the HCV DNA vaccine inducing RANTES expression in splenocytes is unknown. These data also support that the combined plasmid DNA vaccine could elicit mainly the Th_1 type immune response.

However, further study on the cytokine production and cells phenotype by FACS will be required to identify the IL-2 or IL-4 secreting cells of splenocyte. In addition to IL-4, the measurement of other Th_2 representative cytokines will be helpful to confirm the Th_1 dominant response induced by our DNA vaccine.

These cumulative results confirmed that vaccination with DNA sequences derived from the HBV-surface antigen and HCV-envelope antigen could be an effective method for the induction of humoral and Th_1 type immune responses, and moreover, suggest that the combined plasmid DNA vaccine is a potentially attractive possibility for immunization strategies against viral hepatitis, including HBV and HCV. Further evaluation of the CTL immune response and quantitative antibody response will give a more complete picture of the immune response generated in this study. In addition, it will be more useful to study the immunization protocol or safety of DNA vaccines for their practical use.

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