DIFFERENT CONTRIBUTION OF CO-STIMULATORY MOLECULES B7.1 AND B7.2 TO THE IMMUNE RESPONSE TO RECOMBINANT MODIFIED VACCINIA VIRUS ANKARA VACCINE EXPRESSING PRM/E PROTEINS OF JAPANESE ENCEPHALITIS VIRUS AND TWO HEPATITIS B VIRUS VACCINES

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Summary. – This study clarifies the role of co-stimulatory molecules B7.1 and B7.2 in the immune response to 3 types of vaccines: a/ recombinant modified Vaccinia virus Ankara (MVA) (vJH9) expressing prM/E proteins of Japanese encephalitis virus (JEV), b/ recombinant yeast-expressed Hepatitis B virus (YHBV), c/ human plasma-derived Hepatitis B virus (PHBV). We constructed plasmids expressing B7.1 and B7.2 molecules and found that the expression level of B7.2 protein in transfected CHO-k1 cells was higher than that of B7.1 protein. Mice were co-injected with vaccines vJH9, YHBV and PHBV and plasmids expressing B7.1 or B7.2, respectively, and specific antibody titers for each vaccine were monitored at days 7, 14 and 28 post injection (p.i.). In mice injected with vJH9 vaccine and both B7 plasmids, plasmid B7.2 induced a higher anti-JEV immune response than plasmid B7.1. This implies that the stimulation of the B7.2 immune pathway may be a feasible method of boosting protective immunity against a recombinant viral vaccine. Both B7 molecules were able to induce a specific anti-HBV immune response using YHBV vaccine. On the other hand, B7 molecules had little effect to the specific antibody induction in PHBV vaccination. These results suggested that the contribution of B7.1 and B7.2 molecules in an immune response depended on the character and status of the presenting antigen.

Key words: co-stimulatory molecules B7.1, B7.2; Hepatitis B virus; immune response; Japanese encephalitis virus; vaccine

Introduction

Antigen-specific T-cell activation in an immune response requires at least two signals (Sharpe, 1996). Interaction between the antigen and major histocompatibility complex class II (MHC-II) on the antigen-presenting cells (APCs) and T cell receptor (TCR)/CD3 complex on the helper T cell (T_H) are necessary, but not sufficient, for T_H activation (Davis and Bjorkman, 1988). Co-stimulatory signals are also required for antigen-TCR engagement, because the absence of co-stimulatory signals occurs in an anergy state or functional inactivation of mature T cells (Schwartz, 1990). Thus, T-cell co-stimulatory signals play a critical role in determining the fate of a T cell that initiates an immune response (Sharpe, 1996). In a co-stimulation signal pathway, the CD28 molecule together with CD40 and inducible co-stimulator receptor (ICOS) is well characterized to recognize B7.1 (CD80) and B7.2 (CD86) counter-receptors expressed

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Abbreviations: APC(s) = antigen-presenting cell(s); CTLA-4 = cytotoxic T lymphocyte activation antigen 4; HBV = Hepatitis B virus; i.m. = intramuscularly; MVA = modified Vaccinia virus Ankara; p.i. = post injection; TCR = T cell receptor; TNF = tumor necrosis factor; JEV = Japanese encephalitis virus; PHBV vaccine = plasma-derived HBV inactivated vaccine; YHBV vaccine = recombinant yeast-derived HBV subunit vaccine

on APC (Frauwirth and Thomson, 2002). B7.1 and B7.2 provide co-stimulating signals in the early stage of the antigen-specific immune response of T lymphocytes (Azuma *et al*., 1993). The interaction between B7 molecules and CD28 initiates proliferation, cytokine production and differentiation of T cells, whereas their binding to cytotoxic T lymphocyte activation antigen 4 (CTLA-4) delivers a negative signal to T cells (Santra *et al*., 2000b). Both B7 molecules have a high affinity for CTLA-4 and a lower affinity for CD28 (Santra *et al.*, 2000a). Signaling through the B7 co-stimulatory pathway induces cytokines, such as interleukin 2 (IL-2), interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), lymphotoxin, granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin 3 (IL-3), by stabilizing their mRNAs (Fraser *et al*., 1991). The B7 co-stimulatory pathway has been implicated in several important biological responses, including the rejection of allogenic/xenogenic grafts, the control of graft versus host disease, the regulation of pathogens including viruses, the reduction of diabetes, the improvement of arteriosclerosis and the prevention of several autoimmune diseases (Larsen *et al*., 1996; Lenschow *et al*., 1995; Russel *et al*., 1996). The B7 co-stimulatory signal is also associated with B and NK cell activation (Santra *et al*., 2000b). Thus, a B7.1 and B7.2 expression based plasmid vaccine strategy could be used to enhance the immune response (Boyer *et al*., 2002; Kim *et al*., 1997; Santra *et al*., 2000a; Tsuji *et al*., 1997). However, it is not clear whether the requirement of these B7 molecules for an immune response is dependent upon the antigen that is presented by the APC or by a different presentation pathway. Generally, exogenous antigen is processed within endosomes and presented by APCs associated within MHC-II, whereas endogenous antigen, synthesized within a cell, is processed in the cytosol and endoplasmic reticulum and presented in association with MHC-I (Santra *et al*., 2000b).

Therefore, we presume that the contribution of B7 molecules will depend on the type of vaccine and its origin. To clarify this hypothesis we selected three vaccine types: MVA to express JEV prM/E proteins (vJH9) representing endogenous antigen and two types of HBV vaccine, which were derived from YHBV and PHBV representing exogenous antigen. We previously reported that recombinant vJH9 vaccine can induce anti-JEV neutralizing antibodies in mice and swine (Nam *et al*., 1999, 2002). Here, we report the effect of co-administration of the three types of vaccines with B7.1 and B7.2 co-stimulatory molecules in mice.

Materials and Methods

Vaccines. A live recombinant viral vaccine (vJH9) expressing JEV prM/E proteins (Nam *et al*., 1999) was prepared using MVA, a highly attenuated Vaccinia virus with a modified early/late promoter from the H5 gene. Vaccine YHBV contained a purified major HBV surface antigen (HBsAg) without preS1 and preS2 derived from recombinant yeast (Euvax B Inj., Korea). The PHBV vaccine was a plasma-derived HBV inactivated vaccine (Hepaccine-B Inj, Cheil Foods & Chemicals Inc, Korea).

Cloning of murine B7.1 and B7.2 genes. Spleen cells from Balb/c mice were harvested and cultured for 2 days in RPMI-1640 with 10% FBS (Invitrogen) and Concanavalin A (Sigma) as a stimulator. Total RNA was purified from cultured spleen cells by a Trizol method. Total RNA was reverse-transcribed to produce cDNA by using random hexamer primers (660 ng/μl), reverse transcriptase (200 U, Invitrogen), 10 mmol/l dNTP and 100 mmol/l DTT in a 20 μl volume. Samples were incubated for 30 mins at 42°C and 5 mins at 95°C. To clone B7.1 and B7.2 genes, B7.1-F/B7.1-R and B7.2-F/B7.2-R primer sets were used (Table 1). PCR products were cloned into a TA cloning vector (Invitrogen) and sequenced. B7 genes were confirmed by reamplification using B7.1-E-F/B7.1-E-R and B7.2-E-F/B7.2-E-R primer sets (Table 1). Each primer had *Bam*HI and *Hind*III restriction sites near their ends. The forward primer had the Kozak consensus sequence GACC preceding the ATG start codon that is optimal for initiation by eukaryotic ribosomes. Mouse B7.1 and B7.2 cDNAs amplified by RT-PCR were cloned directly into *Bam*HI or *Hind*III sites of pcDNA3.1 plasmid (Invitrogen) producing plasmids B7.1/pcDNA3.1 and B7.2/pcDNA3.1. Both plasmids were sequenced using the dye terminator cycle sequencing core kit with AmpliTaq DNA polymerase (Perkin-Elmer) and the Applied Biosystems model 377 DNA sequencer (Perkin-Elmer).

Table 1. Oligonucleotides used for cDNA synthesis and PCR amplification

Oligonucleotide	Sequence (5'–3')[a]	Polarity
B7.1-F	ATGGCTTGCAATTGTCAGTTG	forward
B7.1-R	CTAAAGGAAGACGGTCTGTTC	reverse
B7.2-F	ATGGACCCCAGATGCACCATG	forward
B7.2-R	TCACTCTGCATTTGGTTTTGC	reverse
B7.1-E-F	GGCC**GGATCC***GACC*ATGGCTTGCAATTGTCAGTTG	forward
B7.1-E-R	GGCC**AAGCTT**CTAAAGGAAGACGGTCTGTTC	reverse
B7.2-E-F	GGCC**GGATCC***GACC*ATGGACCCCAGATGCACCATG	forward
B7.2-E-R	GGCC**AAGCTT**TCACTCTGCATTTGGTTTTGC	reverse

[a]Underlined bases represents start or stop codon, bolded ones restriction enzyme sites, and italicized ones Kozac sequences.

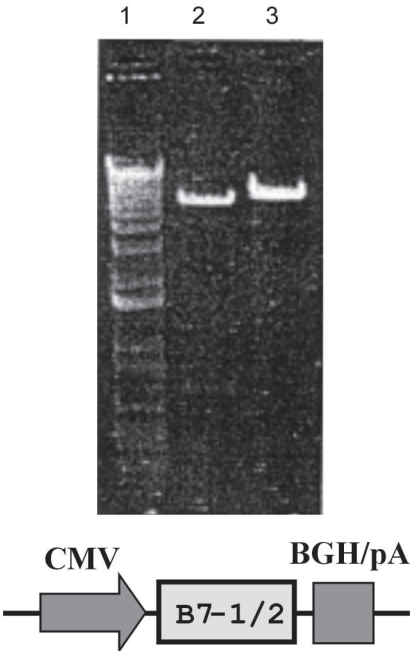

Fig. 1

Construction of pcDNA3.1 vector for expression of mouse B7.1 and B7.2 proteins

100 bp ladder (lane 1), B7-1/pCDNA3.1-*Bam*HI (lane 2), B7-2/pCDNA3.1-*Hind*III (lane3).
CMV = Human cytomegalovirus immediate-early promoter/enhancer, BGH/pA = bovine growth hormone polyadenylation signal.

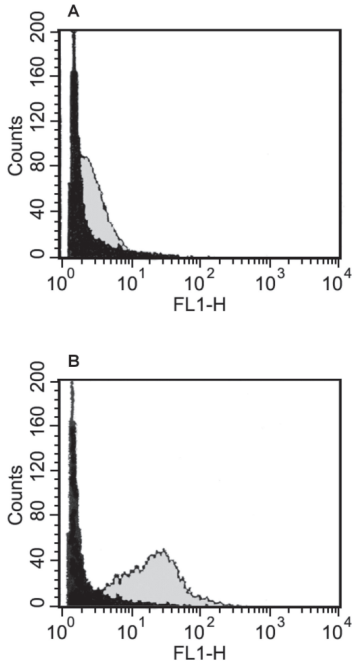

Fig. 2

Expression of the B7.1 (A) and B7.2 (B) molecules on CHO-k1 cells transfected with corresponding plasmids detected by flow cytometry

CHO-k1 cells transfected with control pcDNA3.1 (dark area). CHO-k1 cells transfected with B7.1/pcDNA3.1 and B7.2/pcDNA3.1 plasmids, respectively (bright area).

Expression of B7.1 and B7.2 genes in CHO-k1 cells. 5 x 10^6/ml CHO-k1 cells incubated in 10% FBS/DMEM were placed into an electroporation cuvette (BioRad) and mixed with 15 μg of recombinant plasmid B7.1/pcDNA3.1 and B7.2/pcDNA3.1, respectively, and a control pcDNA3.1. Electroporation was performed in an electroporator (BTX T820) at 480 V, 99 μ and two cycles. Cells were incubated in 10% FBS/DMEM for 3 weeks and assayed by flow cytometry.

Flow cytometry. Transfected CHO-k1 cells were washed three times with PBS and stained with FITC-conjugated goat anti-B7.1 and anti-B7.2 antibodies (Pharmingen) for 30 mins on ice. The cells were then washed three times with PBS and analyzed with a FACSCALIBUR Flow Cytometer (Becton and Dickinson).

Mice and their inoculation. Three-week-old mice (strain ICR) were purchased from Korea Food and Drug Administration (KFDA). Each animal group consisted of five mice. 1 x 10^8 PFU of vJH9 vaccine was injected intramuscularly (i.m.) together with various doses (25, 50, 100, 200 μg) of B7.1/pcDNA3.1 and B7.2/pcDNA3.1 plasmids. Both commercial HBV vaccines (YHBV and PHBV) were diluted 1:10 with PBS as recommended for mouse inoculation by the KFDA. Mice were co-injected i.m. with 50 μl of diluted vaccine and with 100 μg/mouse of B7.1/pcDNA3.1 or B7.2/pcDNA3.1.

ELISA. Inoculated mice were bled at 1, 2 and 4 weeks p.i. For given animal group, equal serum volumes of each mouse were mixed and final serum mixture was tested. The titer of anti-JEV antibodies was determined by ELISA (Cho *et al*., 1995) and the titer of anti-HBsAg antibodies was determined by a commercial HBV detection kit (Korea Green Cross Co., Korea). All procedures followed the manufacturer's recommendation or were modified only slightly. Each sample was assayed in triplicates and ELISA titer was determined from the mean value of absorbance at 495 nm of three wells.

Results

Expression of B7.1 and B7.2 plasmids in CHO-k1 cells

Two recombinant plasmids B7-1/pcDNA3.1 and B7.2/pcDNA3.1 expressing B7-1 and B7-2 proteins, respectively, were constructed. The arrangement and confirmation of the B7 genes are shown in Fig. 1. The sequence of the recombinant plasmids was consistent with the previously reported B7.1 and B7.2 gene sequences. CHO-k1 cells transfected with B7.1/pcDNA3.1 and B7.2/pcDNA3.1 plasmids showed expression of B7.1 and B7.2 molecules in comparison with CHO-k1 cells transfected with control pcDNA3.1 in flow cytometry assay. However, the expression level of B7.2 was much higher than that of B7.1 (Fig. 2A, B).

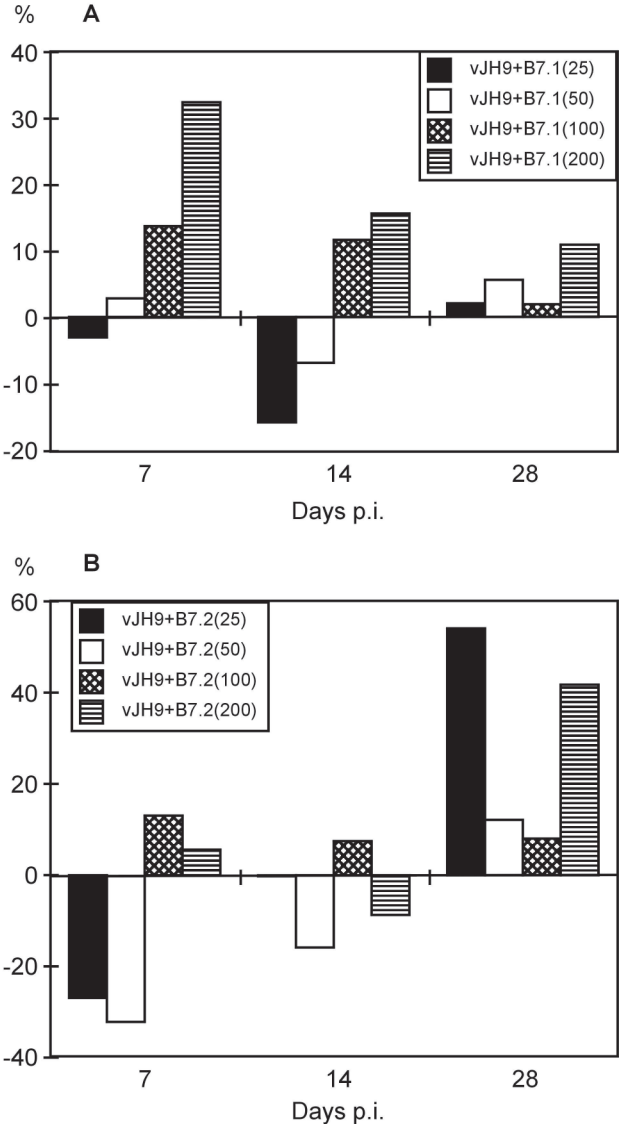

Fig. 3

Increase in ELISA titers (%) of anti-JEV antibody in mice co-injected with vJH9 vaccine and B7.1/pcDNA3.1 (A) and B7.2/pcDNA3.1 (B), respectively

The baseline represented the antibody titer from mice injected with only vJH9 vaccine. The number in parentheses represented the dose μg/mouse of plasmid DNA.

Immune response to vJH9 vaccine co-injected with plasmids expressing B7.1 and B7.2

The comparison of ELISA titers determined in mice co-injected with vJH9 and B7.1 and B7.2 molecules was performed by using the titer from mice injected with vJH9 alone as a baseline (zero value) (Fig. 3). Although a synergistic effect of the B7.1 molecule was observed with a 200 μg dose at day 7 p.i., this long-lasting effect of the B7.1 molecule was not significant at day 28 p.i. (Fig. 3A). However, ELISA titers of sera after co-injection with B7.2 molecule increased by 10–53% at day 28 p.i. compared with that of mice injected with vJH9 alone (Fig. 3B). Although the dose of B7.2/pcDNA3.1 did not precisely correlate with the increase in titer, the B7.2 molecule showed a synergistic effect in boosting the immune response to vJH9 vaccine.

The immune response to YHBV and PHBV vaccines co-injected with plasmids expressing B7.1 and B7.2

The antigen is processed in APC by a different pathway depending on its character and origin and the different effects of co-stimulating molecules. We searched for the effect the B7 molecules would have on the immune response to the vaccines YHBV and PHBV. Generally, HBV vaccines induce an inadequate response in 2.5–5.0% of healthy and immunocompetent vaccine recipients. That means these patients did not acquire protective levels of anti-HBV antibodies following the routine administration of three consecutive HBV vaccine doses (Leroux-Roels *et al.*, 1997). Therefore, if B7 molecules can strengthen the immune response to HBV vaccines, co-administration with B7 molecules will be a useful way to minimize the inadequate responsiveness. To evaluate this possibility, YHBV and PHBV vaccines were co-injected with 100 μg/mouse of B7.1/pcDNA3.1 and B7.2/pcDNA3.1, respectively, At 1, 2, and 4 weeks p.i., the sera were tested for presence of anti-HBsAg antibodies by ELISA.

The mice co-injected with YHBV vaccine and both B7 plasmids showed 80% increase in antibody titer at day 28 p.i. in comparison with mice inoculated only with YHBV vaccine (Fig. 4A). However, PHBV vaccine co-injected with B7 plasmids did not induce an antibody response to HBsAg in mice (Fig. 4B). The increased titer of anti-HBsAg antibodies clearly shows that both B7 molecules can increase substantially the antibody level in mice co-injected with the recombinant subunit YHBV vaccine, but not with inactivated PHBV vaccine at day 28 p.i.

Discussion

In this study, we tested the positive effect of B7.1 and B7.2 molecules on the humoral immune response in mice co-injected with various types of vaccines, such as vJH9 viral vaccine representing endogenous antigen, recombinant yeast-derived HBV subunit vaccine and plasma-derived HBV inactivated vaccine representing exogenous uptake antigen. Since the process of immune response *in vivo* is dependent on the extracellular or intracellular character of the vaccine,

these tests will help in the design of a vaccine or with finding a way of boosting the immune response to the vaccine.

Previously, we reported that viral recombinant vaccine vJH9 successfully induced the humoral immune response to JEV in mice and swine (Nam *et al*., 1999, 2002). In our experiments we co-injected vJH9 vaccine and B7 molecules as plasmids to mice. Molecule B7.1 had little effect on the elicited humoral immune response to recombinant viral vaccine, but B7.2 had distinct effects on the production of anti-JEV antibody. This means that B7.1 is not required for the induction of the humoral immune response to live recombinant Vaccinia virus. On the other hand, B7.2 is required for induction of immune response by vJH9 vaccine.

Our findings were consistent with the previous study in which the induction of humoral and cellular immune responses by recombinant Vaccinia virus expressing gp120 of Human immunodeficiency virus (HIV) had a unique B7.2 requirement (Santra *et al*., 2000b). A critical requirement for B7.2 molecule to elicit both humoral and cellular immune responses to intracellular synthesized antigens such as vaccine vJH9 expressed JEV protein was demonstrated (Santra *et al*., 2000b). There are two possible reasons why B7.2, but not B7.1 induced a high immune response to endogenous antigen in this study. One is the biological character of B7.2 itself with previous reports showing that B7.2 in APC is induced more rapidly than B7.1 and that B7.2 has a higher and stronger effect in anti-tumor immunity and proteoglycan-induced arthritis than does B7.1 (Brennan *et al*., 1995; Yang *et al*., 1995; Sharpe, 1996). The other reason represented the higher expression level of B7.2 than B7.1 in CHO-k1 cells that could lead to a higher impact of B7.2 molecule on induction of antibodies.

The mice injected with vJH9 showed that antibody responses could be different according to the amount of injected plasmid DNA. It seemed that the amount of plasmid DNA higher than 200 μg/mouse could have an opposite effect. In the following experiments we used only 100 μg/mouse of plasmid DNA for co-injection studies with YHBV and PHBV vaccines.

HBV vaccine is well established as a model for study of the immune response to vaccination. Some HBV vaccinations fail to induce specific antibody against HBsAg (Leroux-Roels *et al*., 1997). Since B7 molecules can help to induce the immune response to plasmid DNA vaccine or viral vaccine (Boyer *et al*., 2002; Kim *et al*., 1997; Tsuji *et al*., 1997), it is possible that B7 co-stimulating molecules increase the production of antibodies to HBV vaccine. We tested two types of HBV vaccine – YHBV and PHBV. Although both HBV vaccines are exogenous protein antigens, it is not known whether the HBV vaccines are processed by the same or different immune pathways in APCs. Interestingly, both B7 molecules had little effect in eliciting of humoral immune responses in PHBV. However,

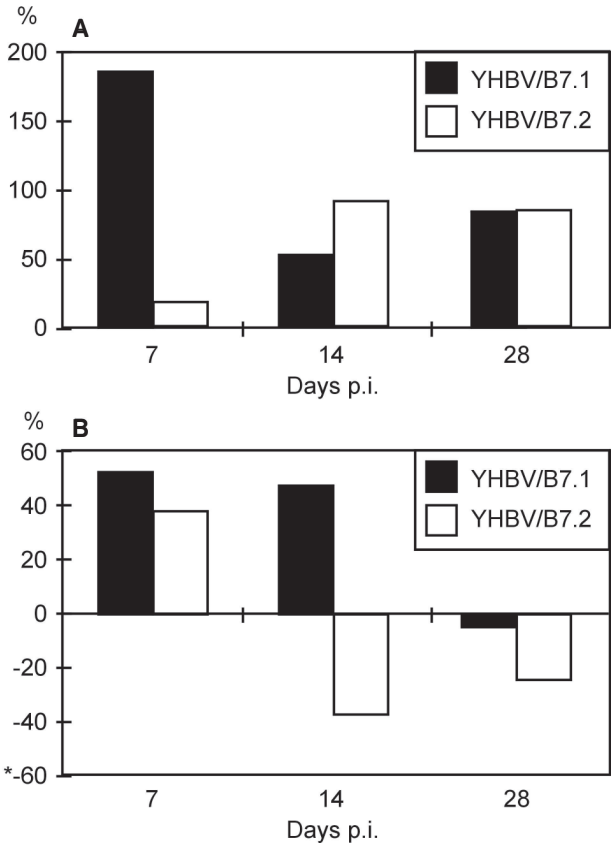

Fig. 4

Increase in ELISA titers (%) of anti-HBsAg antibodies in mice co-injected with YHBV (A) or PHBV (B) vaccines and B7.1/pcDNA3.1 or B7.2/pcDNA3.1, respectively

The baseline represented the antibody titer of mice injected only with YHBV (A) or PHBV(B) vaccines. The injected dose of B7 molecules was 100 μg/mouse of plasmid DNA.

YHBV co-injected with B7.1 and B7.2 molecules showed positive effects in boosting of the humoral immune response to HBV vaccine. The difference between PHBV and YHBV vaccine may reflect the character or status of the viral antigen itself, such as a difference in the method of antigen internalization or antigen-presentation by MHC. The effect of B7.1 and B7.2 molecules on recombinant subunit vaccine YHVB shown in this study contrasted with a previous report where the elicitation of humoral and cellular immune responses by recombinant HIV gp120 protein vaccine did not require B7.1 and B7.2 molecules (Santra *et al*., 2000b). These contrasting results may reflect a different character or processing of the exogenous antigen and the different immune processes of HIV and HBV proteins. Our results showed that the effect of co-stimulating B7 molecules in an immune response were dependent on the specific condition and character of the presenting antigen.

Taken together, our results showed that B7 molecules have a positive effect in the induction of humoral immune response. This is especially true for the B7.2 molecule, which has broad effects in several vaccine types. Generally, the presence of B7 co-stimulating molecules may be useful for the development of a new type of vaccine with the enhanced preventive effect.

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