Efficient Induction of Th1-type Immune Responses to Hepatitis B Virus Antigens by DNA Prime-Adenovirus Boost

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ABSTRACT

Background: Chronic infection with hepatitis B virus (HBV) affects about 350 million people worldwide, which have a high risk of development of cirrhosis and hepatocellular carcinoma. Treatment of chronic HBV infection relies on IFN-α or lamivudine. However, interferon-α is effective in only about 30% of patients. Also, the occurrence of escape mutations limits the usage of lamivudine. Therefore, the development and evaluation of new compounds or approaches are urgent. **Methods:** We comparatively evaluated DNA and adenoviral vaccines expressing HBV antigens, either alone or in combined regimens, for their ability to elicit Th1-type immune responses in Balb/c mice which are believed to be suited to resolve HBV infection. The vaccines were tested with or without a genetically engineered IL-12 (mIL-12 N220L) which was shown to enhance sustained Th1-type immune responses in HCV E2 DNA vaccine. **Results:** Considering the Th1-type cytokine secretion and the IgG2a titers, the strongest Th1-type immune response was elicited by the DNA prime-adenovirus boost regimen in the presence of mIL-12 N220L. In addition, the codelivery of mIL-12 N220L modulated differentially the immune responses by different vaccination regimens. **Conclusion:** Our results suggest that the DNA prime-adenovirus boost regimen in the presence of mIL-12 N220L may be the best candidate for HBV vaccine therapy of the regimens tested in this study and will be worthwhile being evaluated in chronic HBV patients. **(Immune Network 2005;5(1):1-10)**

Key Words: Hepatitis B virus, DNA prime-adenovirus boost, Th1-type immunity

Introduction

Hepatitis B Virus (HBV) is an important cause of human liver diseases with an estimated 350 million chronic HBV carriers worldwide. Patients with chronic HBV infection face high risk of developing cirrhosis and hepatocellular carcinoma, which result in a high rate of mortality (1,2). Upon HBV infection, only 5～10 % of adults become chronic carrier. However, more than 90% of children born to HBV-infected mothers or children infected in the first years of life become chronically infected. Even though the plasma-derived and the recombinant HBV vaccines are currently being used for preventing HBV infection, 5～10% of healthy adult receiving these commercially available HBV vaccines fail to mount an adequate immune response (3). These limitations have prompted the demand for more effective vaccines.

Interferon-α and lamivudine have been used to resolve chronic HBV infection. However, interferon-α is effective in only about 30% of patients. Also, the occurrence of escape mutations limits the usage of lamivudine (4). To overcome this problem, several therapeutic immunization attempts have been made using animal model systems, mouse and duck. Prom-

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ising results in a HBV-transgenic line by DNA vaccine have been reported but may be dependent on the strain used, since these results were not reproducible in other HBV transgenic lines (5,6). It was also reported that the vaccination with DNA encoding S and S1S2 resulted in a lowering of viremia and a decrease in hepatic DNA levels in chronically infected ducks (7). Recently, vaccination by DNA prime-Canarypox boost regimen in a chronically HBV-infected chimpanzee resulted in disappearance of HBV DNA from blood and liver (5). These data raised prospects for curing chronic HBV infection but further studies are needed because only one chronically-infected chimpanzee with relatively low viral load was used in the study.

The clearance of HBV infection is believed to be mediated by multi-specific cytotoxic T lymphocyte (CTL) and T helper responses (2,8,9,10). Even though the clearance of HBV infection by CTL had been thought to be mediated primarily by the destruction of HBV-infected cells, the studies in chimpanzees and HBV-transgenic mice demonstrated that 90 % of the HBV DNA is cleared by noncytolytic antiviral mechanism, such as IFN-γ and TNF-α (11). In addition, Th1 cytokines, especially IFN-γ were predominantly secreted by PBMCs in acute self-limiting hepatitis B following antigen stimulation but not in chronic hepatitis B (12,13). Therefore, the vaccines which can elicit Th1-type cellular immune responses will be good candidates as prophylactic and therapeutic vaccines to resolve HBV infection.

DNA vaccines have been evaluated to be a new approach to the development of effective and safe vaccines (14). DNA vaccines have potential advantages compared to recombinant protein vaccine due to the induction of Th1-type cellular immune responses which may be suited to intracellular pathogen and viral infection, such as HBV infection (14). However, despite of a successful result in the influenza model in mice, DNA vaccine alone has not been proven to induce complete protection against some other infectious pathogens (15-17). One potential strategy to overcome this limitation may be the boosting with viral-vector based vaccines following the DNA (15, 18-21). Replication-defective recombinant adenovirus and highly attenuated live recombinant poxviruses, such as the modified virus Ankara (MVA), the genetically attenuated vaccinia-based vector NYVAC, or the canarypox-based vector designated ALVAC have been proven to be safe and shown to elicit effective immune responses when used either alone or as a booster after DNA priming in mice and primate (19-21).

Cytokines and other molecules involved in costimulation signaling have been used as genetic adjuvants to modulate immune responses elicited by DNA and viral vector vaccines (22-26). Recently, we have reported that co-immunization of a genetically engineered murine IL-12 (mIL-12 N220L) with hepatitis C virus (HCV) E2 DNA elicited higher long-term E2-specific memory $CD8^+$ T-cell response and induced better protection against tumor challenge than that of wild type (27).

In this study, we assessed DNA vector (D) and replication-deficient adenoviral vaccines (A), either alone (DNA prime-DNA boost: D-D, Adenovirus prime-Adenovirus boost: A-A) or as combined regimens (DNA prime-Adenovirus boost: D-A, Adenovirus prime-DNA boost: A-D), expressing HBV S and S1S2X for their ability to elicit Th1-type cellular responses in Balb/c mice. Also, the immune-stimulatory effect of mIL-12 N220L on the immune responses elicited by each vaccine regimen was investigated. The strongest Th1-type immune response was elicited by the DNA prime-adenovirus boost regimen in the presence of mIL-12 N220L. Even though the A-A regimen was much less efficient in eliciting Th1-type cytokine responses, the cytolytic activity elicited by the A-A regimen is comparable to that by the D-A regimen. These results suggest that different effector functions of $CD8^+$ T cells may be induced differentially by different vaccination protocols. Furthermore, we have shown that the codelivery of mIL-12 N220L modulated differentially the immune responses by different vaccination regimens. Therefore, it may be needed to optimize carefully the conditions for codelivering mIL-12 N220L (or wild type IL-12) in different vaccination regimens.

Materials and Methods

Construction of expression vectors. The S, S1S2 and X genes of HBV were amplified by PCR from pGEM-HBV cDNA using these primers: S with S-*AscI* (5' AGGAGGCGCGCCAAATGGAGAACACAACAT CAGG 3') and S-*XhoI* (5' TTATCTCGAGTTAAAT GTATACCCAAAGAT 3'), S1S2 with S1-*AscI* (5' A GGAGGCGCGCCAAATGGGAGGTTGGTCTTC CAA 3') and S2-*NotI* (5' TAATGCGGCCGCTAGT TCGGTGCAGGGTCCCC 3') and X with X-*EcoRI* (5' AGGAGGCGCGCCAAATGGCTGCTAGGGT GTGCT 3') and X-*XbaI* (5' TCAGTCTAGATTAG GCAGAGGTGAAAAAGTT 3') primers, respectively. X gene was fused to 3' of S1S2 gene to generate S1S2X. The DNA fragment encoding gDs (Herpes simplex virus type 1 glycoprotein D signal sequence, amino acid residue 1 to 34) was fused to 5' of S and S1S2X genes to generate gDsS and gDsS1S2X, which were then cloned into pGX10 (pGX10-gDsS and pGX10-gDsS1S2X).

Each plasmid was transfected into COS7 cells. After a 36～48-hour incubation, the cells were harvest-

ed and the expression of S and S1S2X was confirmed by ELISA (Dong-A Pharmaceutical Co., LTD) for S and western blot analysis for S1S2X.

Construction of replication-defective recombinant adenoviruses. An adenovirus with deletion in the E1 and E3 region (Q. Biogene) was used to generate the constructs expressing the HBV S or S1S2X. The DNA fragment encoding S or S1S2X was cloned into pShuttle-gDs, using AscI and XbaI, which comes from pShuttle-gDs△ST (28). Each replication-defective recombinant adenovirus was generated by following the manufacturer's instructions (28). The expression of each HBV antigen in QBI293 cells was confirmed by ELISA for S and western blot analysis for S1S2X.

Peptides. Overlapping peptides spanning a large part of S and the full length of S1S2 were synthesized by PEPTRON (www.peptron.com). The peptides were 20 amino acids long and overlapped by 10 amino acids. HBV S 28～39 (IPQSLDSWWTSL) was also synthesized.

Cell lines. To establish stably transfected syngeneic cell lines expressing S or S1S2 antigens, expression plasmids expressing S (pCIN-S) or S1S2 (pCIN-S1S2) were separately constructed. The S or S1S2 gene was amplified by PCR using these primers: S with S-*XhoI* (5' GCGCTCGAGATGGAGAACACAACATCAG GATTCC 3') and S-*EcoRI* (5' CCCGAATTCTTAA ATGTATACCCAAAGATA 3'), S1S2 with S1-*EcoRI* (5' CCCGAATTCATGGGAGGTTGGTCTTCCAA ACCTCG 3') and S2-*NotI* (5' CGCGCGGCCGCCT AGTTCGGTGCAGGGTCCCCAG 3'), respectively. Using the restriction enzyme sites (S gene: XhoI and EcoRI, S1S2 gene: EcoRI and NotI), the amplified DNA fragments were cloned into the pCI-neo vector (Promega, Cat. No. E1841) which has the neomycin phosphotransferase gene, a selection marker for mammalian cells. P815 or CT26 cells were transfected with *BamHI*-linearized pCIN-S or pCIN-S1S2The and selected with G418 sulfate (Calbiochem, Cat. No. 345810). Selected cells were screened by ELISA for S and Western blot analysis for S1S2. Positive clones were used as stimulator and/or target cells for CTL assays.

Vaccination. Female BALB/c mice were purchased from Japan SLC (Shizuoka, Japan) and kept under specific pathogen-free conditions in the animal care facility in POSTECH. 6 to 8-week-old mice were intramuscularly injected with 60 ug of DNA (20 ug of pGX10-gDsS pGX10-gDsS1S2X and pGX10-mIL-12 N220L or mock vector) or 1.5×10^7 pfu (Plaque Forming Unit) of recombinant adenoviruses (5×10^6 of rAdv-gDsS, rAdv-gDsS1S2X and rAdv-mIL12 N220L or rAdv-mock) as indicated. Boosting was performed 4 weeks after the priming with DNA or adenovirus vaccine. Mice were sacrificed at the indicated time points for immunological assays.

Table I. S and S1S2 peptide pools

S	
1～20	MENIASGLLG PLLVLQAGFF
21～40	LLTKILTIPQ SLDSWWTSLN
31～50	SLDSWWTSLN FLGGTPVCLG
41～60	FLGGTPVCLG QNSQSQISSH
51～60	QNSQSQISSH SPTCCPPICP
61～70	SPTCCPPICP GYRWMCLRRF
101～120	QGMLPVCPLI PGSSTTSTGP
111～130	PGSSTTSTGP CKTCTTPAQG
121～140	CKTCTTPAQG TSMFPSCCCT
131～150	TSMFPSCCCT KPTDGNCTCI
141～160	KPTDGNCTCI PIPSSWAFAK
151～170	PIPSSWAFAK YLWEWASVRF
171～190	SWLSLLVPFV QWFVGLSPTV
201～220	WGPSLYNILS PFMPLLPIFF
S1S2	
1～20	MGGWSSKPRQ GMGTNLSVPN
11～30	GMGTNLSVPN PLGFFPDHQL
21～40	PLGFFPDHQL DPAFGANSNN
31～50	DPAFGANSNN PDWDFNPNKD
41～60	PDWDFNPNKD HWPEANQVGV
51～70	HWPEANQVGV GAFGPGFTPP
61～80	GAFGPGFTPP HGGLLGWSPQ
71～90	HGGLLGWSPQ AQGILTTVPA
81～100	AQGILTTVPA APPPASTNRQ
91～110	APPPASTNRQ SGRQPTPISP
101～120	SGRQPTPISP PLRDSHPQAM
111～130	PLRDSHPQAM QWNSTTFHQA
121～140	QWNSTTFHQA LPDPRVRGLY
131～150	LPDPRVRGLY FPAGGSSSGT
141～160	FPAGGSSSGT VNPVPTTASP
151～174	VNPVPTTASP ISSIFSRTGD PAPN

IFN-γ ELISPOT assay. IFN-γ ELISPOT assays were performed as previously described (28). As stimulators, S pool, S1S2 pool (final concentration: 1 ug/ml for each peptide) or S CTL epitope (S 28～39, 10 ug/ml) was used (Table I). IFN-γ ELISPOT responses to medium controls were subtracted from the responses to the each stimulator. Results are expressed as the number of IFN-γ-secreting cells (SFCs)/10^6 cells.

Intracellular cytokine staining. Splenocytes derived from immunized mice ($1\sim2\times10^6$) were stimulated with S 28～39 peptide (10 ug/ml) in complete media in the presence of Bredfeldin A (1 ug/ml) for 6 hours at 37°C CO_2 incubator. Cells were washed and then labeled with a FITC-conjugated anti-CD8 antibody (Pharmingen, Cat. No. 553031) for 30 min at 4°C. After a wash, cells were permeabilized with Cytofix/Cytoperm for 20 min at 4°C and then stained with a PE-conjugated anti-IFN-γ (Pharmingen, Cat. No.

554412) or anti-TNF-α (Pharmingen, Cat. No. 554419) antibody. Finally, the cells were washed, fixed, acquired on a FACScan flow cytometer, and analyzed by using CellQuest software.

Cytotoxic T-lymphocyte (CTL) assay. Splenocytes derived from immunized mice (3×10^7/well) were stimulated in six-well culture plates for 5～7 days with 1×10^6 mitomycin C-treated stably transfected P815 cells expressing S or S1S2 as stimulator cells in complete medium supplemented with 10 U/ml of recombinant murine IL-2. Serial dilutions of effector cells were cultured with 1×10^4 Cr^{51}-labeled targets in 200 ul round-bottom wells. Specific cytolytic activity of cells was tested in short-term Cr^{51}-release assays against CT26 cells expressing S, S1S2 or peptide-pulsed CT-26 cells. After a 5 hours incubation at 37°C, 100 ul of supernatant was collected for gamma radiation counting. The percentage specific release was calculated as [(experimental release-spontaneous release)/(total release-spontaneous release)]×100. Spontaneous release was determined from target cells incubated without effector cells and total release was determined in the presence of 1% Triton X-100. Data shown are the mean of triplicate cultures.

Antibody ELISA. 96-well-immunoplates (Nunc, Cat. No. 439454) were coated with 50 ul of 0.1 ug/ml S or S1S2 antigen diluted in PBS. After overnight incubation at 4°C, the plates were blocked with 5% Non-fat milk in PBST (0.5% Tween-20) for at least 1 hour. 50 ul of mouse serum diluted at various dilutions in 5% Non-fat milk in PBST was added. After incubation for 2 hours at 37°C or overnight at 4°C, the plates were washed 5 times with PBST. 50 ul of 1 : 3000 diluted anti-mouse total IgG- or IgG2a-HRP conjugate in 5% Non-fat milk in PBST was added. After 90 min incubation at room temperature, the plates were washed 7 times with PBST. 50 ul of TMB substrate (Sigma, Cat. No. T-8665) was added and the color was developed for 5～10 min at room temperature. The reaction was stopped with 2 N H_2SO_4 and the optical density at 450 nm (O.D. 450) was measured with an ELISA plate reader. The cut-off for seroconversion was defined as the optical density+2 standard deviations for 4 serum samples obtained from naïve mice.

Results

Comparison of Th-1 cytokine responses. Homologous prime-boost regimens (D-D, A-A) and heterologous prime-boost regimens (D-A, A-D) were assessed for their ability to induce IFN-γ-secreting lymphocytes. In addition, the effect of the codelivery of mIL-12 N220L was also evaluated. To evaluate the immune responses generated by priming itself, mice were primed and left for 8 weeks without boosting. A plasmid DNA or an adenovirus encoding mIL-12 N220L was codelivered as indicated. Otherwise, a mock plasmid or a mock adenovirus was added. When the S peptide pool was used as a stimulator, the levels of IFN-γ ELISPOT responses elicited by DNA (D) and adenovirus vaccine (A) were similar (Fig. 1A, a). However, adenovirus vaccine was better than DNA vaccine in eliciting IFN-γ ELISPOT responses to the S1S2 peptide pool (Fig. 1A, b). The IFN-γ ELISPOT responses to both peptide pools were not enhanced by codelivery of mIL-12 N220L (Fig. 1A, a & b).

To investigate the IFN-γ ELISPOT responses elicited by each prime-boost regimen, mice primed with DNA or adenovirus vaccine expressing HBV S and S1S2 were boosted 4 weeks after the priming. 4 weeks after the boosting, total splenocytes were prepared and used for IFN-γ ELISPOT assays. To measure IFN-γ ELISPOT responses to S antigen, the S peptide pool or S 28-39 (IPQSLDSWWTSL), an L^d-restricted CTL epitope, was used as a stimulator. The S1S2 peptide pool covering the full length of S1S2 was used to measure IFN-γ ELISPOT responses since there is no defined epitope for S1S2. The D-A regimen elicited the strongest IFN-γ ELISPOT response to S 28～39 in the regimens tested (Fig. 1B, a, open bars). Similar results were obtained when the S peptide pool was used as a stimulator (data not shown). A stronger IFN-γ ELISPOT response to the S1S2 pool was elicited by the D-A regimen than the other regimens (Fig. 1B, b, open bars). The codelivery of mIL-12 N220L enhanced the IFN-γ ELISPOT responses to both S 28-39 and the S1S2 peptide pool (Fig. 1B, a & b, open bar vs. closed bar). In contrast, no dramatic effect on the IFN-γ ELISPOT responses elicited by the other regimens was generated by the codelivery of mIL-12 N220L.

Since an L^d-restricted CTL epitope (S 28～39) of S antigen is available, intracellular cytokine staining assays were also performed to measure the $CD8^+$ T cell responses to S antigen elicited by the prime-boost regimens. The highest percentage of IFN-γ-positive T cells was generated in the D-A regimen upon stimulation with S 28～39, which is consistent with the results of the IFN-γ ELISPOT assay (Fig. 2A). The populations of TNF-α-positive $CD8^+$ T cells were slightly lower than those of IFN-α-positive $CD8^+$ T cells. Similarly with the intracellular IFN-γ staining assay, the D-A regimen led to the highest frequency of TNF-α-positive $CD8^+$ T cells and the populations of IFN-γ and TNF-α-positive $CD8^+$ T cells were slightly enhanced by the codelivery of m IL-12 N220L (Fig. 2A and B). However, the codelivery of mIL-12 in the A-A and the A-D regimens led to

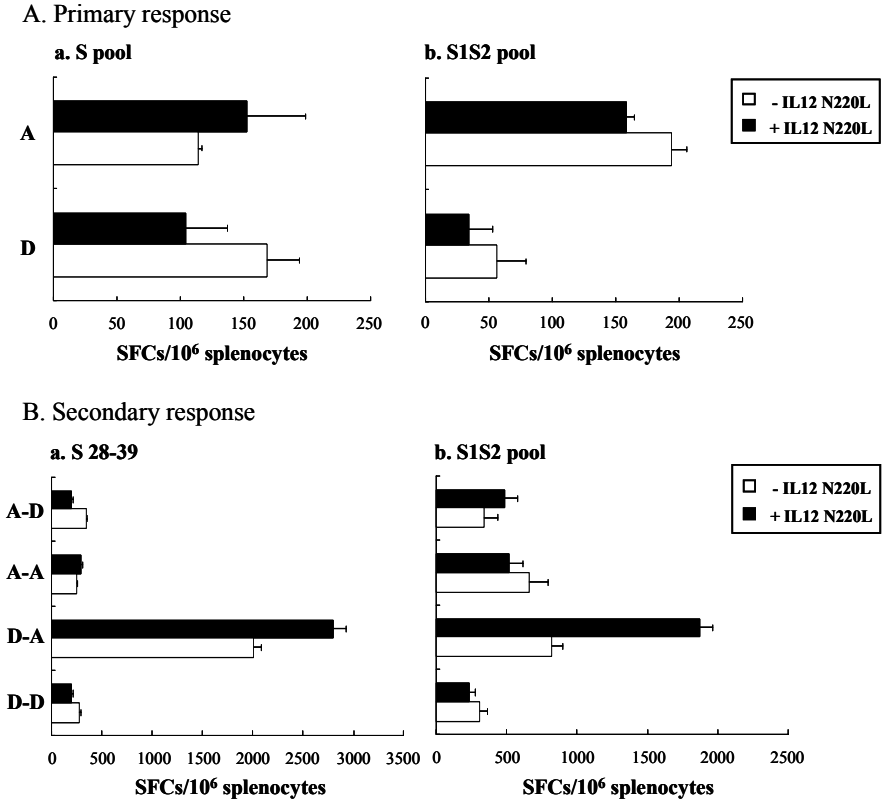

Figure 1. IFN-γ ELISPOT responses of total splenocytes to S and S1S2. Mice were primed with DNA or adenovirus vaccine encoding HBV antigens with or without mIL-12 N220L. Some of them were left for 8 weeks to investigate primary immune responses (A) and the others were boosted 4 weeks after the priming (B). IFN-γ ELISPOT analyses were performed 4 weeks after the boosting with S, S1S2 peptide pool (1 ug/ml for each peptide) or S 28～29 (10 ug/ml) as a stimulator. The IFN-γ ELISPOT responses to medium controls were subtracted from the responses to the each stimulator. Results are expressed as the number of IFN-γ-secreting cells/10^6 cells (SFCs: Spot Forming Cells). Open bar: without mIL-12 N220L, Closed bar: with mIL-12 N220L, D: DNA, A: Adenovirus, D-D: DNA prime-DNA boost, D-A: DNA prime-Adenovirus boost, A-A: Adenovirus prime-Adenovirus boost, A-D: Adenovirus prime-DNA boost.

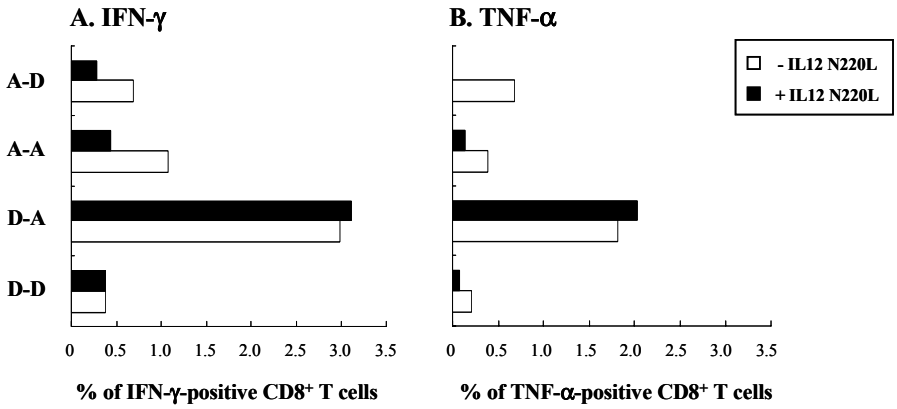

Figure 2. Intracellular cytokine staining. Splenocytes were prepared 4 weeks after the boosting and used for intracellular cytokine staining assays as described in the materials and methods. Results were expressed as the percent of S 28-39-specific IFN-γ (A) or TNF-α (B)-ositive $CD8^+$ T cells over all $CD8^+$ T cells. Open bar: without mIL-12 N220L, Closed bar: with mIL-12 N220L, D-D: DNA prime-NA boost, D-A: DNA prime-Adenovirus boost, A-A: Adenovirus prime-Adenovirus boost, A-D: Adenovirus prime-DNA boost.

decrease in the frequencies of both IFN-γ and TNF-α-positive $CD8^+$ T cells. Together with the results of IFN-γ ELISPOT assay, the D-A regimen in the presence of mIL-12 N220L appeared to be the best in eliciting Th1-type cytokine responses, which are believed to play important roles in resolving HBV infection.

CTL responses. The CTL assays were performed in parallel with the ELISPOT assays. The D-A regimen elicited the strongest CTL activity against S antigen, which is consistent with the IFN-γ and TNF-α responses (Fig. 3A, open circle). The CTL activity elicited by the A-A regimen is slightly lower than that by the D-A but still much higher than those by the D-D and A-D regimens (Fig. 3A, open circle). This is unexpected since the frequency of T cells specific to S 28～39 by the A-A regimen determined from the IFN-γ ELISPOT and intracellular IFN-γ staining was much lower than that by the D-A and comparable to those in the D-D and the A-D regimens. The CTL responses to S1S2 were also consistent with the IFN-γ ELISPOT responses (Fig. 3B, open circle).

The codelivery of mIL-12 N220L did not significantly affect the CTL activities both to S and S1S2

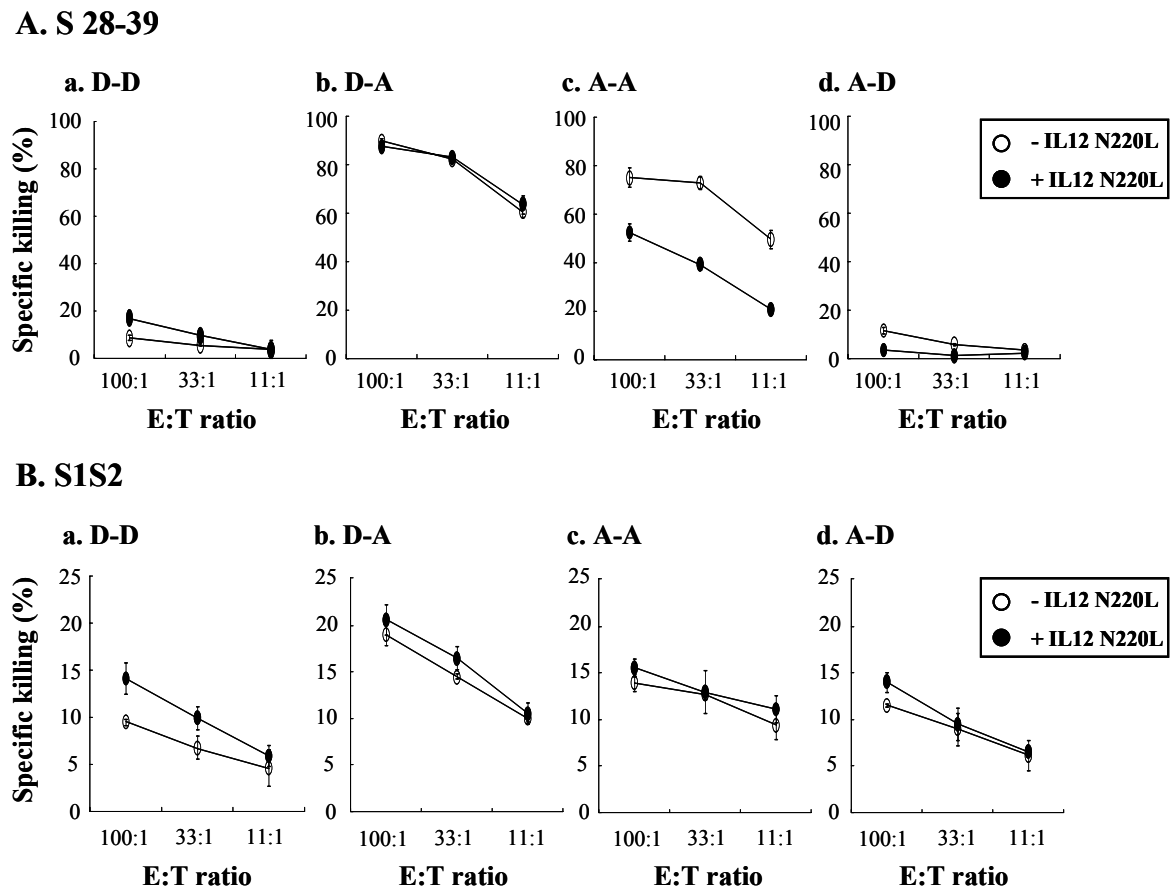

Figure 3. CTL activity. Cytolytic activities to S (A) and S1S2 (B) elicited by prime-boost regimens were measured using the splenocytes prepared 4 weeks after the boosting as indicated in the materials and methods. Open circle: without mIL-12 N220L, Closed circle: with mIL-12 N220L, D-D: DNA prime-DNA boost, D-A: DNA prime-Adenovirus boost, A-A: Adenovirus prime-Adenovirus boost, A-D: Adenovirus prime-DNA boost.

by all regimens except the CTL activity to S by the A-A regimen (Fig. 3A & B, open vs. closed circle). The CTL activity to S by the A-A regimen was dramatically decreased in the presence of mIL-12 N220L (Fig. 3A, c). This is consistent with the results of intracellular cytokine staining assays where the codelivery of mIL-12 N220L reduced the frequencies of IFN-γ and TNF-α-positive $CD8^+$ T cells specific to S 28-39 by 50% compared to those in the absence of mIL-12 N220L (Fig. 2A & B).

Antibody responses to S and S1S2. To further compare the ability of the D-A and the A-A regimen to elicit immune response, antibody response to S and S1S2 were characterized. Since the D-A and the A-A regimens are generally better than the other regimens, we further characterized these two regimens. Higher titers of total IgG responses to both S and S1S2 were elicited by the D-A regimen than the A-A regimen (Fig. 4A & B). The codelivery of mIL-12 N220L did not modulate substantially the total IgG responses except that to S1S2 by the A-A regimen. Similarly with total IgG responses, the D-A regimen either with or without mIL-12 N220L was better than the A-A regimen in eliciting IgG2a responses to both S and S1S2, suggesting better Th1-type responses (Fig. 4A & B). Taken together with the results of the Th1-type cytokine responses, the D-A regimen appeared to be better than the A-A regimen in eliciting Th1-type immune responses.

Identification of CTL epitopes in S1S2. Since any CTL epitope in Balb/c mice has not been identified for S1S2 antigen, it is difficult to analyze the $CD8^+$ T cell responses to S1S2 in detail. To identify CTL epitopes, the splenocytes stimulated with P815-S1S2 were used for IFN-γ ELISPOT assays with each peptide as a stimulator. A significant IFN-γ ELISPOT response was obtained only with the aa 71～90 peptide (Fig. 5A). To confirm that a CTL epitope (s) is (are) included in the aa 71～90 peptide, the peptide was used for CTL assay. As shown in Fig. 5B, the

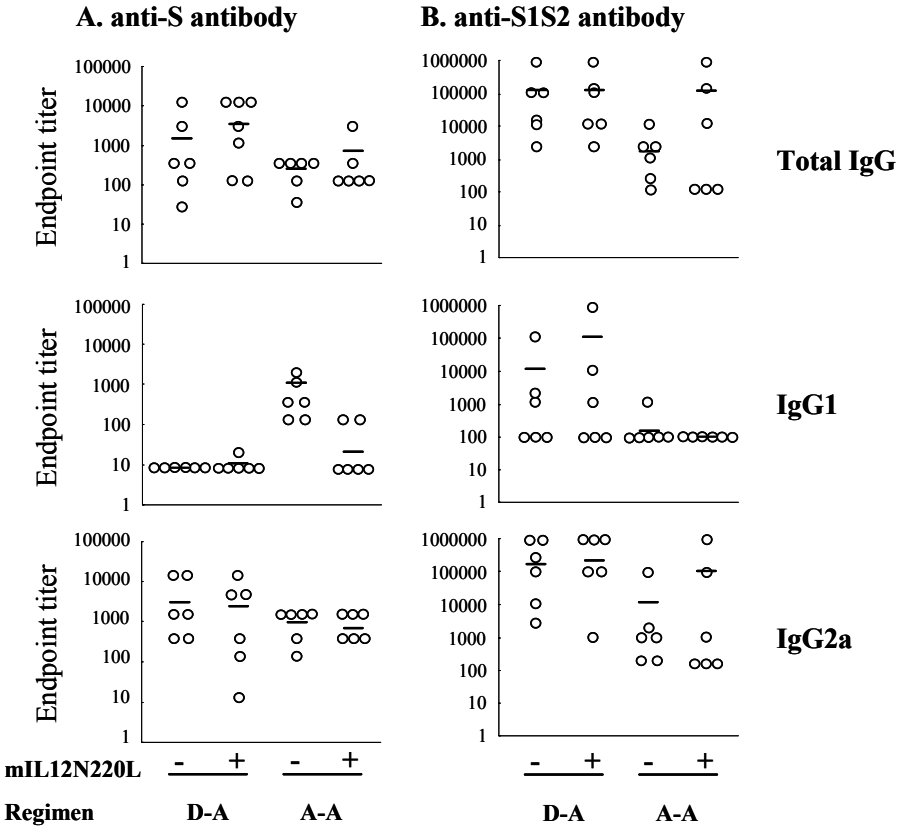

Figure 4. Antibody responses to S and S1S2. Serum was taken to determine the titers of anti-S (A) and S1S2 (B) antibodies, total IgG, IgG1 and IgG2a subclasses. The serially diluted serum samples were used for ELISA and the cutoff for seroconversion was defined as the optical density+2 standard deviations for 6 serum samples obtained from naive mice. The results are expressed as endpoint dilutions (open circle) for 6 mice per group and as the average value (bar) for each group. D-A: DNA prime-Adenovirus boost, A-A: Adenovirus prime-Adenovirus boost.

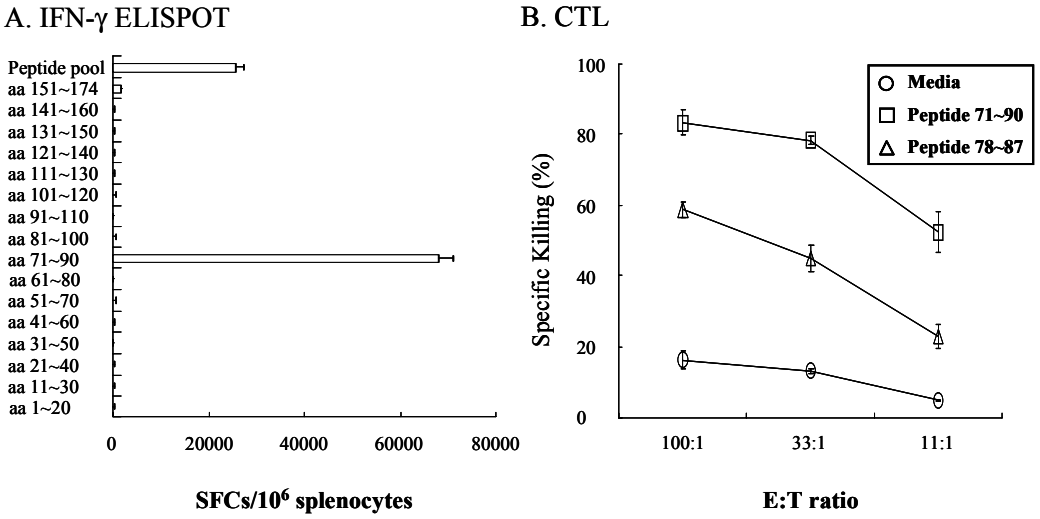

Figure 5. Mapping of S1S2 CTL epitopes. A. Splenocytes stimulated with P815-S1S2 were used for IFN-γ ELISPOT assays with each peptide (10 ug/ml) as a stimulator. The IFN-γ ELISPOT responses to medium controls were subtracted from the responses to the each peptide. Results are expressed as the number of IFN-γ-secreting cells/10^6 cells (SFCs: Spot Forming Cells). B. Splenocytes were stimulated with P815-S1S2 and used for CTL assays using CT26 cells pulsed with aa 71～90 as target cells.

CT26 cells pulsed with the aa 71～90 peptide could be recognized by the splenocytes stimulated with P815-S1S2. Possible CTL epitopes in the aa 71～90 peptide were searched using a peptide binding prediction algorithm (http://.bimas.dcrt.nih.gov/molbio/hla_bind/). As a result, two putative CTL epitopes were identified in the aa 76～87 peptide, aa 76～84 (K^d restricted) and aa 78～87 (L^d restricted). Further

experiments will be needed to confirm that the defined epitopes can work as CTL epitopes.

Discussion

In the present study, we comparatively assessed DNA vaccine and adenoviral vector vaccine, either alone (the D-D and the A-A) or in combined regimen (the D-A and the A-D), for their ability to elicit Th1-type cellular immune responses to HBV S and S1S2 in Balb/c mice. The strongest Th1-type cellular immune responses to S and S1S2 were elicited by the D-A regimen in the presence of mIL-12 N220L. These results suggest that the D-A regimen may be a good candidate for prophylactic and therapeutic HBV vaccine. To our knowledge, it is the first report that the D-A regimen either in the presence or absence of mIL-12 N220L is better than the other regimens in eliciting Th1-type cellular immune responses against HBV S and S1S2.

Several recombinant viral vectors have been used for vaccine trials since they are very efficient in inducing both humoral and cellular immune responses, often after a single inoculation (29). The efficacy of the DNA prime-viral vector boost regimen was demonstrated first in the influenza virus vaccine study in mice using the hemagglutinin (HA) antigen (30,31). Since that, the DNA-viral vector regimen has been shown to elicit higher cellular immune responses than the DNA-DNA and the viral vector-viral vector regimens in malaria, simian immunodeficiency virus, ebola virus and Hepatitis C virus (15,18-21,29,32). In despite of strong immunogenicity of viral vectors, the immunity induced against the vector-specific antigens can limit the immune responses to the transgene products when multiple injections are required. In the viral vector-viral vector regimen, the immune responses to not only the desired antigens but also the vector-specific antigens could be induced. The immunity to the vector-specific antigens can interfere with the amplification of the immune responses to the desired antigens on the boosting steps with the recombinant viral vector (21). However, in the DNA-viral vector regimen, the immune responses to the desired antigens elicited by DNA priming can be selectively amplified by the boosting with recombinant viral vector. Furthermore, as mentioned above, the intramuscular injection of DNA vaccine generally directs the immune responses to Th1-type but the viral vectors do not always result in the induction of Th1-type immunity. Especially, adenoviruses are known to activate dendritic cells but do not polarize them toward a Th1-inducing subset (33). These facts may explain why the D-A regimen elicited relatively stronger Th1-type immune responses to both S and S1S2 than the A-A.

Even though the IFN-γ and the TNF-α responses to the CTL epitope of S (S 28～39) by the A-A regimen were similar to those by the D-D and A-D regimens, the CTL activity by the A-A regimen was much stronger than those by the D-D and the A-D regimens and further comparable to that by the D-A regimen. IFN-γ ELISPOT responses to CTL epitopes are believed to be correlated to CTL activities. However, it has been also reported that cytolytic activities and cytokine production of $CD8^+$ T cells are largely segregated and also differentially regulated (34-36). These reports further showed that $CD8^+$ T cells are functionally heterogeneous in terms of production of cytokines and cytolytic effector molecules. So, it may be possible that the immune responses elicited by different vaccination regimens are different not only in the strength but also in the characteristics of effector functions. Since it has been shown that a pool of $CD8^+$ T cells induced by DNA vaccination are less differentiated compared to virus-primed $CD8^+$ T cells, the boost with the same adenovirus vaccine could result in differences in phenotypes and functions of the $CD8^+$ memory between the D-A and the A-A regimens (37).

Murine IL-12 N220L was shown to enhance HCV E2-specific long-term $CD8^+$ T cell responses when codelivered with E2 DNA vaccine in mice (27). The codelivery of mIL-12 N220L generally enhanced Th1-type cytokine responses of the D-A regimen. Since the immune-stimulatory effect of mIL-12 N220L became more obvious at later time points (more than 6 weeks after the last vaccination) more significant differences between the groups with and without mIL-12 N220L would be expected if measured at later time points. In contrast to the positive effect of mIL-12 N220L on the Th1-type cytokine responses by the D-A, the Th1-type cytokines and the CTL response to S elicited by the A-A regimen were instead decreased by the codelivery of mIL-12 N220L (Fig. 2, 3). The IFN-γ ELISPOT response to S1S2 by the A-A regimen was also slightly reduced. Even though IL-12 has been known to promote strong cell-mediated immune responses when used as an adjuvant in DNA and viral vector vaccine there are some reports that IL-12 suppresses the immune responses elicited by vaccines under certain circumstances and particularly at high doses (38-40). Especially, Lasarte et al. showed that cellular immune responses were suppressed when recombinant adenovirus expressing IL-12 (rAdv-IL12) was used at doses of 10^6～10^8 pfu (38). The doses of rAdv-IL12 for the suppressive effect may be different depending on the antigens and also the types of immune responses. The adenovirus expressing mIL-12 N220L was used at doses of 5×10^6 pfu in our study. The doses were

just above the doses at which rAdv-IL-12 can suppress the cellular immune responses. That may explain why, in our study, the suppressive effect was observed only in the A-A regimen not in the D-A regimen. Even though a single injection of rAdv-mIL-12 N220L may not cause suppression, a boost injection with rAdv-mIL-12 N220L could result in suppression in the cellular immune responses. However, the suppressive effect was observed by the plasmid expressing IL-12, only when high doses of the plasmid expressing IL-12 was used after the pretreatment of cardiotoxin which was reported to enhance gene expression from DNA vaccine by $>$10-fold. So, a single injection of rAdv-mIL-12 N220L in the D-A regimen may not be enough to suppress the cellular immune responses in our study.

In this study, we have assessed the efficacy of several HBV vaccine regimens in mice and have demonstrated that the DNA prime-adenovirus boost in the presence of mIL-12 N220L appeared to be most effective in eliciting Th1-type cellular immune responses to HBV S and S1S2 in Balb/c mice. Thus, the DNA prime-adenovirus boost regimen with mIL-12 N220L may be a promising candidate for effective prophylactic and therapeutic vaccines against HBV. We have also shown that despite the positive effect of rAdv-IL-12 N220L on the immune responses elicited by the D-A regimen, it suppressed cellular immune responses elicited by the A-A regimen. Thus, the conditions for codelivering rAdv-IL12 N220L (or wild type IL-12) need to be carefully optimized for each vaccination protocol.

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