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Costimulatory protein B7–1 enhances the cytotoxic T cell response and antibody response to hepatitis B surface antigen

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ABSTRACT **There is a need for more effective therapy for chronic virus infections. A principle natural mechanism for elimination of virus-infected host cells is activation of viral antigen-specific cytotoxic T lymphocytes (CTL). In an effort to develop methods of inducing virus-specific CTL responses that might be utilized in therapy of virus infections, we have investigated the effect of B7, a costimulatory factor for T-cell activation. In this study we show that delivery of genes encoding human B7–1 and a viral antigen in the same recombinant viral vector to cells of mice induces a greater viral antigen-specific CTL response than does similar delivery of the viral antigen gene alone. Two recombinant adenovirus vectors were constructed with the foreign genes inserted in the early region 3. One of them (Ad1312) directed expression of the surface antigen gene of hepatitis B virus (HBS); the other (Ad1310) directed coexpression of HBS and human B7–1 (CD80) by means of an internal ribosomal entry site placed between the two coding sequences. When inoculated into BALB/c mice, both vectors induced a viral surface antigen-specific CTL response. The response induced by Ad1310 was stronger than that by Ad1312 as measured by a chromium release assay for CTL activity and limiting dilution analysis for CTL precursor frequency, indicating that the B7–1 gene co-delivered with the HBS gene had an enhancing effect on the CTL response against surface antigen. Ad1310 also induced a higher titer of antibody against surface antigen than did Ad1312. This result suggests that expression of a costimulatory protein and a viral antigen in the same cells *in vivo* induces stronger immune responses than expression of the antigen alone. This could be a novel strategy for development of both preventive and therapeutic vaccines against infectious agents.**

Activation of naive T cells requires not only recognition of an antigen-major histocompatibility complex on the surface of antigen-presenting cells (APC) by the T-cell receptor but also costimulatory signals, which can be provided by spleen cells of immunized mice (1). One such signal has been shown to result from interaction of costimulatory factors on the surface of APCs with specific molecules on the surface of T cells. Costimulatory factors on professional APCs include the B7–1 protein (CD80), which specifically interacts with the CD28 and CTLA-4 proteins on the surface of T cells resulting in a costimulatory signal for T-cell activation (2–9). B7–1 appears to be expressed only by professional APC (macrophages, dendritic cells, and activated B cells) and not other cells *in vivo* (3, 10, 11).

The experiments described here were done to determine whether the cytotoxic T lymphocyte (CTL) response and antibody response are enhanced when cells expressing a foreign antigen *in vivo* are made to also express B7–1. This was done by delivering both genes for a viral antigen (hepatitis B surface antigen or HBsAg) and B7–1 in a recombinant viral vector to cells of BALB/c mice and comparing the immune responses with that with a vector carrying the HBsAg gene (HBS) alone. The recombinant viral vector used was human adenovirus type 5, which infects a wide variety of cell types in the mouse (12) and has been used as a vector to deliver several different heterologous vaccine antigen genes to mice and other animals (13–21). The results showed that both the CTL response, as measured by chromium release assay for CTL activity and limiting dilution analysis for CTL precursor (CTLp) frequency, and the antibody response against HBsAg were enhanced when the B7–1 gene was co-delivered with the viral antigen gene.

MATERIALS AND METHODS

Construction of Recombinant Adenovirus Vectors. Recombinant adenovirus vectors (AdV) were constructed by cotransfection of plasmid p199 carrying different inserts and *Spe*I-digested viral DNA of the adenovirus type 5 strain Ad5*Marietta* into 293 cells (22), essentially as described (23). Both Ad5*Marietta* and p199 were gifts from P. Hung (Wyeth-Ayerst Research, Philadelphia). Recombinant viruses were isolated from single plaques, amplified in 293 cells, and screened by the ability of infected cells to secrete HBsAg into the medium. Viral DNA of HBsAg-producing candidates were prepared (24) and characterized by restriction enzyme analysis. Expression of foreign genes carried by the recombinant AdVs were confirmed in both the human cell line 293 and murine fibroblast cell line BALB/3T3 infected with the vectors.

Large Scale Preparation of AdV. Large scale preparation of recombinant AdVs was performed as described (25). Briefly, monolayers of the human cell line 293 grown in DMEM supplemented with 10% bovine calf serum were infected with virus stock. The infected monolayers were maintained in the same medium with 2% calf serum until extensive cytopathic effect was observed. Virus particles were released by the addition of Nonidet P-40, precipitated with PEG 8000, and further purified by CsCl density centrifugation. The titers of purified virus stocks were determined on 293 cells by plaque assay. HBsAg was undetectable in the purified virus stocks.

Detection of B7–1 Protein on the Surface of Recombinant AdV-Infected Cells. B7–1 protein presented on the surface of infected cells was detected by an indirect immunofluorescence assay. In brief, BALB/3T3 cells infected with AdVs at a multiplicity of infection of 20 and incubated for 36 h were detached from the tissue culture dishes by brief incubation in 5 mM EDTA/phosphate-buffered saline and dispersed into single cells by repeated pipetting. The suspended single cells

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Abbreviations: APC, antigen presenting cell; AdV, adenovirus vector; CTL, cytotoxic T lymphocyte; CTLp, CTL precursor; HBS, hepatitis B surface antigen gene; HBsAg, hepatitis B surface antigen; IRES, internal ribosomal entry site; pA, SV40 polyadenylylation signal; PCMV, cytomegalovirus immediate-early promoter.

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were stained with monoclonal antibody to human B7–1 (Becton–Dickinson) (10 μg/ml) followed by incubation with fluorescein-labeled secondary antibody and flow cytometric analysis, using a FACScan system (Becton–Dickinson).

Construction of HBsAg-Producing Cell Line A20.1203. A retrovirus vector carrying the HBS gene was created by the following procedure. The coding sequence for the small HBsAg protein from a cloned hepatitis B virus genome of subtype adw_2 (26) preceded by the tripartite leader sequence of adenovirus [from pWS4 (27), a gift from R. Dornburg (University of Medicine and Dentistry of New Jersey, Piscataway, NJ)], was inserted into the plasmid pMV7 (28) and used to transfect the ecotropic packaging cell line Ψ2 (29). The virus transiently produced by the transfected Ψ2 cells was used to infect the amphotropic packaging cell line PA317 (30). The infected PA317 cells were selected with neomycin to generate the cell line PA317.1203, which produced the retrovirus vector RV1203. Murine B cell lymphoma cell line A20 (H-2^d) was infected with RV1203 and selected with neomycin to generate the cell line A20.1203, which produced HBsAg stably. A control cell line A20.918 was similarly constructed from A20 with the use of another retroviral vector RV918, which carried the core antigen gene of hepatitis B virus.

CTL Activity and CTLp Frequency Analysis. BALB/c mice (H-2^d, female, 6–8 weeks old) immunized with different AdVs were sacrificed at different time points after immunization. Spleen cells from the mice were cocultured with γ-irradiated (20,000 rad) syngeneic stimulator cells A20.1203 or control cells A20.918 (both H-2^d) in RPMI 1640 supplemented with 10% fetal calf serum and 10% T-STIM culture supplement (Collaborative Biomedical, Bedford, MA) for 7 days. Alternatively spleen cells were stimulated with the synthetic 12-mer peptide IPQSLDSWWTSL (S_{28-39}), which is a defined CTL epitope of HBsAg in H-2^d BALB/c mice (31, 32) or a control peptide GFADLMGYIPLVGAPL for 7 days. Both peptides were used at a concentration of 2 μg/ml in the same medium. CTL activity was determined with a standard chromium release assay, using ^{51}Cr-labeled A20.1203 or A20.918 as target or control, at a different effector/target ratio. The percentage specific release was calculated as [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100. Total release was measured by target cells lysed with Triton X-100. Spontaneous release was routinely 10–20% of total release.

Limiting dilution analysis was used to determine frequency of HBsAg-specific CTLp. Serially diluted responder spleen cells from immunized mice were cocultured with 5×10^4 γ-irradiated stimulator cells A20.1203 in 16-well replicates, in the same medium as described above. After 7 days, ^{51}Cr-labeled A20.1203 cells were added into each well and incubated for 4 h before supernatants from each well were measured in a γ counter. Individual wells were considered positive when the amount of ^{51}Cr release was greater than 10% specific release or three times the standard deviation of spontaneous release, whichever was higher. The data were analyzed using a computer program to estimate the frequency of HBsAg-specific CTLp within 95% confidence limits, based on the Poisson model (33).

RESULTS

Construction of Recombinant AdVs Carrying One or Two foreign Genes. Recombinant AdVs, which carry the HBS gene alone (Ad1312) or the HBS plus B7–1 genes (Ad1310) were constructed, as shown in Fig. 1. Foreign gene fragments as a transcription cassette were inserted in place of a deletion between 79.5 and 84.7 map units within the E3 gene region of the adenovirus type 5 genome. This deletion destroys an adenovirus gene, the product of which (gp19K) functions in down-regulating class I major histocompatibility complex expression and interfering with recognition and lysis by $CD8^+$ CTLs (35). In both vectors, the foreign gene(s) to be delivered were flanked by a cytomegalovirus immediate-early promoter (PCMV) and a SV40 polyadenylylation signal (pA). The insertion was made in such a way that the transcription from PCMV was in the same direction as those from the E3 and major late promoters of adenovirus. In the cassette for Ad1310, the internal ribosomal entry site (IRES) of encephalomyocarditis virus (36) was placed between HBS and B7–1 coding sequences to form a dicistronic DNA fragment. The IRES functions as an internal initiation site for the translation of B7–1 from mRNA. It has been suggested that transcription of inserted genes at the E3 region were mostly initiated from the E3 promoter or the major late promoter of adenovirus and subjected to splicing of various patterns, even in the presence of a flanked SV40 promoter (37). However we have made another AdV construct in which a similar dicistronic transcription cassette was inserted in E3 in the opposite orientation and have observed expression of both genes in the insert (data not shown), suggesting both the PCMV and IRES may be functioning in the context of the E3 region.

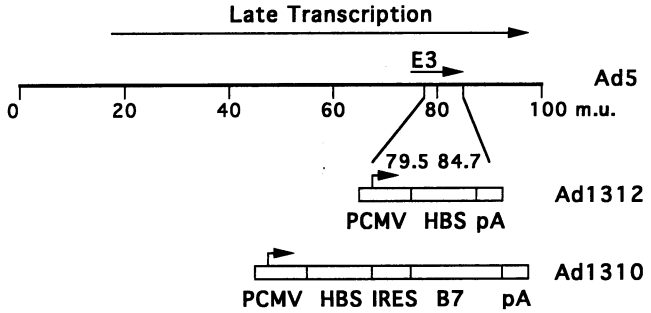

FIG. 1. Recombinant AdVs Ad1312 and Ad1310 are derivatives of the Ad5*Marietta* strain, in which the E3 gene region between map units (m.u.) 79.5 and 84.7 was replaced with different transcription cassettes for HBS or HBS plus B7–1 genes. PCMV was from pACCMVpLpA (34), a gift from R. Gerald (University of Texas, Dallas); HBS was from a cloned HBV genome of adw_2 subtype (26); IRES was from pCITE-2a (Novagen); B7, human B7–1 gene was from piLNhuB7, a gift from P. Linsley (Bristol–Myers Squibb); pA was from pACCMVpLpA (34).

Expression of the HBS gene (data not shown) and B7–1 gene carried by Ad1310 and/or Ad1312 was confirmed in human and murine cell lines infected with each vector. Fig. 2 demonstrates that the B7–1 protein was presented on the surface of BALB/3T3 cells infected by Ad1310, but not on those infected by Ad1312, indicating B7–1 gene carried by Ad1310 was expressed and the product was translocated correctly.

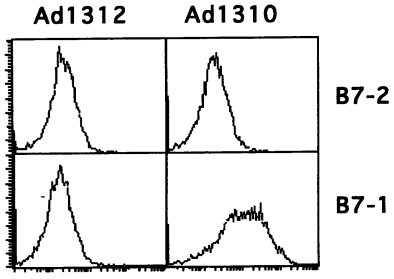

FIG. 2. Expression of the B7–1 gene directed by Ad1310. BALB/3T3 cells infected with Ad1312 or Ad1310 were stained with monoclonal antibodies against human B7–1 or B7–2 (control), followed by incubation with fluorescein-labeled secondary antibody and flow cytometric analysis, using a FACScan system (Becton-Dickinson). Murine B7–1 were not detectable from the infected cells when stained with a monoclonal antibody against murine B7–1, which did not cross-react with human B7–1 (data not shown).

***In Vivo* Expression of the HBS Gene Carried by Recombinant AdVs in BALB/c Mice.** BALB/c mice (H-2^d) were injected i.m. with 10^8 plaque-forming units of different recombinant AdVs. Blood was drawn 1 and 3 days after injection to assay serum HBsAg levels. As shown in Fig. 3*A*, on day 1 after inoculation of Ad1312, the mean serum HBsAg concentration for this group was 23.6 ± 5.4 ng/ml compared with 9.2 ± 3.8 ng/ml for the group receiving Ad1310. By day 3 the HBsAg levels of the Ad1312 group dropped to 2.8 ± 1.3 ng/ml, while that of the Ad1310 group dropped to 1.4 ± 0.9 ng/ml. These results indicate that HBsAg was efficiently produced and secreted in the mice receiving recombinant AdVs carrying HBS gene. By day 7 HBsAg was no longer detectable in both groups.

B7–1 Enhanced Antibody Response to HBsAg. Starting one week after i.m. inoculation of AdV, serum samples were obtained from the immunized mice and assayed for antibodies against HBsAg. As shown in Fig. 3*B*, the serum antibody level of both groups was high at week 1, dropped significantly by week 2, and then rose again at weeks 4 and 6. Although the serum HBsAg level of mice receiving Ad1310 (HBS plus B7–1) was lower than those receiving Ad1312 (HBS alone) (Fig. 3*A*), the antibody level in the former was higher than in the latter at all time points tested, indicating that the B7–1 gene co-delivered with HBS has an enhancing effect on the antibody response against the expression product of HBS. In comparison, mice receiving 4 μg of the alum-adjuvanted recombinant hepatitis B vaccine (Engerix-B, SmithKline Beecham Phamaceuticals, Philadelphia, PA) by i.m. injection at the same time generated HBsAg-specific antibody, which increased steadily from an $A_{492\,nm}$ of 0.96 (week 1) to 9.6 (week 6), without any decrease at week 2 (not shown in Fig. 3*B*).

B7–1 Enhanced CTL Response to HBsAg. Two weeks after i.m. injection, spleen cells from the immunized mice of each group were assayed for HBsAg-specific CTL activity and CTLp frequency. The splenocytes were co-cultured with the

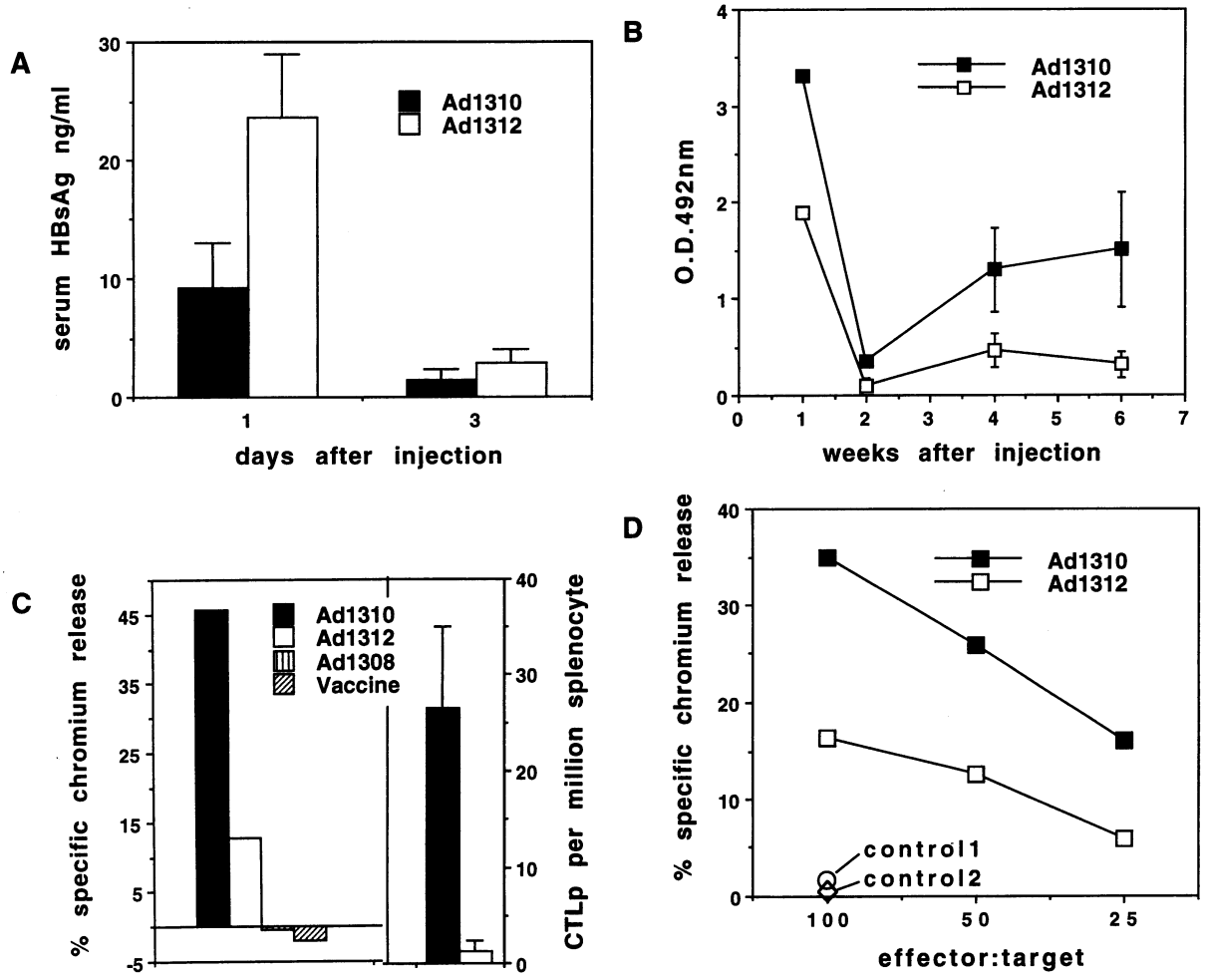

FIG. 3. (*A*) Groups of 5 BALB/c mice (female, 6–8 weeks old) were injected i.m. in the quadricep muscles with 10^8 plaque-forming units of Ad1310 or Ad1312. Serum samples of day 1 and day 3 after injection were assayed for HBsAg concentration, using the Auszyme Monoclonal EIA kit (Abbott). The positive control in the kit (9 ng/ml) was used as reference to estimate the concentration of HBsAg in the sera. (*B*) Starting 1 week after injection, serum samples were assayed for antibody against HBsAg, using the AUSAB EIA kit (Abbott). The positive control in the kit (50–150 mIU/ml) gave an $A_{492\,nm}$ of 1.0 when assayed in parallel with the serum samples. Cutoff value was 0.055. The week 1 data are from an equal-volume pool of five mouse serum samples; the week 2 data are mean ± SD of two serum samples, while those for weeks 4 and 6 are mean ± SD of three serum samples. (*C*) Two weeks after injection, spleen cells from mice immunized with Ad1310, Ad1312, Ad1308, or recombinant hepatitis B vaccine (pool of two spleens from each group) were co-cultured with A20.1203 cells for 7 days, followed by a chromium release assay, to determine HBsAg-specific CTL activity (left). ^{51}Cr-labeled A20.1203 was used as target cell at an effector/target ratio of 100:1. The same splenocytes were also analyzed by limiting dilution to assess CTLp frequency (right). Ninety-five percent confidence limits are indicated by the error bars. (*D*) Six weeks after injection, spleen cells from mice immunized with Ad1310 or Ad1312 (pool of three spleens from each group) were stimulated with the HBsAg CTL epitope peptide IPQSLDSWWTSL (S_{28-39}) or the control peptide GFADLMGYIPLVGAPL (control 1) for 7 days, followed by chromium release assay, to determine the epitope-specific CTL activity. ^{51}Cr-labeled A20.1203 (HBsAg positive) was used as the target cell, while ^{51}Cr-labeled A20.918 (HBsAg negative) was used as a negative control (control 2).

HBsAg-producing cell line A20.1203 (H-2^d) as stimulator. After 7 days of *in vitro* stimulation, a standard chromium release assay was performed, using ^{51}Cr-labeled A20.1203 as target cells to determine HBsAg-specific CTL activity. As shown in the left panel of Fig. 3*C*, while specific release of ^{51}Cr was observed in spleen cells from both groups receiving Ad1310 or Ad1312, the activity of the former was 3.6 times higher than that of the latter. CTL activity was not observed in mice immunized with a control vector Ad1308 that did not carry HBS gene, nor in mice immunized with the alum-adjuvanted recombinant hepatitis B vaccine, a result in agreement with a previous report (38). A similar result was observed at week 6 (Fig. 3*D*), when spleen cells from immunized mice were stimulated with a peptide representing a defined CTL epitope of HBsAg in BALB/c mice. The epitope-specific CTL activity of spleen cells from mice receiving Ad1310 was more than two times higher than those receiving Ad1312.

To determine HBsAg-specific CTLp frequency in the splenocytes of the immunized mice, the same spleen cells used for CTL activity assay were serially diluted and co-cultured with A20.1203 for 7 days, followed by ^{51}Cr release assay. As shown in the right panel of Fig. 3*C*, 2 weeks after immunization the Ad1310-immunized mice had 30 times more HBsAg-specific CTLp in spleen than the Ad1312-immunized mice. These results confirm that BALB/c mice immunized with Ad1310 carrying both HBS and B7–1 genes mounted a stronger CTL response than mice immunized with Ad1312 carrying the HBS gene alone.

To investigate whether there is an enhancing effect of B7–1 when the recombinant AdVs were given by different routes, groups of BALB/c mice were injected i.v. or i.p. with 10^8 plaque-forming units of Ad1310 or Ad1312. Pairs of mice from each group were sacrificed 2–6 weeks after immunization, and their spleen cells were assayed for HBsAg-specific CTL activity. As shown in Table 1, while the value of specific release varied from experiment to experiment, the CTL activity of splenocytes from Ad1310-immunized mice was consistently higher than splenocytes of Ad1312-immunized mice, with a difference ranging between 1.4 and 5 times. This result confirmed that HBsAg-specific CTL response was enhanced when B7–1 gene was co-delivered with HBS gene by the same vector.

DISCUSSION

Hepatitis B virus is one of several viruses that can persist after primary infection in man. Such persistent infections can cause significant morbidity and early death. Therapeutic approaches to eliminate the virus and terminate chronic infections have not been very successful. Most commonly, inhibitors of virus replication such as nucleoside analogues have been used, and while they suppress virus replication during drug administration, virus replication most often returns to pretreatment levels when drug is stopped. Thus different and improved therapeutic approaches are needed. An important natural mechanism for elimination of virus-infected cells *in vivo* is an antigen-specific CTL response. Viruses persist when this and other relevant host responses are not vigorous enough to eliminate all infected cells. If a CTL response directed at virus-infected cells could be enhanced during persistent virus infection, it might lead to elimination of virus-infected cells and control of the infection. We are seeking ways of inducing strong viral antigen-specific CTL responses that can be investigated for therapeutic effects on persistent virus infections. To that end we have investigated the effects of B7–1 on the CTL response directed at cells producing viral antigen by delivering genes encoding both the viral antigen and B7–1 in a recombinant adenovirus vector to cells of mice infected with the vector. The results indicate that the HBS gene delivered by an AdV as a single dose can induced both CTL response and antibody response and that the B7–1 gene co-delivered in the same vector can enhance the CTL response to viral antigen-producing cells and antibody response to the antigen. In contrast, a single dose of the recombinant hepatitis B vaccine currently in use failed to induced any CTL response against HBsAg, although it induced a higher antibody titer than the recombinant AdVs.

Table 1. CTL activity of spleen cells from mice injected i.v. or i.p. with recombinant AdVs

Experiment	Weeks after injection	Route	Specific Release (%)* Ad1310	Specific Release (%)* Ad1312	Ad1310/ Ad1312
1†	2	i.v.	47 ± 7	16 ± 10	2.9
2	2	i.v.	18	3.5	5.1
3	3	i.v.	61	12	5.1
4‡	2	i.p.	20	14	1.4
5§	6	i.p.	13.4	6.6	2.0

*Effector/target = 100:1.
†Two mice from each group. Data shown are mean ± SD.
‡Pool of two spleens.
§Pool of three spleens.

HBsAg is a protein that is very efficiently secreted by cells. After translation, the HBsAg protein is exported from the cell in the form of 22-nm membrane particles. "Soluble" or unadjuvanted HBsAg particles given to BALB/c mice have been reported to undergo processing in a novel endosomal pathway for major histocompatibility complex class I-restricted epitope presentation and to induce a $CD8^+$ CTL response that was dose-independent in the dose range tested (38–41). When we infected mice with recombinant AdVs that directed expression of the HBS gene, HBsAg particles were synthesized and secreted by infected cells and could be detected in the blood. The secreted HBsAg particles can be taken up and processed by professional APCs, which are capable of providing a costimulatory signal resulting in the CTL response that we observed after inoculation of mice with Ad1312, which directed production of HBsAg alone. Since expression of the B7–1 gene appears to be confined to professional APCs *in vivo*, many cells infected with the vector Ad1312 (HBS alone) would not be expected to naturally express B7–1. However, B7–1 gene co-delivered with the HBS gene in the vector Ad1310 (HBS plus B7–1)-infected cells might supply the essential costimulatory signal on the surface of all cells expressing viral antigen gene (including cells that are not professional APCs) for T-cell activation. This hypothesis is supported by our results, which show that although higher level of serum HBsAg was detected in mice injected i.m. with Ad1312 than those injected with Ad1310, Ad1310 induced a stronger CTL response than Ad1312. Thus the enhancing effect of the B7–1 gene delivered with the viral antigen gene to all cells of a mouse infected with viral vector was clearly apparent above the CTL response induced by professional APC-presented HBsAg epitopes from secreted HBsAg particles.

Our results also demonstrate a moderate enhancing effect of B7–1 on the antibody response against HBsAg. The antibody response against HBsAg is known to be T-cell dependent (42). Therefore this enhancing effect on antibody response may be attributed to the activation of HBsAg-specific helper T cells by the costimulatory protein B7–1.

The experiments described here employed human B7–1 and demonstrated that it can exert an effect on CTL induction in the mouse. This is consistent with the findings of others that human B7–1 can provide a costimulatory signal for activation of murine T cells. We are now comparing human and murine B7–1 for their relative effects on the immune response to viral antigens in our system.

Since the CTL response and antibody response directed against viral antigen bearing cells are important in the prevention of infection and recovery from infection by many

viruses and we have shown that delivery of a costimulator gene along with a viral antigen gene results in stronger immune responses against the viral antigen, we plan to investigate this strategy for both therapy and prevention of viral infections.

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