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J Immunol (2005) 174 (1): 195–204.

<https://doi.org/10.4049/jimmunol.174.1.195>

Generation of Murine CTL by a Hepatitis B Virus-Specific Peptide and Evaluation of the Adjuvant Effect of Heat Shock Protein Glycoprotein 96 and Its Terminal Fragments¹

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Previously, we reported that a 7-mer HLA-A11-restricted peptide (YVNTNMG) of hepatitis B virus (HBV) core Ag (HBcAg₈₈₋₉₄) was associated with heat shock protein (HSP) gp96 in liver tissues of patients with HBV-induced hepatocellular carcinoma (HCC). This peptide is highly homologous to a human HLA-A11-restricted 9-mer peptide (YVNVNMGGLK) and to a mouse H-2-K^d-restricted 9-mer peptide (SYVNTNMGL). To further characterize its immunogenicity, BALB/c mice were vaccinated with the HBV 7-mer peptide. It was found that a specific CTL response was induced by the 7-mer peptide, although the response was ~50% of that induced by the mouse H-2-K^d-restricted 9-mer peptide, as detected by ELISPOT, tetramer, and ⁵¹Cr release assays. To evaluate the adjuvant effect of HSP gp96, mice were coimmunized with gp96 and the 9-mer peptide, and a significant adjuvant effect was observed with gp96. To further determine whether the immune effect of gp96 was dependent on peptide binding, the N- and C-terminal fragments of gp96, which are believed to contain the putative peptide-binding domain, were cloned and expressed in *Escherichia coli*. CTL assays indicated that only the N-terminal fragment, but not the C-terminal fragment, was able to produce the adjuvant effect. These results clearly demonstrated the potential of using gp96 or its N-terminal fragment as a possible adjuvant to augment CTL response against HBV infection and HCC. *The Journal of Immunology*, 2005, 174: 195–204.

Infection by hepatitis B virus (HBV)³ represents a significant medical problem. There are >350 million individuals infected with HBV globally, >120 million in China alone. HBV infection is a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). Evidence exists that a strong cytotoxic T cell response was capable of clearing the virus and that viral persistence in chronic HBV infection might be due to the failure of this response (1, 2). Several studies have suggested the involvement of heat shock protein (HSP) gp96 in the antigenic peptide presentation process (3–9). Previously, we isolated an HBV-specific 7-mer peptide (YVNTNMG) that was bound with gp96 from the liver tissue of HCC patients infected with HBV (10) using a three-step purification method (11). This peptide shares sequence homology with a naturally processed peptide Ag, P2 (YVNVNMGGLK), that was isolated from the HLA class I molecules on the cell membrane of HBV-infected liver (12). The P2 peptide exhibited activity in sensitizing target cells for lysis by CD8⁺ CTLs. The P2 sequence, similar to that of HBcAg₈₈₋₉₆, contained the HLA-A11 binding motifs (12). Moreover, the 7-mer

peptide epitope was also found in H-2d mice immunized with HBcAg₈₇₋₉₅ peptide (SYVNTNMGL) (13). Despite these findings, it is not clear whether this HBV-specific 7-mer peptide can elicit a specific CTL response.

Tumor-derived, gp96-rich cell lysates can elicit antitumor immune responses against a variety of cancers (14). This observation has prompted investigations into tumor-derived chaperones as potential immunotherapeutics for human cancers (15, 16). According to this hypothesis, gp96-peptide complexes are transported to the MHC class I Ag presentation pathway of APCs, which results in representation of the gp96-bound peptides on APC MHC class I molecules and, subsequently, activation of the tumor-directed CD8⁺ T lymphocytes (17, 18). However, under some circumstances, peptide-independent activities can also account for gp96-elicited tumor rejection (19, 20). After removing endotoxin contamination, gp96 was found to induce extracellular signal-regulated phosphorylation of kinases in macrophages, and the MAPK cascade has been implicated in the peptide-independent, antitumor response (21). Despite the difference between the two hypotheses, however, it is clear that gp96 can help stimulate the CTL response through either a direct or an indirect mechanism.

The immune effect of gp96 is probably dependent on its ability to bind peptide epitopes. It was reported that both the N- and C-terminal fragments of gp96 are able to bind peptides (22, 23), with the N-terminal fragment behaving at a similar capacity as the full-length gp96 (19). However, whether the terminal fragments can serve as an adjuvant as effectively as the full-length gp96 remains to be determined.

To analyze the immunogenicity of this small HBV-specific peptide and determine whether gp96 and its terminal fragments can enhance the peptide-specific CTL response, gp96 protein was isolated from murine liver tissue, and its terminal fragments were produced in *Escherichia coli* by recombinant DNA technology. BALB/c mice were vaccinated with either peptide alone or in

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Received for publication April 16, 2004. Accepted for publication October 19, 2004.

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¹ This work was supported by a grant from the National Frontier Research Program (Project 973) of the Ministry of Science and Technology of the People's Republic of China (Grant 2001cb510001).

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³ Abbreviations used in this paper: HBV, hepatitis B virus; FPLC, fast protein liquid chromatography; HBcAg, HBV core Ag; HCC, hepatocellular carcinoma; HSP, heat shock protein; β_2m , β_2 -microglobulin; SFC, spot-forming cell.

combination with gp96 or its terminal fragments. Murine CTL responses were then analyzed, and the adjuvant effects of gp96 and its terminal fragments were determined.

Materials and Methods

Peptide synthesis

The 7-mer peptide, YVNTNMG; the 9-mer peptide, SYVNTNMGL; HBcAg_{88–94}; HBcAg_{87–95}; and the non-K^d-restricted control peptide, FLPS-DFFPSV, were synthesized by Sigma-Aldrich (St. Louis, MO). The purity (>95%) of the peptides was confirmed by HPLC and mass spectrometry.

Refolding of the peptide with the murine K^d H and L chain

Refolding was performed as described by Ogg and Altman (24, 25). In brief, murine MHC H chain H-2K^d and β_2 -microglobulin (β_2m) were expressed in *E. coli* using a prokaryotic expression system (pET; R&D Systems) and were isolated from the inclusion bodies. A sequence containing the BirA enzymatic biotinylation site was added to the COOH terminus of the H chain protein. Both proteins and peptide were refolded by the dilution method (26).

Preparation of gp96

HSP gp96 was purified as described previously (10). Thirty healthy murine livers were homogenized until > 95% of the cells were lysed. After precipitation with ammonium sulfate (50–70%), the solubilized protein was applied to a Con A-Sepharose column (Pharmacia Biotech). The eluted material was applied to POROS 20QE column (4.6–100 mm; BD Biosystems) on an AKTA fast protein liquid chromatography (FPLC) system (Pharmacia Biotech), then eluted with 300–1000 mM NaCl. The high purity fractions were applied to a gel filtration Superdex G200 column (Pharmacia Biotech) for further purification. The identity of the gp96 protein was confirmed by Western blotting using anti-gp96 mAb (NeoMarkers). The quantities of gp96 were estimated by the protein concentration test kit (Bio-Rad). Additionally, all material for gp96 preparations was treated before use to remove possible contaminating endotoxin. All buffers were made in pyrogen-free water.

Plasmid construction and expression of the terminal fragments of gp96

The DNA encoding the N-terminal fragment N333 (22–355 aa) and the C-terminal fragment C190 (561–751 aa) of gp96 were cloned into the *Bam*HI and *Xho*I sites of the GST fusion expression vector pGEX-6p-1 (Pharmacia Biotech). The plasmids were verified by direct DNA sequencing. After induction, bacterial cells were lysed by sonication in PBS. The clarified supernatants were passed through a glutathione-Sepharose 4B column to purify the GST fusion protein. The fusion proteins were then cleaved by rhinovirus 3C protease, and the resultant protein mixture was applied to a gel filtration Superdex G200 column on an AKTA FPLC Workstation (27). SDS-PAGE and Western blotting analyses were used to confirm the identity of the protein. A *Limulus* amoebocyte lysate for endotoxin detection (Chinese Horseshoe Crab Reagent Manufactory) has been used in our laboratory. The endotoxin in all samples was <10 endotoxin units/ml.

Peptide binding assays

Assays of peptide binding to gp96 and its terminal fragments were routinely performed in 500 μ l of sodium phosphate buffer with 10–50 μ g of proteins and 1–100 μ M FITC-labeled peptide (Meilian). GST served as the control protein. The reactions were incubated at room temperature for up to 20 h. Gel filtration Superdex G200 was used to remove the free peptide and purify the HSP-peptide (FITC) complex. After collecting the mean peak of the gel filtration, fluorescence was measured by a fluorometric method (490 nm excitation/510 nm emission).

Immunization of mice and harvest of splenocytes

Female BALB/c mice (6–8 wk old) were immunized s.c. at multiple sites with either peptides alone or a mixture of peptides and BSA, gp96, or N333/C190. The injection volume was adjusted to 100–200 μ l. Spleens were removed 7 days after the last immunization. The cells were dispersed with a syringe plunger. The cell suspension was then filtered through cell strainers, and erythrocytes were lysed with 0.83% ammonium chloride lysis solution. Splenocytes were washed and resuspended. The splenocytes were stimulated with peptides for 7 days. CTL assay cells were cultured in RPMI 1640 medium containing 10% FCS, 2-ME, L-glutamine, and antibiotics.

ELISPOT assay

Ninety-six-well polyvinylidene difluoride-backed plates (Diacclone) were precoated with 15 μ g/ml anti-IFN- γ mAb overnight at 4°C. Plates were blocked for 1 h at 37°C. Purified splenocytes was dispensed at a predetermined density in duplicate wells. Peptide (10 μ M) was used to stimulate the cells. The plates were incubated at 37°C for 18–40 h. After washing, the plates were incubated for another 1.5 h at 37°C after addition of the second biotinylated anti-IFN- γ Ab. A 1/1000 dilution of streptavidin-alkaline phosphatase conjugate was added to the wells and incubated for 1 h, after which the chromogenic alkaline phosphatase substrate was added. After 30 min, the colorimetric reaction was terminated by washing with tap water. After drying, the spots were counted.

Tetramer preparation and flow cytometric analysis

The 45-kDa refolded protein was isolated by FPLC and biotinylated with the enzyme BirA. Streptavidin-PE conjugate (Sigma-Aldrich) was added in a 1:4 molar ratio, and the tetramer product was concentrated to 1 mg/ml. Peptide-specific splenocytes were incubated at 37°C for 30 min in staining buffer (PBS with 0.1% BSA and 0.1% sodium azide) containing 3 μ l of PE-labeled tetrameric complex. Cells were washed once in warm staining buffer, then incubated at 4°C in staining buffer containing saturating amounts of anti-CD8 $\alpha\beta$ mAb conjugated to FITC (BD Biosciences) and anti-CD3 mAb conjugated to PE-Cy5 (BD Biosciences). Samples were analyzed by FACS using CellQuest software (BD Biosciences). Lymphocytes were gated on CD8⁺, CD3⁺, and tetramer⁺ cells. More than 10⁵ events were acquired for each sample.

⁵¹Cr release cytotoxicity assays

P815 (mouse mastocytoma cell line) cells were seeded at 5000 cells/well in a 96-well, round-bottom plate and used as target cells. Target cells were incubated overnight at 37°C in RPMI 1640 medium supplemented with L-glutamine plus 10% heat-inactivated, pooled FCS loaded with Ag (10 μ g/ml peptide) and pulsed with ⁵¹Cr. Purified splenocytes were added as effector cells to the 96-well plate at various E:T cell ratios (100:1, 50:1, 25:1, and 12.5:1) in a final volume of 100 μ l/well and incubated at 37°C for 4 h. Supernatants (100 μ l) were harvested, and ⁵¹Cr release was measured. Spontaneous release was measured in wells containing target cells alone. Triton X-100 was used to lyse the target cells maximally. The percentage of specific lysis was calculated by the following formula: (experimental release cpm – spontaneous release cpm / maximum release cpm – spontaneous release cpm) \times 100 = percent of specific lysis.

Results

Preparation of HSP gp96 and its terminal fragments

In this study we purified HSP gp96 to homogeneity from healthy murine livers. Approximately 20–30 μ g of gp96 was obtained from each gram of liver tissue. The protein was first purified on a POROS 20QE column that eluted between 400 and 600 mM salt concentration (sodium chloride). Fractions containing gp96 were pooled and additionally purified on a gel filtration column (Fig. 1, A and D).

The immune effects of gp96 may depend on its ability to bind peptides (8, 17). It was reported that gp96 might contain a peptide-binding element within its N- and C-terminal domains (22, 23), but whether the peptide binding domains can generate immune effects is not known. To address this issue, two terminal fragments of gp96, N333 (22–355 aa) and C190 (561–751 aa), were cloned into pGEX-6p1 expression vector, expressed in bacteria, and purified on a gel filtration column after removing GST as described in *Materials and Methods* (Fig. 1, A–C). The purified proteins were analyzed by Coomassie Blue staining to be at least 90% pure (Fig. 1, D–F, lanes 2) and immunoblotted with an anti-gp96 mAb (Fig. 1, D–F, lanes 1). Only gp96 and N333, but not C190, were recognized by the mAb (compare lanes 1 in Fig. 1, D–F), indicating that the epitope sequence recognized by the Ab was located within the N-terminal region of the protein.

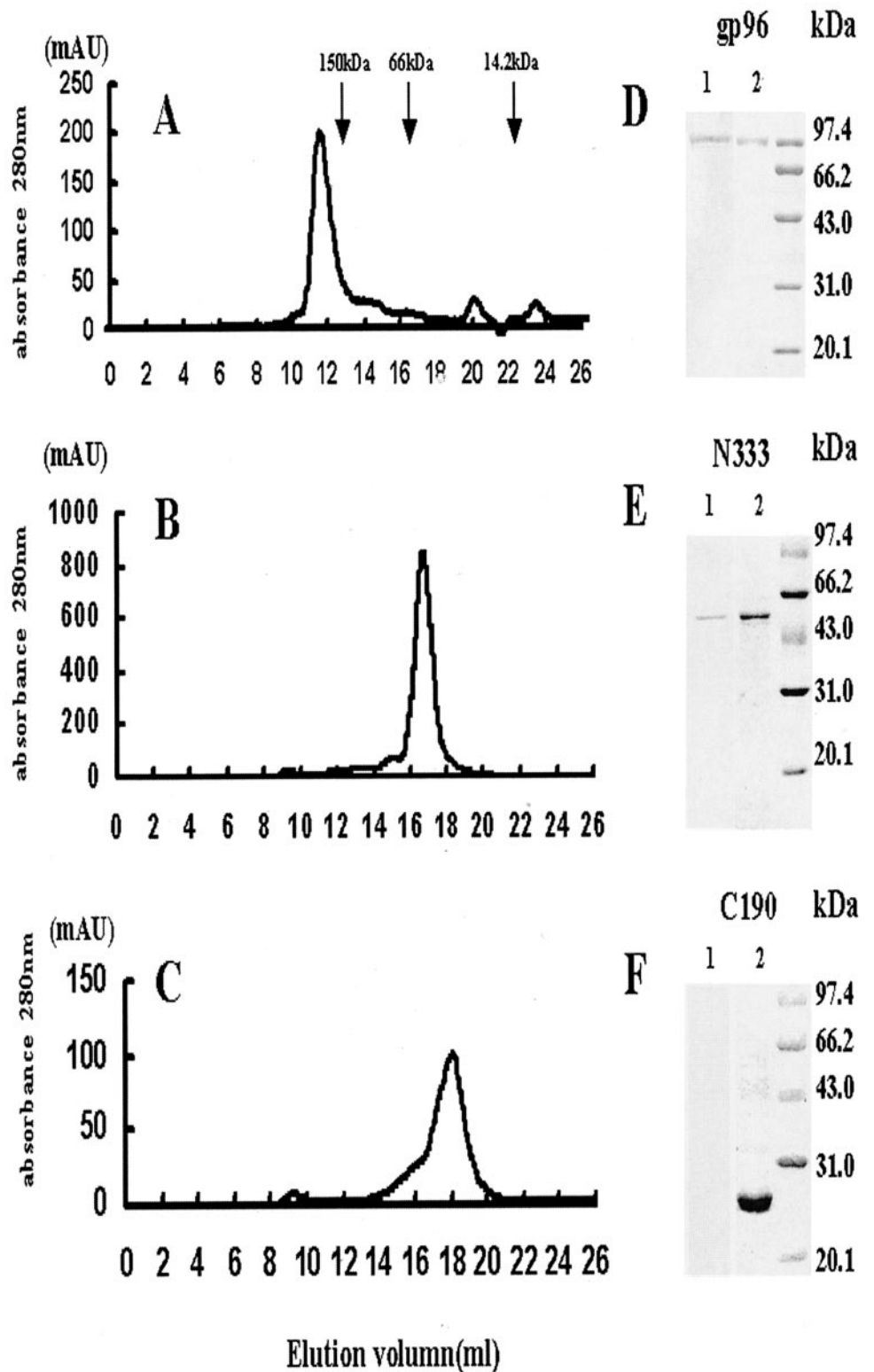


FIGURE 1. Gel filtration and SDS-PAGE analysis of gp96 and its terminal fragments N333 and C190. Purified gp96 from healthy murine liver tissue (A) and the bacterially expressed N-terminal fragment N333 (B) and C-terminal fragment C190 (C) were subjected to Superdex G200 gel filtration profile analysis. Peak fractions collected from the column were also analyzed on a 12% SDS-PAGE gel (D–F) and stained with Coomassie Blue (lanes 2) or immunoblotted with an anti-gp96 mAb (lanes 1).

Complex formation by refolding murine H-2K^d H chain and β₂m in the presence of the HBV-specific, 7-mer peptide or mouse H-2-K^d 9-mer peptides

Both HBV-specific, 7-mer peptide (YVNTNMG) and the mouse H-2-K^d-restricted, 9-mer peptide (SYVNTNMG) contain sequences resembling the anchor sites for the murine MHC class I molecule. However, peptides with putative MHC-restricted sequence motifs may not necessarily bind to the corresponding MHC

molecule or induce CTL (28). Before directly evaluating their T cell immunogenic potential, we first tested the binding capability of the peptides with class I MHC molecule in a refolding assay. The 7-mer, 9-mer, and a non-K^d-restricted control peptide (FLPS-DFFPSV) were refolded with the murine MHC H chain H-2K^d and β₂m at the molar ratio of 1:4.8:18 for peptide:MHC H chain:β₂m. Folding efficiency was determined by gel filtration analysis. As shown in Fig. 2A, binding of the HBV-specific 7-mer peptide to

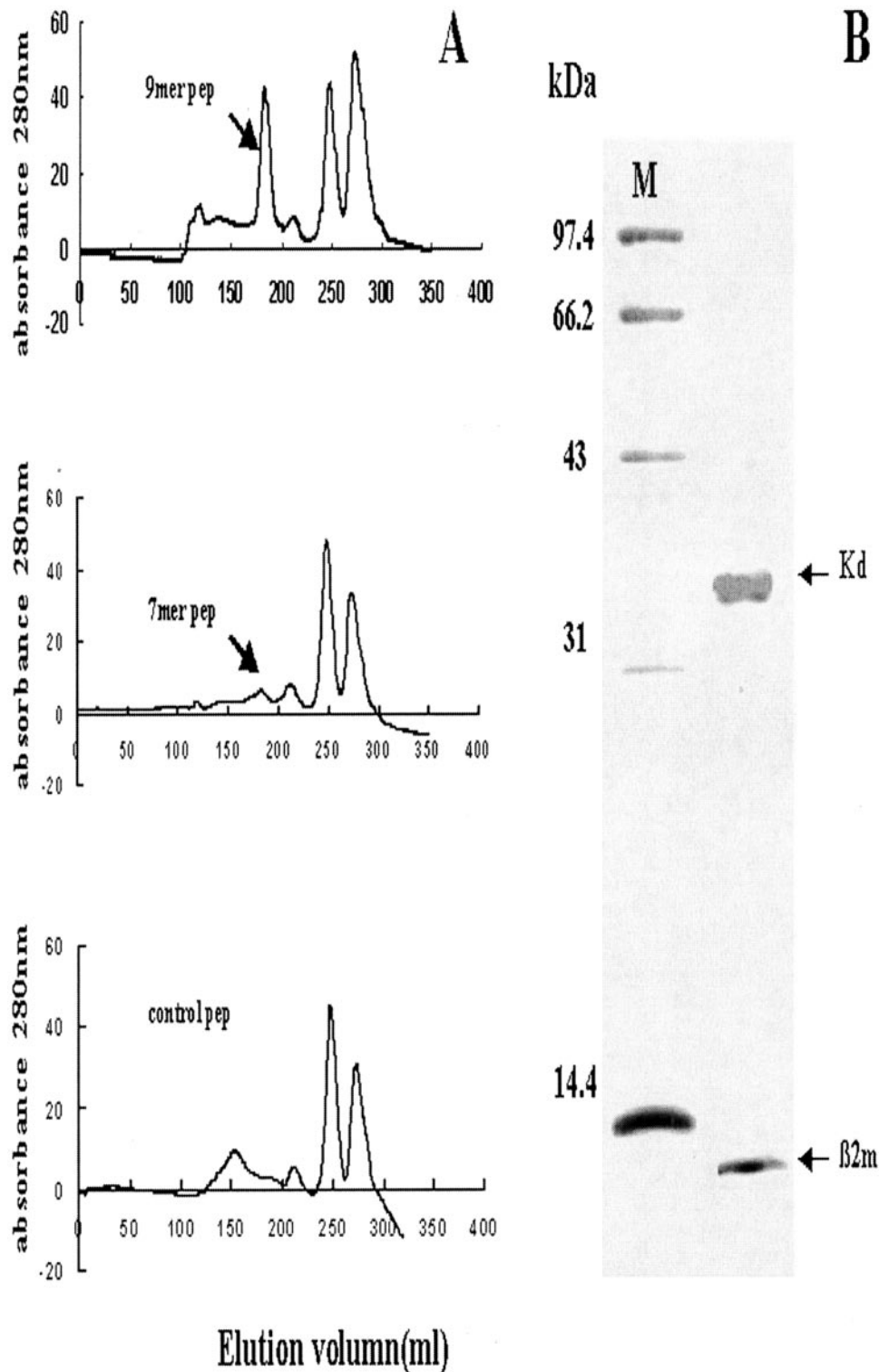


FIGURE 2. Refolding of murine H-2K^d H chain and β ₂m with the HBV-specific 7-mer peptide (YVNTNMG), murine H-2K^d-restricted 9-mer peptide (YVNVNMGK), or a non-K^d-restricted control peptide (FLPSDFF-PSV). **A**, The refolded complexes (pointed to by arrows), eluted with the expected M_r (45 kDa), were analyzed by gel filtration chromatography. **B**, A 15% SDS-PAGE gel was used to confirm the components of the refolded complex. The result showed the representative SDS-PAGE for both 9-mer and 7-mer MHC complexes.

the class I MHC molecule was detected, but was much weaker than that of mouse H-2-K^d 9-mer peptide, whereas no specific binding was observed for the control peptide. The MHC molecules specific for the 7-mer and 9-mer peptides were also confirmed by SDS-PAGE analysis (Fig. 2B). The refolding result indicated that these two peptides possess affinity for the murine MHC molecule, which prompted us to investigate whether they could stimulate a CTL response in mice.

Murine CTL response to the peptides, as detected by ELISPOT, tetramer, and ⁵¹Cr release assays

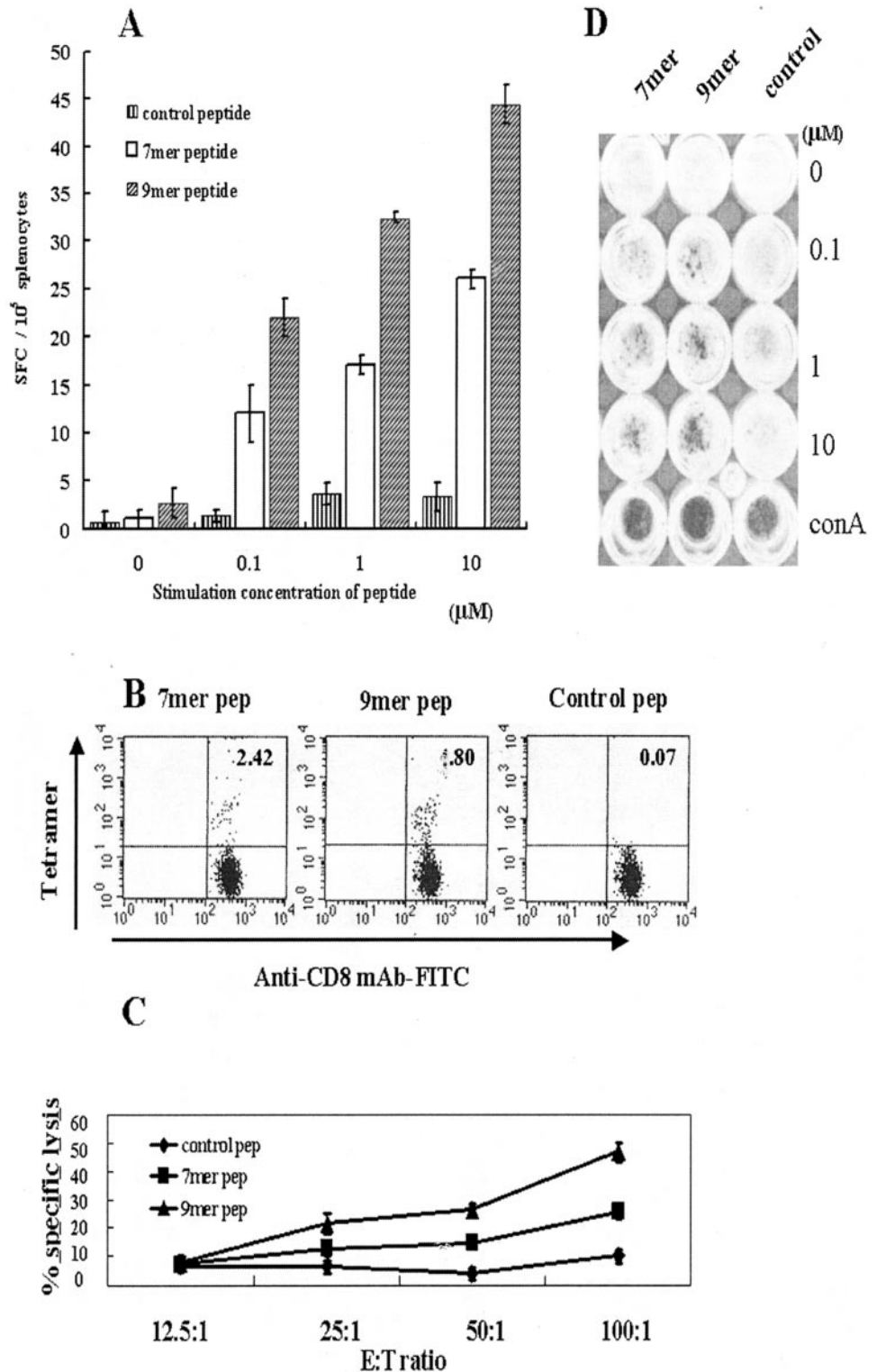
To determine whether these peptides can elicit a CTL response in vivo, female BALB/c mice (6–8 wk old) were immunized s.c. with 100 μ g of 7-mer, 9-mer, or the control peptide emulsified in IFA, and CTL responses were monitored weekly thereafter. Freshly isolated splenocytes from the immunized mice were assayed in an ex vivo ELISPOT assay. Mouse splenocytes isolated

after the first two rounds of peptide immunizations did not produce visible spots with all three peptides. However, CTL was detected after the third injection for the two specific peptides, but not the control peptide. To further demonstrate the specificity of the CTL response, increasing concentrations of peptides (0, 0.1, 1, and 10 μM) were used to stimulate the cells. Splenocytes from the mice immunized with the control peptide were used as the negative control, and splenocytes stimulated with Con A served as the positive control. As shown in Fig. 3A, the mean frequency of the CTL

response for the HBV 7-mer peptide was $\sim 50\%$ of that for the mouse H-2-K_d 9-mer peptide at all tested concentrations. The number of spot-forming cells (SFCs) increased for both peptides in a dose-dependent fashion. Minimal changes were observed for the control peptide. The results were representative of at least three independent experiments.

The HBV 7-mer peptide and the mouse 9-mer peptide were then used to generate tetramer complexes, H-2K_d/ $\beta_2\text{m}$ /7-mer and H-2K_d/ $\beta_2\text{m}$ /9-mer. Both tetramer complexes were able to stain

FIGURE 3. Detection of murine CTL using ELISPOT, tetramer, and ^{51}Cr release assays. **A**, BALB/c mice were immunized three times with 100 μg of the synthetic 7-mer, 9-mer, and the control peptides in IFA. Seven days after the last inoculation, splenocytes were isolated from the immunized mice and were tested for 40 h by ELISPOT assay in the presence of 0, 0.1, 1, and 10 μM of the respective peptides. Bars represent the mean number of SFC in 10^5 splenocytes, with the SDs indicated. **B**, Splenocytes were stained with K^d/ $\beta_2\text{m}$ /7-mer or K^d/ $\beta_2\text{m}$ /9-mer tetramer together with anti-CD8^{FITC} and anti-CD3^{PE-Cy5} Abs. The analyzed splenocytes were gated at CD3⁺, CD8⁺, and tetramer⁺ cells. The numbers shown represent the percentage of tetramer⁺ cells within CD8⁺CD3⁺ T lymphocytes. Control groups were stained with K^d/ $\beta_2\text{m}$ /9-mer tetramer. **C**, Splenocytes from the same mice were restimulated in vitro with 10 μM peptides. Five days later, a standard ^{51}Cr release assay was performed on P815 target cells pulsed with the same peptide. **D**, Photograph showing the representative wells in ELISPOT assay.



peptide-specific CTL from the stimulated mouse splenocytes. The production of H-2K^d/β₂m/7-mer tetramer complex is less than that of H-2K^d/β₂m/9-mer tetramer, probably due to the lower binding efficiency of the 7-mer peptide, as observed in the previous refolding assay. Splenocytes from mice immunized with the control peptide were also stained with H-2K^d/β₂m/9-mer tetramer. Fresh splenocytes from the three doses of peptide-immunized mice were stained with the respective tetramers, and the proportions of peptide-specific CD8⁺ T cells were determined. As shown in Fig. 3B, ~2.42% of the CD8⁺ T cells were specific for the HBV 7-mer peptide, compared with 4.80% for the 9-mer peptide. No specific staining was observed with the splenocytes isolated from mice immunized with the control peptide.

Furthermore, splenocytes from these immunized mice were restimulated *in vitro* with the corresponding peptides. A standard ⁵¹Cr release CTL assay was performed, 5 days poststimulation, on the murine K^d-restricted blast cell line P815 pulsed with the same peptide. Control cells were splenocytes from mice immunized with the nonspecific peptide. As shown in Fig. 3C, the peptide-specific CD8⁺ T cell was K^d-dependent, and the CTL produced was functional. CTL from the splenocytes restimulated with the 7-mer peptide resulted in ~23% cytolysis of the target cells when the E:T cell ratio was 100:1, whereas CTL from the splenocytes restimulated with the 9-mer peptide caused ~45% cytolysis at the same E:T cell ratio (Fig. 3C). The results from the three independent CTL assays were highly consistent, showing that the HBV-specific peptide was immunogenic and produced a functional CTL response. CTL specific for the 7-mer peptide was ~50% of that induced by the 9-mer peptide, probably due to the loss of an anchor site at its C terminus for binding with the MHC molecule or increased sensitivity to proteolysis.

Adjuvant effects of gp96 and its terminal fragments

It has been reported that HSP-peptide complexes reconstituted *in vitro* could elicit a peptide-specific CTL response (29). Therefore, we performed the peptide-binding assay according to the methods described before with some alteration (29). The results showed that both 7-mer and 9-mer FITC-conjugated peptides could bind to either gp96 or its terminal fragments. The control protein GST had no binding ability (data not shown). We used the purified HSP-peptide complex to immunize the mice. The results were negative. Neither the gp96-7/9-mer peptide complex nor its terminal fragment-7/9-mer peptide could elicit obvious peptide-specific CTL in mice no matter how large the amount of the complex used (0–50 μg). The lack of reconstituted HSP-peptide complex to prime the CTLs in mice might be because the amount of HSP-bound peptide is too low for cross-presentation in this study. Sufficient cross-presentation may only occur with highly abundant peptides. It was reported that mixtures of HSP and peptide could also result in a specific CTL response and functional immunity (30). To investigate whether gp96 could enhance peptide-specific CTL response as soluble protein, BALB/c mice were coimmunized with the purified murine gp96 or its terminal fragment N333 or C190 and the 9-mer peptide at a suboptimal dose (10 μg) using gp96, N333, and C190 alone as controls. As shown in Fig. 4A, a dose-dependent adjuvant effect was observed for gp96 in the ELISPOT assay, with the peak activity at 10 μg, but gp96 alone had no CTL response. Higher amounts (50 μg) of gp96 were detrimental to the adjuvant effect, presumably due to the fact that excess gp96 might be immunosuppressive and decreased the capability of mice to generate an immune T cell response, which was also consistent with a previous report (31). BSA, as a negative control, was unable to provide any adjuvant effect. The difference between gp96 and BSA

was statistically significant ($p < 0.05$). For the terminal fragments, N333 showed a clear adjuvant effect when used in combination with the 9-mer peptide to immunize the mice, but C190 did not (Fig. 4B). This effect was dose dependent up to the highest amount tested (50 μg). The fact that N333 was not as effective as full-length gp96 in enhancing the CTL response, might be due to 1) the reduced binding affinity for peptide or less efficiency in stimulating the innate immune system due to less optimal conformation, and/or 2) the loss of one or more peptide binding sites. Nonetheless, this result indicated that the N-terminal domain of HSP gp96 was important for the observed adjuvant activity.

Splenocytes from the immunized mice were further analyzed in a tetramer staining assay (Fig. 4C). Consistent with the ELISPOT assay, the highest percentage of CD8⁺ T cells (4.95%) specific for the peptide was observed when 10 μg of gp96 was used. In the case of N333, the percentage of peptide-specific CD8⁺ T cells continued to rise with increasing amounts of protein (2.29% at 50 μg of N333; Fig. 4C). Again, this result confirmed the adjuvant effects of gp96 and, to a lesser extent, its N-terminal fragment in eliciting a peptide-specific CTL response.

Interestingly, when we performed the same experiments with the HBV 7-mer peptide, no significant adjuvant effect was observed for gp96 or N333 (data not shown). It is conceivable that this lack of immune effect was due to a reduced affinity in binding between gp96 and the 7-mer peptide, and this hypothesis is currently being investigated in our laboratory.

Correlation between CTL response and peptide concentrations

Peptide-specific CTL responses in splenocytes isolated from mice immunized with different amounts of peptide (0, 0.4, 4, 10, and 50 μg) and a constant amount (10 μg) of the adjuvant proteins were compared. As shown in Fig. 5, increasing amounts of the immunizing peptide resulted in dose-dependent increases in the CTL response when gp96 or N333 was used as the adjuvant in an ELISPOT assay (Fig. 5A) as well as in tetramer staining assay (Fig. 5B), indicating that their adjuvant effects were peptide concentration dependent. No significant CTL response was observed when BSA or the C-terminal fragment (C190) was used as the adjuvant protein. Taken collectively, these results demonstrated that HSP gp96 and its N-terminal fragment could serve as potential adjuvants to enhance the peptide-specific CTL response in mice.

Discussion

We previously reported the isolation of a gp96-bound, HBV-specific, HLA-A11-restricted 7-mer peptide (YVNTNMG) from liver tissues of HCC patients infected with HBV (10, 11). After refolding with the mouse K^d H and L chains, the peptide was able to bind with murine MHC class I molecules. MHC class I molecules, possessing high degrees of polymorphism as the result of the evolutionary process, are able to present various antigenic determinants to host T cells for induction of host immune responses to combat the invading foreign pathogens, such as viruses (32). The epitopes recognized by MHC molecules are believed to be between eight and 12 aa. However, whether a shorter virus-specific peptide can associate with the MHC molecule and elicit a specific immune response has not been reported to the best of our knowledge. In this report we demonstrated that the 7-mer HBV-specific peptide was indeed immunogenic in mice and produced peptide-specific CTL. The magnitude of the specific CTL response for the 7-mer peptide was approximately half that for its homologous 9-mer peptide. In addition, we demonstrated the adjuvant effects of gp96 and its N-terminal fragment for eliciting a peptide-specific CTL response. This study for the first time demonstrated the antigenic property of

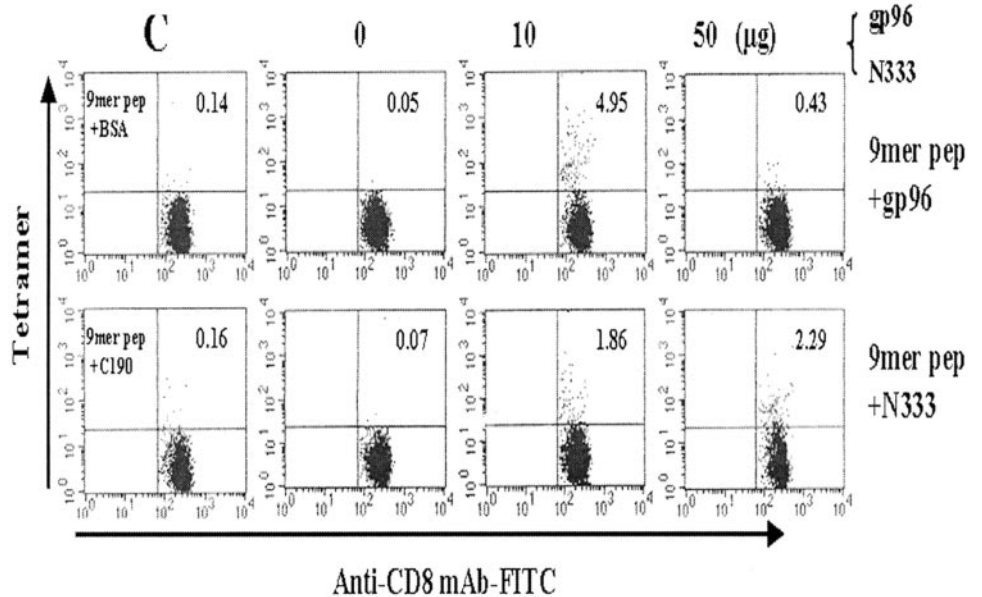
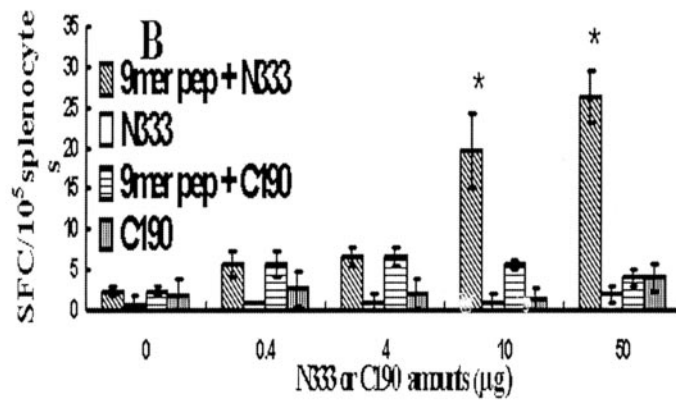
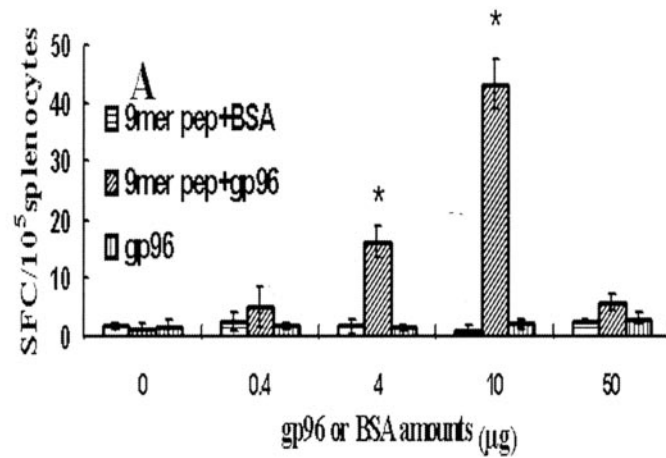


FIGURE 4. Murine CTL response to the 9-mer peptide in the presence of different amounts of gp96 and its terminal fragments as adjuvants. Four groups of mice were immunized twice a week with 10 μg of the 9-mer peptide and different amounts (0, 0.4, 4, 10, and 50 μg) of BSA, gp96, N333, or C190, respectively. Splenocytes were obtained 7 days after the last immunization. *A* and *B*, ELISPOT assay. Splenocytes at 10^5 cells/well were tested in the presence of 10 μM peptide. Bars represent the mean number of SFC from three independent experiments. *, Statistically significant difference ($p < 0.05$). *C*, Tetramer staining. Splenocytes isolated from the immunized mice were stained with K^d/ $\beta_2\text{m}$ /9-mer tetramer along with anti-CD8^{FITC} and anti-CD3^{PE-Cy5} Abs. The analyzed splenocytes were gated at CD3⁺, CD8⁺, and tetramer⁺ cells. Numbers indicate the percentage of tetramer⁺ cells within the CD8⁺ and CD3⁺ T lymphocytes.

the gp96-derived, HBV-specific 7-mer peptide in binding with murine MHC class I molecules to induce specific CTL in vivo.

Because gp96 can bind with the entire repertoire of antigenic peptides generated in the cell, it is conceivable that, besides the 7-mer peptide, gp96 may also bind with homologous, but larger, peptides or precursor molecules. Gp96 has been reported to associate not only with the final antigenic peptides (i.e., the epitopes), but also with their precursors (33). Although the functional implications for this type of binding are still unclear, it is unlikely to be a random association. The 9-mer and 7-mer HBV peptides bound

to gp96 and terminal fragments of gp96 might explain the mechanism of the adjuvant effects. The results showed that both 7-mer and 9-mer FITC-conjugated peptides could bind to either gp96 or its terminal fragments. The association of gp96 with MHC ligands may indicate its role in MHC class I peptide processing and presentation. Additional investigations are warranted to understand the detailed mechanisms involved in the interaction among gp96, MHC molecules, and peptide epitopes.

Gp96, as one of the most abundant intracellular HSPs, possesses multiple functions. Among these functions, its ability to

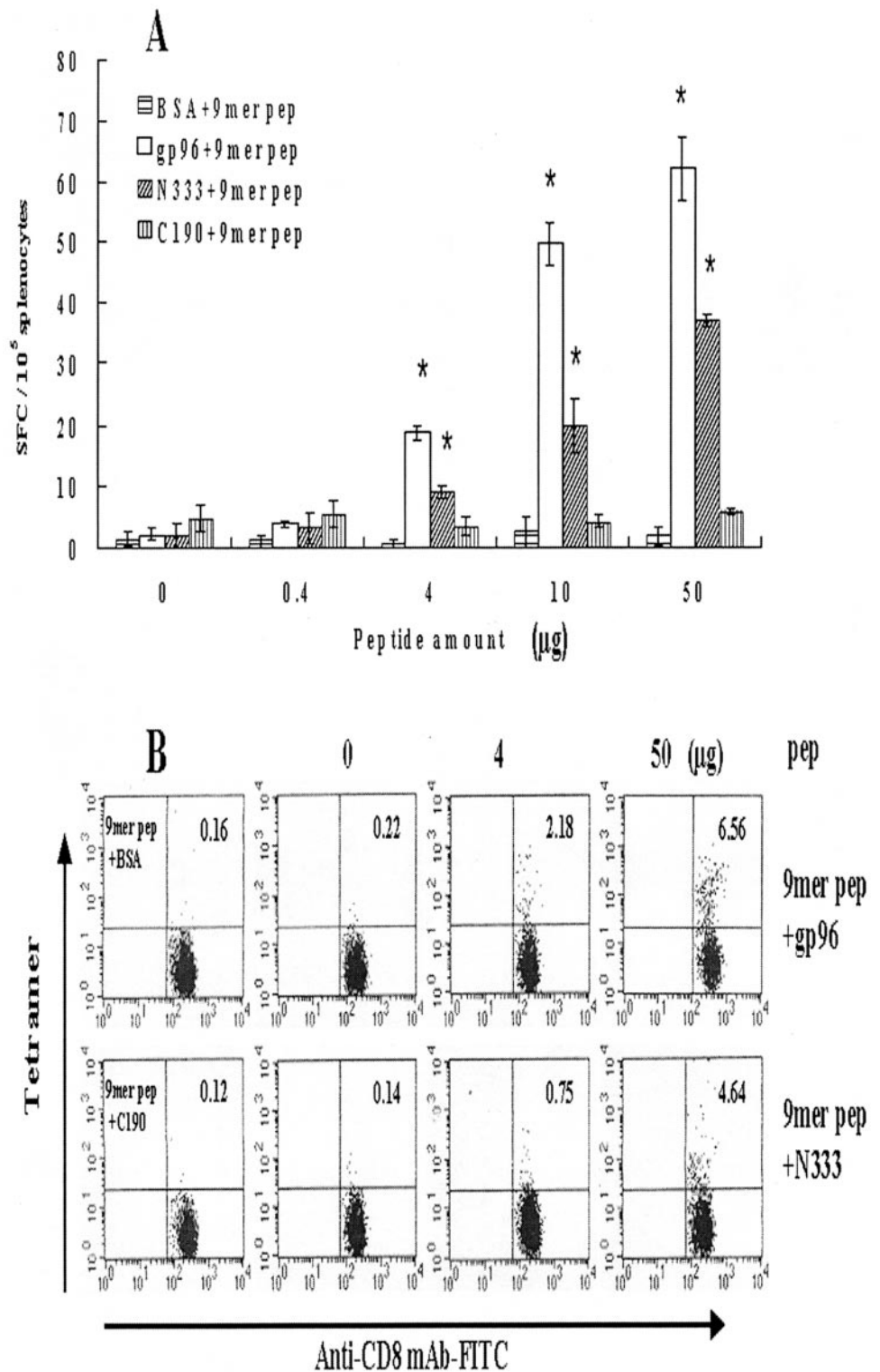


FIGURE 5. Correlation of CTL response with increasing amounts of peptide doses. Four groups of mice were immunized twice a week with 10 µg of BSA, gp96, N333, or C190, with increasing amounts (0, 0.4, 4, 10, and 50 µg) of the 9-mer peptide. Splenocytes were obtained 7 days after the last immunization. *A*, ELISPOT assay. Splenocytes at 10^5 cells/well were tested in the presence of 10 µM peptide. Bars represent the mean number of SFC from three independent experiments. *, Statistically significant difference ($p < 0.05$). *B*, Tetramer staining. Splenocytes from immunized mice were stained with K^d/β₂m/9-mer tetramer along with anti-CD8^{FITC} and anti-CD3^{PE-Cy5} Abs. The analyzed splenocytes were gated at CD3⁺, CD8⁺, and tetramer⁺ cells. Numbers indicate the percentage of tetramer⁺ cells within CD8⁺ and CD3⁺ T lymphocytes.

bridge the innate and adaptive immune systems has attracted extensive interest (34–38). HSPs are involved in eliciting a potent, specific, cellular adaptive immune response, which was suggested to be dependent on their ability to chaperone a large variety of peptides. Immunization of mice with tumor-derived HSP generates protective immunity against subsequent tumor challenges. Immunotherapies using HSPs to generate specific antitumor responses have been evaluated in clinical studies (16, 39–41). In this report the gp96–7/9-mer peptide complex and its terminal fragment–7/9-mer peptide complex could not elicit

obvious peptide-specific CTL in mice, consistent with recent observations in vitro (42). However, when mixed with different amounts of peptides directly, gp96 and its N-terminal fragment (N333) showed adjuvant effects in enhancing the peptide-specific CTL response, and this response was peptide concentration dependent. One explanation is that the increased amount of peptide may form more HSP-peptide complex to obtain sufficient cross-presentation. Additionally, HSPs might induce an innate immune response to produce an immunological environment optimal for the adequate peptide to be cross-presented (19). The

findings implied that the N-terminal part of gp96 might contain the very motif to stimulate the innate immune system. However, additional work is necessary to completely rule out the possibility that proteins copurified with gp96 may contribute to its immunological effect, as suggested by several recent publications (42, 43).

Ideal therapeutic vaccines for infectious diseases and cancer should elicit not only the humoral response, but also the cellular response. An optimized peptide epitope specific for the MHC class I molecules can potentially boost the Ag-specific cytotoxic T cell response. However, without suitable adjuvants, a small synthetic peptide can elicit only a weak CTL response (44). At the present time, alum is the only adjuvant suitable for clinical applications. However, alum cannot efficiently switch on the innate responses and is thus unable to direct the adaptive immune responses. HSP70, another member of the HSP family, has been found to promote immunogenic APC function, elicit a strong CTL response, and prevent the induction of tolerance (45–49). Our results together with these previous findings point to the potential of using HSPs as a T cell adjuvant to induce CTLs targeting viral pathogens or cancer cells.

Acknowledgments

We are grateful to Drs. Weidong Zhong and Robert Tam (Valeant Pharmaceuticals International, Costa Mesa, CA) for helping prepare the manuscript, to Dr. Songdong Meng for critical reading of the manuscript, to Fulian Liao for technical assistance, to Prof. Pramod K. Srivastava for providing the mouse gp96 clone, to Prof. Wei-feng Chen (Peking University) and Prof. Xue-tao Cao (Second Military Medical University) for help with various stages of the project, and to Weihua Zhuang for graphic preparation.

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