tiate acute infection or 2×10^6 PFU of the mutant strain of LCMV Arm, clone 13 (Cl.13), intravenously to initiate chronic infection (45). The virus titers were determined by plaque assay on Vero cells, as described elsewhere (2). Vaccinia virus recombinant VVGP33 (30), which expresses the LCMV GP33-41 epitope, was provided by J. Lindsay Whitton (Scripps Research Institute, La Jolla, CA).

Reagents. Synthetic CpG ODN (5002: TCCATGACGTTCTTGATGTT) was purchased from Hokkaido System Science (Sapporo, Japan) and was protected with phosphorothioate to avoid nuclease-dependent degradation. The LCMV peptides used were GP33-41 (KAVYNFATC, *H-2D^b^*), NP396-404 (FQPQNG QFI, *H-2D^b^*), GP283-291 (GYCLTKWMI, *H-2K^d^*), GP61-80 (GLKGPDIYKG VYQFKSVEFD, *I-A^b^*), and NP309-328 (SGEGWPYIACRTSIVGRAWE, *I-A^b^*). They were synthesized by Operon Biotechnology (Tokyo, Japan) by using 9-fluorenylmethoxy carbonyl chemistry. The peptides were desalted and then analyzed by high-performance liquid chromatography. Mouse fibrosarcoma cell line MC57G (*H-2^b^*) was obtained from ATCC and was maintained in Eagle's minimal essential medium containing 10% fetal calf serum (FCS) until use.

Coupling of peptides to liposomes. The oleoyl liposomes consisted of dioleoyl phosphatidylcholine, dioleoyl phosphatidylethanolamine, dioleoyl phosphatidylglycerol acid, and cholesterol in a 4:3:2:7 molar ratio (29). Each peptide was coupled to the surfaces of the liposomes by the use of disuccinimidyl suberate, as described previously (42). The final preparations contained 0.7 mg of the peptide and 10 mg of the liposome per ml. Liposomes conjugated with the GP33-41 peptide and NP396 (Lip-GP33/NP396), Lip-GP33/GP61, and Lip-GP33/NP309 were made by mixing peptide GP33-41 with peptide NP396-404, peptide GP61-80, and peptide NP309-328, respectively, in equal amounts before conjugation.

Immunization. The mice received the liposomal peptides at the indicated amounts together with 5 μg of CpG in 100 μl of phosphate-buffered saline (PBS) by one of the three different routes: subcutaneously (s.c.), intramuscularly (i.m.), or i.p. The i.n. immunization was performed by introducing 20 μl of a liposome solution (14 μg of peptide) and 5 μg of CpG (5 μl) into the nasal cavity. Mice that had recovered from an acute infection of LCMV Arm served as positive controls.

^{51}Cr-release assay. Spleen cells were prepared 7 days after immunization to obtain effector cells. The cytotoxic activities of the GP33-specific CTLs were measured by standard 4-h ^{51}Cr-release assays. For peptide-pulsed target cells, 1×10^6 MC57G cells were pulsed with 10 μM of peptide GP33 for 2 h and then labeled with 100 μCi of $Na_2{}^{51}CrO_4$ for 1 h. For virus-infected targets, the MC57G cells were infected with VVGP33 or wild-type vaccinia virus at a multiplicity of infection of 5. After 1 h of infection, the cells were washed, resuspended in medium, and incubated overnight. The infected targets were then labeled with ^{51}Cr, as described above. The labeled target cells were plated in wells of a round-bottom 96-well plate at 2×10^3 cells/well with effector cells at various effector-cell-to-target-cell (E/T) ratios. Blocking by antibodies was performed by adding anti-CD4 or anti-CD8 (5 μg/ml; eBioscience, San Diego, CA) to the mixture. Percent lysis was calculated as $[(cpm_{sample} - cpm_{spontaneous})/(cpm_{maximum} - cpm_{spontaneous})] \times 100$. The maximum amount of ^{51}Cr released was determined with the supernatants of cells that were lysed by addition of 2% Nonidet P-40. The spontaneous release of ^{51}Cr from target cells incubated without effector cells was measured in the supernatants. Percent specific lysis was calculated by subtracting the percent lysis with unpulsed targets from that with peptide-pulsed targets.

In vivo CTL assay. The in vivo CTL assay was carried out as reported elsewhere (42). Briefly, spleen cells from naive C57BL/6 mice were split into two equal populations. One population was pulsed with a peptide at a final concentration of 10 μM for 1 h at 37°C and then labeled with a high concentration (2.5 μM) of carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) for 10 min at 37°C. The other was unpulsed and labeled with a lower concentration (0.25 μM) of CFSE. An equal number of cells (1×10^7) from each population was mixed and transferred into immunized mice intravenously. Twelve hours later, spleen cells were prepared and analyzed by flow cytometry. Percent specific lysis was calculated as $1 - [$(number of $CFSE^{low}$ cells in healthy mice/number of $CFSE^{high}$ cells in healthy mice)/(number of $CFSE^{low}$ cells in immunized mice/number of $CFSE^{high}$ cells in immunized mice)$] \times 100$, where $CFSE^{low}$ and $CFSE^{high}$ represent low and high CFSE concentrations, respectively.

IFN-γ ELISPOT assay. The detection of gamma interferon (IFN-γ)-secreting cells was performed by using a mouse IFN-γ enzyme-linked immunospot (ELISPOT) assay set (BD Biosciences–Pharmingen, San Diego, CA). Each well of a sterile 96-well ImmunoSpot ELISPOT assay plate (BD Biosciences–Pharmingen) was precoated with 0.5 μg of unlabeled anti-IFN-γ capture antibody (clone R4-6A2; BD Biosciences–Pharmingen) per well at 4°C overnight. The plates were washed and then blocked with RPMI 1640 containing 10% FCS for 2 h at room temperature. Spleen cells from individual immunized mice were prepared, and red blood cells were removed by treatment with ammonium chloride. After the blocking solution was discarded, effector spleen cells were added to the wells of the plates at two cell densities (10^5 and 10^6 cells/well), along with 10^6 gamma-irradiated (40 Gy) syngeneic spleen cells. The cells were incubated in the presence or the absence of an appropriate peptide at a final concentration of 10 μM at 37°C for 2 days. The cells were then removed by five washes with PBS containing 0.05% Tween 20, followed by the addition of biotinylated anti-mouse IFN-γ detection antibody (clone SMG1.2; BD Biosciences–Pharmingen) at a concentration of 0.5 μg/well. After a 2-h incubation at room temperature, the detection antibody was removed by three washes with PBS containing 0.05% Tween 20. Avidin-horseradish peroxidase was added to the wells. After the unbound avidin-horseradish peroxidase was washed, the spots were developed by using freshly prepared substrate buffer (0.3 mg of 3-amino-9-ethylcarbazole/ml and 0.015% H_2O_2 in 0.1 M sodium acetate).

Intracellular IFN-γ staining. Intracellular cytokine staining was performed as described previously (26). Briefly, the spleen cells of four mice per group were pooled and resuspended in RPMI 1640 containing 10% FCS. In each well of a 96-well round-bottom plate, 2×10^6 spleen cells were incubated with 1×10^5 cells of syngeneic spleen cells which had been pulsed with an appropriate peptide for 3 h in the presence of 0.2 μl/well brefeldin A (GolgiPlug; BD Biosciences) for 5 h at 37°C. The cells were then washed once and incubated for 10 min at 4°C with a rat anti-mouse CD16/CD32 monoclonal antibody (MAb; Fc Block; BD Biosciences) at a concentration of 1 μg/well. Following incubation, the cells were stained with a fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD8α MAb (clone 53-6.7; BD Biosciences) at a concentration of 0.5 μg/well for 30 min at 4°C. After the cells were washed twice, they were fixed and permeabilized by using a Cytofix/Cytoperm kit (BD Biosciences) and stained with a phycoerythrin (PE)-conjugated rat anti-mouse IFN-γ MAb (clone XMG1.2; BD Biosciences). After the cells were washed, flow cytometric analyses were performed with a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ), and the data were analyzed with CellQuest software (Becton Dickinson). The experiment was repeated three times.

Tetramer and CD62L staining. Spleen cells were prepared and treated with an anti-mouse CD16/CD32 MAb as described above for intracellular IFN-γ staining and were then stained with a PE-conjugated GP33 *H-2D^b^* tetramer (Medical and Biological Laboratories, Nagoya, Japan), peridinin chlorophyll protein-conjugated anti-CD8a (BD Bioscience), and FITC-conjugated anti-CD62L (BD Bioscience) for 30 min at 4°C. After the cells were washed twice, they were fixed with PBS containing 1% formaldehyde and 2% FCS and analyzed by flow cytometry.

Statistical analysis. Statistical analyses were performed by Student's *t* test. A P value of <0.05 was considered statistically significant.

RESULTS

Induction of LCMV-specific CD8$^+$ T-cell response in mice immunized with liposome-coupled GP33 peptide. Ag chemically coupled to the surfaces of liposomes composed of unsaturated fatty acids has been shown to be presented to CD8$^+$ T cells (42) and to induce protective immunity against influenza virus (27). For further evaluation of the use of surface-linked liposomal peptide as an antiviral vaccine, we chose an immunodominant CTL epitope, GP33-41, of LCMV, which is presented by the *H-2D^b^* molecule (10, 16, 36). *H-2K^d^*-restricted epitope GP283-292 (10) is not presented in C57BL/6 mice (44) and served as the negative control in our experiments. We conjugated liposomes with GP33-41 peptide (Lip-GP33) or GP283-292 (Lip-GP283) and immunized C57BL/6 mice by one of three different routes (s.c., i.m., or i.p.) together with CpG. The immune responses were evaluated by ^{51}Cr-release assays and ELISPOT assays. As shown in Fig. 1A and B, single i.m. and s.c. immunizations induced substantial levels of CTL activity and IFN-γ-producing cells, whereas i.p. immunizations induced much lower responses, and the unconjugated peptide induced no detectable response in either assay (see Fig. 6 for data only for the ELISPOT assay). In the absence of CpG, the responses were very low (data not shown), in accordance with the findings presented in a previous report (27). Therefore, in

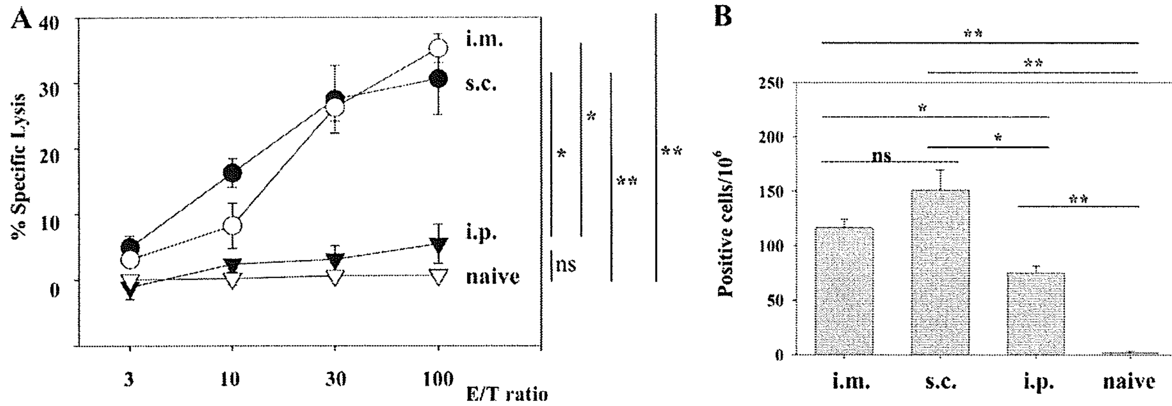

FIG. 1. Comparison of three different routes of immunization with Lip-GP33. Each C57BL/6 mouse received 20 μl of Lip-GP33 diluted in 100 μl of PBS containing 5 μg of CpG. Spleen cells were prepared 7 days after immunization for the ^{51}Cr-release assays (A) and ELISPOT assays (B). (A) ^{51}Cr-release assays were performed to detect GP33-specific CTL activity at various E/T ratios by using MC57G cells pulsed with peptide GP33 as targets. (B) IFN-γ-producing cells responding to peptide GP33 were detected by ELISPOT assays, as described in Materials and Methods. Spleen cells from nonimmunized (naive) mice were used as a negative control. The data are representative of those from two independent and reproducible experiments. The results are shown as the means of four mice per group ± standard errors of the means. *, $P < 0.05$; **, $P < 0.01$; ns, not statistically significant.

the following experiments, the immunizations with CpG were done mainly by the i.m. route. We confirmed the specificities of the reactions observed in the experiments described above as follows. Intracellular IFN-γ staining experiments revealed the peptide-specific IFN-γ production by CD8$^+$ T cells (Fig. 2A). ^{51}Cr-release assays with target cells expressing the GP33 epitope by infection with recombinant vaccinia virus VVGP33 demonstrated that the effector CTLs induced by the GP33-liposome conjugates recognized the epitope, which was endogenously processed and presented by major histocompatibility complex class I molecules (Fig. 2B). Blocking experiments with anti-CD4 and anti-CD8 antibodies demonstrated that the CTL activities were exerted by CD8$^+$ T cells (Fig. 2B). The CTL activities induced by the liposome conjugate were also confirmed by in vivo CTL assays (see Fig. 4B).

Experimental challenge of immunized mice with LCMV. To examine the protective efficacy of Lip-GP33, the immunized mice were challenged with LCMV Arm. Four days after the

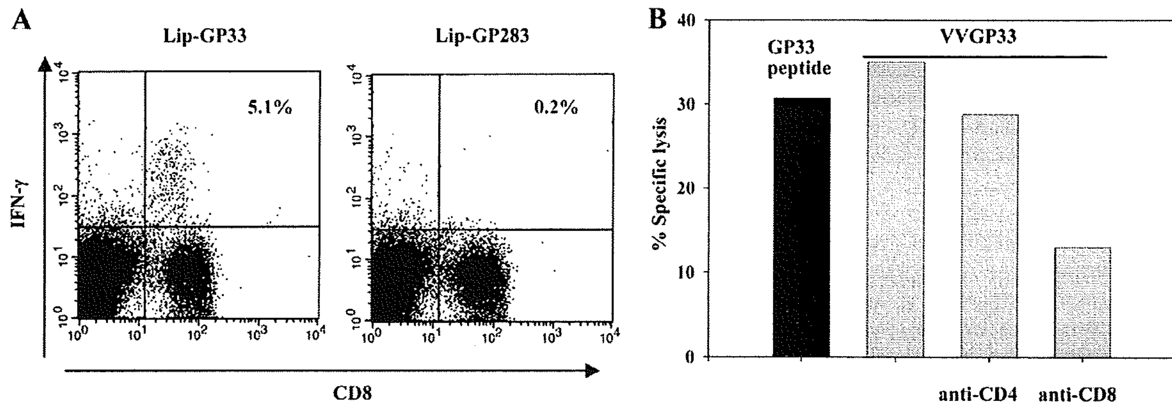

FIG. 2. Confirmation of GP33-specific CD8$^+$ T-cell responses of immunized mice. C57BL/6 mice were injected i.m. with 20 μl of Lip-GP33 or Lip-GP283 diluted in 100 μl of PBS containing 5 μg of CpG, and spleen cells were prepared 7 days later for analysis. (A) The numbers of IFN-γ-producing CD8$^+$ T cells specific to GP33 were determined by intracellular IFN-γ staining. Spleen cells from mice immunized with Lip-GP33 or Lip-GP283 were stimulated with either GP33-pulsed or unpulsed syngeneic spleen cells for 5 h and were then stained to detect their surface expression of CD8 (*x* axis) with FITC-conjugated MAb and their intracellular expression of IFN-γ (*y* axis) with PE-conjugated MAb. All lymphocytes were gated and analyzed on a FACScan flow cytometer by the use of CellQuest software (BD Biosciences). The values shown in the upper right quadrants indicate the percentage of CD8$^+$ cells that are positive for intracellular IFN-γ after stimulation with GP33-pulsed spleen cells. The results of stimulation with unpulsed spleen cells were almost zero (data not shown). Each experiment used four mice per group, and the spleen cells of the mice in each group were pooled. The data shown are representative of those from three independent and reproducible experiments. (B) ^{51}Cr-release assays with MC57G cells infected with VVGP33 as targets were performed to detect CTL activity against the endogenously expressed GP33 epitope (gray bars). As control targets, MC57G cells were infected with wild-type vaccinia virus. The same effector cells were used as GP33 peptide-pulsed and unpulsed targets as well for comparison (black bar). Lysis was also tested in the presence of an anti-CD4 or an anti-CD8 MAb (5 μg/ml) in the CTL assay. Specific lysis is demonstrated at an E/T ratio of 50. Each experiment used three mice, and the spleen cells of the three mice were pooled. The data are representative of those from two independent and reproducible experiments.

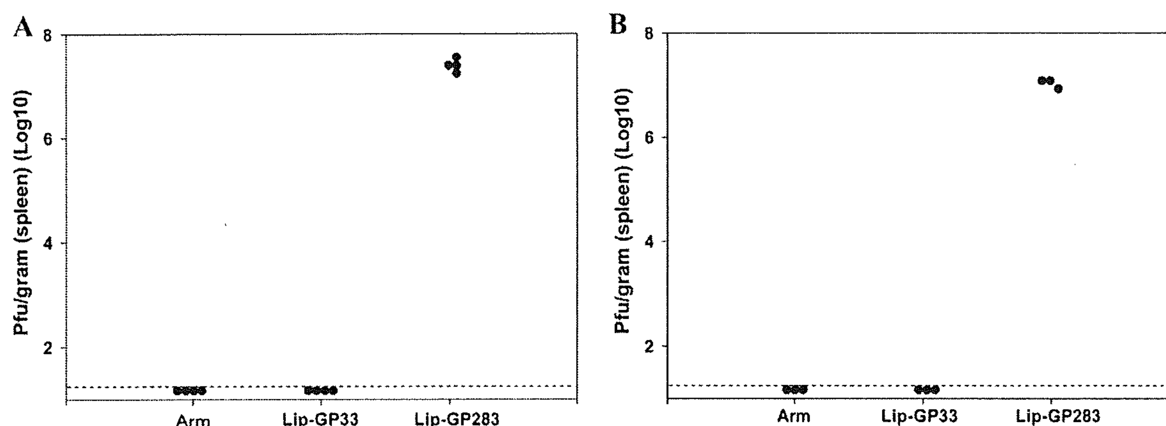

FIG. 3. Challenge experiments with vaccinated and control mice. C57BL/6 mice were immunized by injecting Lip-GP33 or Lip-GP283, as described in the legends to Fig. 1 and 2, or by i.p. inoculation of 2×10^5 PFU of LCMV Arm. After 2 weeks, the mice received 2×10^5 PFU of LCMV Arm i.p. to initiate acute infection (A) or 2×10^6 PFU of Cl.13 intravenously to initiate chronic infection (B). The virus titers in the spleens were quantitated by plaque assay on Vero cells at day 4 (A) or day 8 (B) postchallenge. Virus titers are indicated for each animal in the study. A dotted line represents the lower limit of detection (2×10^1 PFU/g [spleen]). The data are representative of those from three independent and reproducible experiments.

infection, the virus titers in the spleens of the immunized mice were determined. As LCMV Arm is cleared from all tissues during the first week of infection and infection with this virus results in the formation of functional and protective memory $CD8^+$ T cells (2), the mice which recovered from the LCMV Arm infection were used as positive controls. As shown in Fig. 3A, immunization with Lip-GP33 as well as LCMV Arm infection conferred complete protection to the mice, and the titers in the spleen were below the detection limit (2.0×10^1 PFU/g), whereas the mice immunized with the negative control peptide (Lip-GP283) showed titers of $(1.69 \pm 0.40) \times 10^7$ PFU/g (Fig. 3A). Again, in the absence of CpG, the immunized mice were not protected at all (data not shown). The protective efficacy of Lip-G33 with CpG was further tested by challenging the immunized mice with LCMV Cl.13, a mutant strain of LCMV Arm which is known to induce viremia for ~3 months, with the virus persisting in some tissues for life (1). Eight days after infection, the mice immunized with Lip-GP33 or LCMV Arm completely cleared the Cl.13 virus, whereas those which received the negative control peptide (Lip-GP283) had a high titer of the virus in the spleen (Fig. 3B), and the persistence of the virus was confirmed 4 weeks later (data not shown).

Liposome-coupled peptide NP396 induces low CTL responses but complete protection. The bulk of the CTL response to LCMV in *H-2^b^* mice is directed against three dominant epitopes, GP33-41, GP276-286, and NP396-404, which are presented by major histocompatibility complex class I *H-2D^b^* molecules (10, 16, 36). We investigated whether peptides other than GP33 were able to induce antiviral T-cell responses when they were coupled to the surfaces of liposomes. Peptide NP396-404 was coupled to liposomes by itself (Lip-NP396) or with peptide GP33 (Lip-GP33/NP396). Mice were immunized with either Lip-GP33, Lip-NP396, Lip-GP33/NP396, or a mixture of Lip-GP33 and Lip-NP396 (Lip-GP33–Lip-NP396), and the responses were compared by ex vivo CTL assays (Fig. 4A), in vivo CTL assays (Fig. 4B), ELISPOT assays (Fig. 4C), and challenge experiments with LCMV Arm (Fig. 4D).

The level of induction of CTL activities by Lip-NP396 was very low in both the ex vivo and the in vivo assays, and Lip-GP33/NP396 and Lip-GP33–Lip-NP396 induced intermediate levels of CTL activity compared to the levels induced by Lip-GP33 and Lip-NP396 (Fig. 4A and B). On the other hand, Lip-NP396 induced substantial levels of IFN-γ-producing cells, and Lip-GP33/NP396 and Lip-GP33–Lip-NP396 induced even higher, although not significantly higher, numbers of IFN-γ-producing cells compared with the numbers induced by Lip-GP33 and Lip-NP396 (Fig. 4C). When these immunized mice were challenged with LCMV Arm, all the liposomal conjugates except Lip-GP283 were found to have the potency necessary to confer complete protection to mice (Fig. 4D).

i.n. immunization of mice with liposome-coupled peptide. LCMV is transmitted through contact with secretions from infected animals or by inhalation of dried particles from them. Therefore, LCMV infection is also suitable for the study of the capacity of liposomal peptides to induce mucosal immunity and protect mice from infection by viruses through the mode of transmission described above. We introduced Lip-GP33 and CpG by the i.n. or i.m. route, and 1 week later, the numbers of IFN-γ-producing $CD8^+$ T cells in draining cervical lymph nodes and spleens were measured (Fig. 5A). The i.n. immunizations resulted in the induction of IFN-γ-producing $CD8^+$ T cells at high levels in the cervical lymph nodes but at only low levels in the spleens, indicating that effector $CD8^+$ T cells were induced locally near the nasal cavity. On the other hand, the i.m. immunizations induced high numbers of IFN-γ-producing $CD8^+$ T cells in the spleens but only small numbers in the cervical lymph nodes (Fig. 5A). When the immunized mice were challenged with LCMV Arm via the i.n. route, both i.m. and i.n. immunizations with Lip-GP33 were found to provide complete protection to the mice (Fig. 5B).

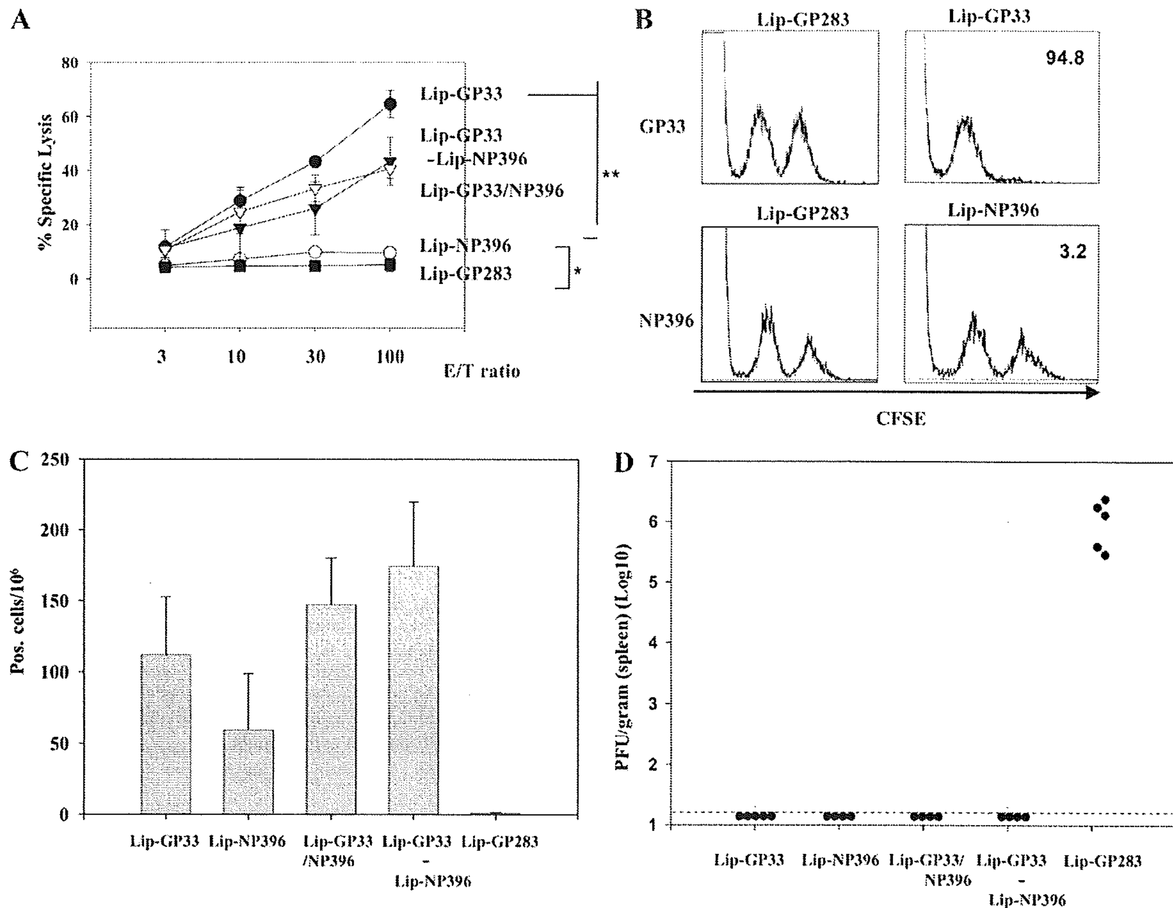

FIG. 4. Comparison of GP33 and NP396 peptides as vaccine components. C57BL/6 mice were immunized i.m. with either Lip-GP33, Lip-NP396, Lip-GP283, or Lip-GP33/NP396 (20 μl each) or with Lip-GP33–Lip-NP396 (10 μl each) in 100 μl of PBS containing 5 μg of CpG. Seven days later, ^{51}Cr-release assays (A), in vivo CTL assays (B), ELISPOT assays (C), and challenge experiments with LCMV Arm (D) were performed. The ^{51}Cr-release assays (A) and ELISPOT assays (C) were done as described in the legend to Fig. 1. The in vivo CTL assays (B) were performed only for the mice immunized with either Lip-GP33, Lip-NP396, or Lip-GP283. One week after immunization, the mice received an equal number of a relevant peptide (GP33 or NP396)-pulsed CFSE^{high} targets and unpulsed CFSE^{low} targets. The numbers show the percentages of specific lysis. The experiment was repeated twice. The challenge experiments with LCMV Arm (D) were performed by the methods described in the legend to Fig. 3A. *, $P < 0.05$; **, $P < 0.01$. No statistically significant difference was observed between the four groups by the ELISPOT assays (C).

Dose-response study of liposome-coupled peptides. To evaluate further the efficacies of the liposomal peptides, we made serial fivefold dilutions of Lip-GP33 and injected them into mice in the presence of CpG. To our surprise, as little as 0.08 μl of Lip-GP33, which contained only 56 ng of the peptide, induced a significant level of IFN-γ-producing CD8$^+$ T cells (Fig. 6A); and half of the immunized mice were completely protected against LCMV challenge (Fig. 6B) in two independent experiments. Immunizations with 0.4 μl or more of Lip-GP33 (≥280 ng peptide) conferred complete protection to all the mice, but the peptide (35 μg) not conjugated to liposomes did not induce either IFN-γ-producing CD8$^+$ T cells or protection against the virus challenge (Fig. 6), which suggests the essential role of the liposome on the induction of the antiviral responses. The results were confirmed in three independent and reproducible experiments.

Effector and memory CD8$^+$ T-cell induction by Lip-GP33 without the help of CD4. Several studies have demonstrated that CD4$^+$ T cells play critical roles in generating primary CD8$^+$ T cells or maintaining memory CD8$^+$ T cells (5, 17, 19, 37, 39). When mice were immunized with minimal CTL epitope peptides, no functional CD8$^+$ T cells could be detected past day 20 in an IFN-γ ELISPOT assay (8). Therefore, we wished to know how long the antiviral T-cell response persists and whether memory CD8$^+$ T cells are established after immunization with Lip-GP33. We also wished to rule out the possibility that CD4$^+$ T cells are involved in the induction of an antiviral T-cell response by Lip-GP33. We immunized both C57BL/6 and CD4-KO mice with a single dose of Lip-GP33 and compared the number of Ag-specific IFN-γ-producing T cells in their spleens by ELISPOT assay (Fig. 7A). When the primary CD8$^+$ T-cell responses to GP33 were compared at

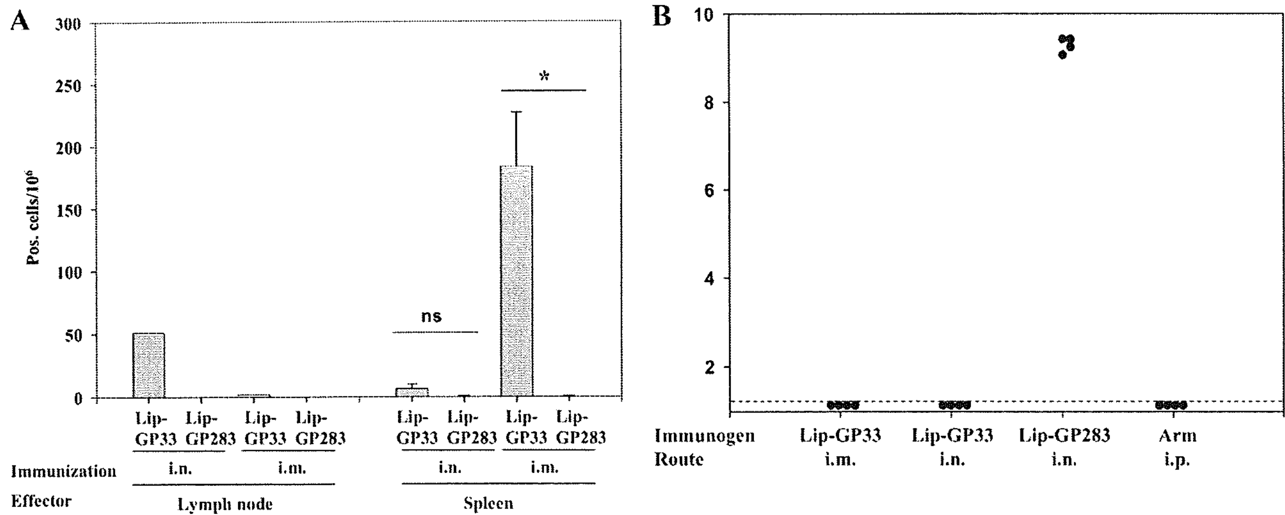

FIG. 5. i.n. immunizations with Lip-GP33. C57BL/6 mice were immunized by the i.n. or i.m. route with 20 μl of Lip-GP33 or Lip-GP283 and 5 μg of CpG. One week later, lymphocytes from cervical lymph nodes and spleens were prepared and subjected to ELISPOT (IFN-γ) assays (A). The immunized mice were also challenged with 2×10^5 PFU of LCMV Arm via the i.n. route, and the virus titers in the spleens were quantitated (B). (A) ELISPOT assays of IFN-γ-producing $CD8^+$ T cells from cervical lymph nodes (left) and spleens (right) of the mice immunized via the i.n. or i.m. route with Lip-GP33 or Lip-GP283. The data are representative of those from two independent and reproducible experiments. The results for one pool of cells from the lymph nodes and the means for spleen cells from four mice per group ± standard errors of the means are shown. *, $P < 0.05$ compared with the results obtained with Lip-GP283 immunization; ns, not statistically significant. (B) One week after immunization, the mice received 2×10^5 PFU of LCMV Arm i.n., and the virus titers in the spleens were quantitated by plaque assay on Vero cells at day 4 postchallenge. The virus titers are indicated for each animal in the study. A dotted line represents the lower limit of detection (2×10^1 PFU/g [spleen]). The data are representative of those from three independent and reproducible experiments.

1 week postimmunization, there was no significant difference between the two groups. Twelve weeks after priming, when the Ag-specific IFN-γ-producing T cells in the spleen diminished, the mice were boosted in the same manner used to prime them. One week postboosting, clear recall responses of 6- and 10-fold expansions of Ag-specific IFN-γ-producing T cells could be seen in the two groups, respectively, and the difference was not significant between the two groups. The recall responses of the C57BL/6 mice were also assessed 18 weeks after priming by tetramer staining and compared with those of

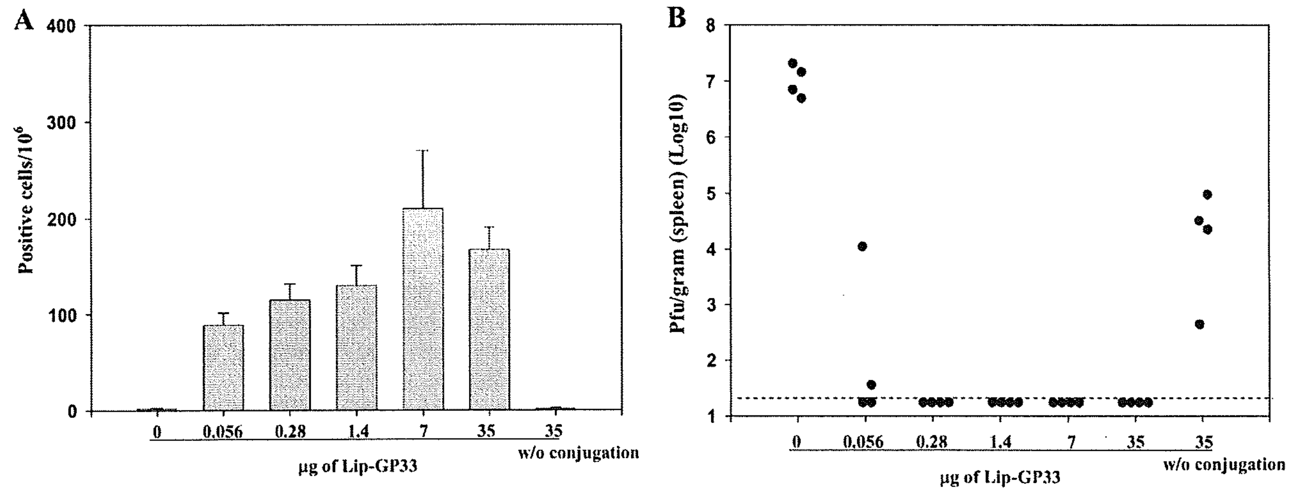

FIG. 6. Dose-response experiments. Serial fivefold dilutions of Lip-GP33 starting at 50 μl were prepared in 100 μl of PBS containing 5 μg of CpG and injected i.m. to each C57BL/6 mouse. Seven days later, ELISPOT (IFN-γ) assays (A) and challenge experiments with LCMV Arm (B) were performed. (A) ELISPOT assays were performed with spleen cells from immunized mice. One group of mice received peptide GP33 (35 μg) without (w/o) conjugation, and one group received GP33 at the same dose conjugated with 5 μg of CpG as one of the Lip-GP33 immunizations. The data are representative of those from three independent and reproducible experiments. The results are shown as the means for four mice per group ± standard errors of the means. (B) The immunized mice received 2×10^5 PFU of LCMV Arm i.p., and the virus titers in the spleens were quantitated by plaque assay on Vero cells at day 4 postchallenge. Virus titers are indicated for each animal in the study. The dotted line represents the lower limit of detection (2×10^1 PFU/g [spleen]). The data are representative of those from two independent and reproducible experiments.

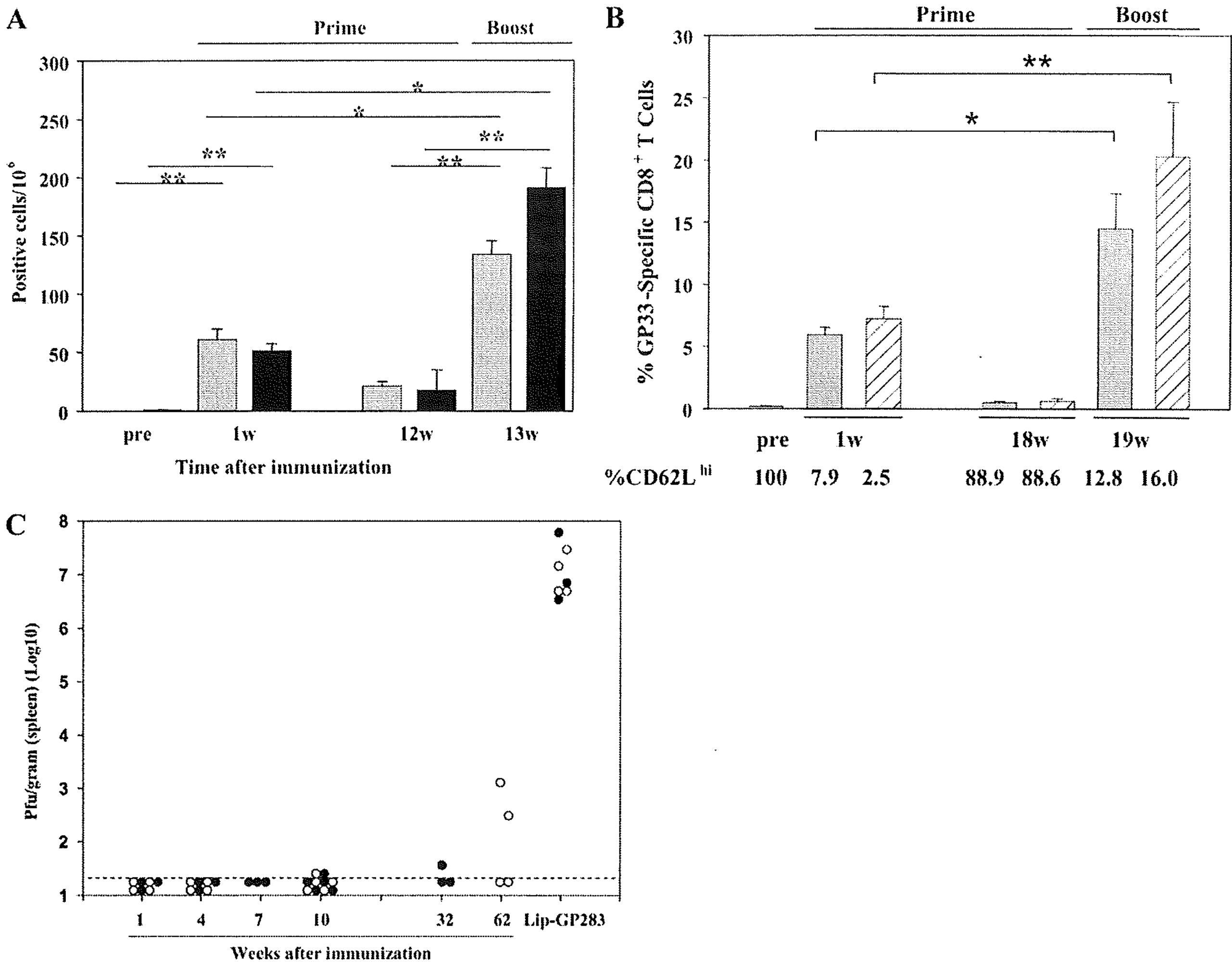

FIG. 7. Analyses of effector and memory $CD8^+$ T-cell induction in the absence of CD4 help. Each of C57BL/6 and CD4-KO mice received 20 μl of Lip-GP33 or Lip-GP283 diluted in 100 μl of PBS containing 5 μg of CpG; and ELISPOT assays (A), tetramer and CD62L staining (B), and challenge experiments with LCMV Arm (C) were performed at different times. (A) At 1 and 12 weeks postimmunization, IFN-γ-producing cells responding to peptide GP33 were detected by ELISPOT assays of spleen cells from C57BL/6 (gray bars) and CD4-KO (black bars) mice. At week (w) 12, the mice were boosted in the same manner in which they received the primary immunizations, and ELISPOT assays were performed 1 week later. (B) GP33 $H\text{-}2D^b$ tetramer staining was performed 1 and 18 weeks postimmunization for C57BL/6 mice (gray bars). For comparison, mice immunized by inoculating 2×10^5 PFU of LCMV Arm i.p. were included in these experiments (shaded bars). At week 18, both groups of mice were boosted with Lip-GP33 and were analyzed for their recall responses 1 week later. The expression of CD62L on gated GP33 $H\text{-}2D^b$ tetramer-positive $CD8^+$ T cells was also determined by flow cytometry. (C) Challenge experiments were performed by the i.p. injection of 2×10^5 PFU of LCMV Arm at the indicated times. The virus titers are shown for each of the C57BL/6 (open circles) and the CD4-KO (closed circles) mice. The data are shown as the means for at least three and five mice per group ± standard errors of the means for the ELISPOT and tetramer assays, respectively. *, $P < 0.05$; **, $P < 0.01$.

the mice which acquired sterile immunity by LCMV Arm infection (Fig. 7B). One week after the boost, 22- and 33-fold expansions of GP33-specific $CD8^+$ T cells were observed in the two groups, respectively. We also analyzed the expression of CD62L, a surface molecule associated with T-cell memory function, of GP33-specific $CD8^+$ T cells in both Lip-GP33- and LCMV Arm-immunized mice. As shown in Fig. 7B, CD62L was downregulated after the mice were primed. Eighteen weeks after the mice were primed, more than 88% of the GP33-specific $CD8^+$ T cells recovered their ability to express CD62L and were again downregulated after the boost. To confirm the establishment of memory $CD8^+$ T cells, the immunized mice were challenged with LCMV Arm at different times after vaccination. As shown in Fig. 7C, complete protection was seen even in the absence of $CD4^+$ T cells at 10 weeks after priming. In the 62nd week, half of the C57BL/6 mice showed perfect protection, and on average, about 4-log-titer reductions were observed.

We then examined if the presence of $CD4^+$ T-cell help would augment the induction of effector and memory $CD8^+$ cells by Lip-GP33. As helper T-cell epitopes, $I\text{-}A^b$-restricted LCMV GP61-80 and NP309-328 were chosen and conjugated to liposomes by themselves (Lip-GP61 and Lip-NP309) or with peptide GP33 (Lip-GP33/GP61 and Lip-GP33/NP309). Mice

were immunized with either Lip-GP33, Lip-GP33/GP61, Lip-GP33/NP309, or a mixture of Lip-GP33 and either Lip-GP61 or Lip-NP309 (Lip-GP33–Lip-GP61 and Lip-GP33–Lip-NP309, respectively). One and 18 weeks later, IFN-γ ELISPOT assays and challenge experiments with LCMV Arm were performed, but the results of the three immunizations (four mice per group) did not show any significant differences (data not shown).

Taken together, these findings suggest that Lip-GP33 generated functional effector and memory $CD8^+$ T cells without the evident help of T cells.

DISCUSSION

In the study described in this report, we demonstrate that antigenic peptides coupled to the surfaces of liposomes serve as efficient vaccine vehicles for the induction of antiviral immunity mediated by $CD8^+$ T cells. We used LCMV infection of mice as a model system because LCMV has been extensively used to study $CD8^+$ T-cell-mediated antiviral immunity (21, 25, 43). In addition, LCMV causes respiratory tract as well as percutaneous infections; and some LCMV variants, such as Cl. 13, WE, and Docile, induce persistent infections in immunocompetent mice. These features of LCMV enabled us to evaluate the efficacy of our vaccine preparation by comparing the findings obtained with this preparation with those obtained in many other studies in which the same epitope was presented as the peptide (3, 7, 18, 31) or in which liposome-encapsulated peptide (23), plasmid DNA (4, 15, 24, 34), recombinant vaccinia virus (14, 20, 30, 44), recombinant lentivirus (46), and recombinant *Listeria* (38) vaccines were used.

Lip-GP33 efficiently induced CTLs, IFN-γ-producing $CD8^+$ T cells, and complete protection against LCMV challenge. Although the numbers of positive cells in the ELISPOT assays were low compared to the results of the intracellular cytokine staining (Fig. 2A), that result seems to be simply due to technical factors because spleen cells from the same immunized animal showed such differences, and these were also observed in mice immunized by LCMV Arm infection.

The minimum amount of GP33 peptide (0.28 μg) required to yield perfect protection in this study was extremely low. Peptide vaccines with adjuvants, cytokines, CpGs, etc., usually require multiple injections in amounts ranging from 10 to 500 μg (3, 7, 18, 31). When peptide GP33 was encapsulated within liposomes and injected with CpG, two immunizations with 80 μg were required to obtain perfect protection, while a smaller dose gave only partial protection (23). In distinction, we expressed Ags on the surfaces of liposomes and speculate that they might be more efficiently recognized by Ag-presenting cells, which resulted in an enhanced presentation to T cells. In direct comparisons, we have found that surface-linked liposomal Ags induced a significantly higher level of Ag-specific IgG production than that induced by liposome-encapsulated Ags in mice (our unpublished observation). In addition, a significant difference was observed between liposomes with different lipid components; more Ags coupled to the unsaturated liposomes were engulfed by macrophages in vitro, and a higher level of Ag-specific antibody production was induced in vivo when unsaturated liposomes were used than when saturated liposomes were used (29). These lines of evidence indicate that the adjuvant effects of liposomes would depend on both the antigenic topology to the liposomal membrane and the membrane mobility of the liposomes. The efficient induction of antiviral immunity with a very low dose of peptide demonstrated in the present study (Fig. 6) could be an advantage of the use of surface-coupled liposomal peptides for the development of peptide-based vaccines.

It has been shown that peptide vaccination of mice immune to LCMV or vaccinia virus causes tumor necrosis factor-dependent shock-like signs (22). This indicates the need for caution in the development of antiviral peptide vaccines. Antiviral vaccines are currently administered to millions of people, and preexisting immunity to viruses is common. These potential problems with peptide vaccines may be even more important in the application to immunotherapy of a persistent infection (6, 13). However, the induction of the shock-like signs by peptide vaccination is dose dependent; less than 4 μg of peptide showed no evident effects (22). Therefore, the high level of efficiency of surface-linked liposomal peptides may circumvent these issues.

Of note, Lip-NP396 also conferred perfect protection, although only low levels of cytolytic activity were detected in both the ex vivo and the in vivo CTL assays (Fig. 4). This is in contrast to the immunization with GP33 peptide encapsulated in liposomes, which induced very strong cytolytic activity by $CD8^+$ T cells but very low protective immunity (23). This may be explained by the difference between GP33- and NP396-specific CTLs. NP396-spepcific CTLs have been found to be ~1,000 times more sensitive to Ag and to have more protective capacity than GP33-specific CTLs (9). Moreover, LCMV and hepatitis B virus have been found to be susceptible to noncytopathic antiviral control mechanisms that depend on local IFN-γ and tumor necrosis factor alpha induction (11, 12, 32, 41). This noncytopathic mechanism may underlie the protective immunity exerted by Lip-NP396. It is unclear why the two immunodominant epitopes, GP33 and NP396, exhibited different $CD8^+$ T-cell responses when they were coupled on the surfaces of liposomes. In our further study of liposomal vaccines with hepatitis C virus epitopes, some immunodominant CTL epitopes also showed such differences when they were used as conventional peptide vaccines and surface-linked liposomal peptide vaccines (unpublished data). Further elucidation of the mechanisms underlying the differences between the induction of CTL activity and cytokine production by surface-linked liposomal peptides may lead to the development of more beneficial vaccines.

Several studies have demonstrated that while the primary expansion of antiviral $CD8^+$ T cells can occur independently of $CD4^+$ T-cell help, CD4 help is required for the long-term (>2-month) survival of memory $CD8^+$ T cells (17, 39, 40). Unhelped memory populations have been reported to be defective in their ability to generate a recall response following secondary challenge (39). LCMV peptide GP33 has been reported to induce effector $CD8^+$ T cells by being emulsified in incomplete Freund adjuvant (3), mixed with CpG (18, 31), or encapsulated in liposomes (23); however, the induction and maintenance of memory $CD8^+$ T cells by those immunizations have not been elucidated. In the present study, it was demonstrated that IFN-γ production and protective immunity were induced and maintained for more than 1 year after a single

immunization with Lip-GP33 (Fig. 7). Significant recall responses were observed in ELISPOT assays and staining of the tetramer after the booster injection with Lip-GP33. These findings as well as the analysis of CD62L expression indicate that the immunization with Lip-GP33 and CpG induced the maturation and maintenance of Ag-specific memory $CD8^+$ T cells. To our knowledge, this is the first time that long-lasting protective immunity against LCMV infection has been induced by the use of minimum CTL epitope peptides. These findings may be useful in the development of vaccines against human pathogens.

In summary, we report that the peptides coupled on the surfaces of liposomes consisting of unsaturated fatty acids very efficiently induce protective antiviral immunity in mice. The mode of action is still unclear, but it seems to be very unique on the basis of the differences in the responses depending on the epitope peptides used and the induction/maturation of memory $CD8^+$ T cells without the help of CD4 cells.

ACKNOWLEDGMENTS

This work was supported by a grant from the Ministry of Health, Labor, and Welfare of Japan and a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

We thank Hiroe Akatsuka for technical assistance. We are also grateful to J. Lindsay Whitton (The Scripps Research Institute) for providing us VVGP33 and technical advice, Rafi Ahmed (Emory University School of Medicine) for providing LCMV Arm and Cl.13, and Stephen M. Feinstone (CBER, FDA) for helpful discussions and preparing the manuscript.

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Vaccine 27 (2009) 3912–3920

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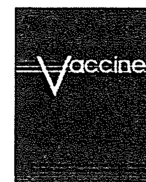

Synthetic peptides coupled to the surface of liposomes effectively induce SARS coronavirus-specific cytotoxic T lymphocytes and viral clearance in HLA-A*0201 transgenic mice

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ARTICLE INFO

Article history:
Received 19 September 2008
Received in revised form 5 March 2009
Accepted 2 April 2009
Available online 23 April 2009

Keywords:
SARS coronavirus
Liposomal peptides
Cytotoxic T lymphocytes

ABSTRACT

We investigated whether the surface-linked liposomal peptide was applicable to a vaccine based on cytotoxic T lymphocytes (CTLs) against severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV). We first identified four HLA-A*0201-restricted CTL epitopes derived from SARS-CoV using HLA-A*0201 transgenic mice and recombinant adenovirus expressing predicted epitopes. These peptides were coupled to the surface of liposomes, and inoculated into mice. Two of the liposomal peptides were effective for peptide-specific CTL induction, and one of them was efficient for the clearance of vaccinia virus expressing epitopes of SARS-CoV, suggesting that the surface-linked liposomal peptide might offer an effective CTL-based vaccine against SARS.

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1. Introduction

Severe acute respiratory syndrome (SARS) is a novel infectious disease that emerged in southern China in late 2002 and spread to several countries in early 2003. More than 8000 cases of SARS had been identified worldwide, and nearly 800 patients had died before the epidemic ended [1]. The etiologic agent of SARS has turned out to be a novel coronavirus termed SARS-associated coronavirus (SARS-CoV) [2–4], which is a plus-stranded RNA virus with an approximately 30-kb long genome encoding replicase gene products and the structural proteins containing spike (S), envelop (E), membrane (M), and nucleocapsid (N). Until now, the viral genome has been sequenced [5] and the viral receptor has been identified [6]. However, the pathogenesis of SARS remains poorly understood, and the apparent latency of SARS-CoV in animal reservoirs continuously provides us a serious threat of reemergence. Therefore, it is urgent to develop a new prophylactic and therapeutic strategy against SARS.

Among the four structural proteins of SARS-CoV, S protein interacts with the cellular receptor to mediate membrane fusion, allowing the virus to enter host cells [6]. Accordingly, S protein is a major target for neutralizing antibodies [7]. High titers of neutralizing antibodies to SARS-CoV were detected in sera of the recovered patients [8], and further, humoral immunity induced by a DNA vaccine contributed to the protection against SARS-CoV challenge in mice [7]. These data imply that neutralizing antibodies play a critical role in the clearance of SARS-CoV. On the other hand, a rapid loss of both $CD4^+$ and $CD8^+$ T cells was observed in patients suffering from severe SARS, and the cell counts gradually returned to normal ranges as the patients recovered [9]. Furthermore, certain HLA class I alleles have been reported to correlate with SARS susceptibility [10,11]. These data strongly suggest that, as with many other viral infections, virus-specific cytotoxic T lymphocytes (CTLs) should be important for viral elimination in SARS as well.

A synthetic peptide vaccine is a potential candidate for a CTL-based vaccine against pathogenic viruses on account of several advantages over conventional vaccines. First, synthetic peptides rarely cause undesirable responses including general toxicity, immunosuppression and autoimmunity. Second, it is possible to select short peptides in the absence of amino acid mutations. Third, synthetic peptides can easily be prepared as a pure immunogen in large quantities. However, a major disadvantage is the weak immunogenicity. Therefore, it is critical to search for adjuvant vehi-

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doi:10.1016/j.vaccine.2009.04.001

Table 1
Predicted CTL epitopes for SARS-CoV nucleocapsid protein.

Name	Position	Sequence	SYFPEITHI[a]	BIMAS[b]	BL_{50} (μM)[c]
N-113	113–122	YLGTGPEASL	98.3	26	25.7 ± 11.2
N-159	159–168	VLQLPQGTTL	309.1	29	235.3 ± 68.7
N-222	222–231	LLLLDRLNQL	69.6	24	52.6 ± 10.5
N-223	223–231	LLLDRLNQL	98.3	20	46.1 ± 4.9
N-227	227–235	RLNQLESKV	36.3	23	165.1 ± 36.6
N-317	317–325	GMSRIGMEV	1267.1	30	72.8 ± 37.7
N-331	331–340	WLTYHGAIKL	50.2	21	90.4 ± 39.2
N-352	352–360	ILLNKHIDA	31.2	19	140.5 ± 44.6

[a] Peptide binding scores to HLA-A2.1 were determined by the SYFPEITHI database [16] at http://www.syfpeithi.de/.
[b] Peptide binding scores to HLA-A2.1 were determined by the BIMAS database [17] at http://www-bimas.cit.nih.gov/molbio/hla_bind/.
[c] Data of peptide binding assays are shown as BL_{50}, indicating a concentration of each peptide that yields the half-maximal MFI of T2 cells pulsed with a control peptide, NS3-1585. Data are given as mean values ± SD of three independent experiments.

cles which are non-immunogenic themselves but which enhance the immunogenicity of peptides.

Liposomes have extensively been investigated as a delivery system for antigen [12]. In most cases, it has been prepared by antigen entrapment within the aqueous lumen of liposomes. In contrast, we have previously shown that ovalbulim (OVA) chemically conjugated on the surface of liposomes induced OVA-specific IgG production but not OVA-specific IgE production in mice [13], suggesting that the surface-linked liposomal antigen could offer a safe antigen delivery system without allergic side effects. Furthermore, we have demonstrated that an OVA-derived peptide, $OVA_{257-264}$ conjugated on the surface of liposomes made from unsaturated, but not saturated fatty acid, induced $OVA_{257-264}$-specific CTLs in mice more effectively than did liposomes containing $OVA_{257-264}$ inside [14,15]. In addition, it was shown that surface-linked liposomal peptides were able to provide tumor eradication [15] and protection against viral challenge [14] in mice. Taken together, these data suggest that liposomes would become an excellent adjuvant vehicle for a synthetic peptide vaccine when a peptide(s) is chemically coupled to the surface of liposomes.

In the current study, we explored the possibility that the surface-linked liposomal peptide might serve as an effective CTL-based vaccine against SARS. Firstly, we attempted to identify HLA-A*0201-restricted CTL epitopes derived from N protein of SARS-CoV (SARS-CoV-N) using computational algorithm, recombinant adenovirus and HLA-A*0201 transgenic mice. N protein was chosen for the analyses because this is more conserved than S protein. Peptides identified were then chemically conjugated on the surface of liposomes and evaluated for their abilities to induce SARS-CoV-N-specific CTLs and to clear virus using recombinant vaccinia virus expressing SARS-CoV-derived epitopes.

2. Materials and methods

2.1. Prediction of CTL epitopes

To define potential HLA-A*0201-binding peptides derived from SARS-CoV-N (Urbani strain) (GenBank accession number: AY278741), we used two computer-based programs, SYFPEITHI (http://www.syfpeithi.de/) [16] and BIMAS (http://www-bimas.cit.nih.gov/molbio/hla_bind/) [17]. As shown in Table 1, eight peptides (N-113, N-159, N-222, N-223, N-227, N-317, N-331, and N-352) with high scores were selected, and synthesized by Operon Biotechnologies (Tokyo, Japan). An I-A^b-restricted helper T cell peptide, hepatitis B virus (HBV) core 128 (amino acid sequence: TPPAYRPPNAPIL) [18] was also synthesized and used for immunization. Synthesized peptides were desalted, and analyzed by high performance liquid chromatography (HPLC).

2.2. Mice

Mice express a transgenic HLA-A*0201 monochain, designated as HHD, in which human beta-2 microglobulin (β2m) is covalently linked to a chimeric heavy chain composed of HLA-A*0201 (α1 and α2 domains) and H-$2D^b$ (α3, transmembrane, and cytoplasmic domains) [19,20]. Eight- to 12-week-old mice were used for all experiments. Mice were housed in appropriate animal care facilities at Saitama Medical University, Saitama, Japan, and handled according to international guidelines for experiments with animals.

2.3. Cell lines

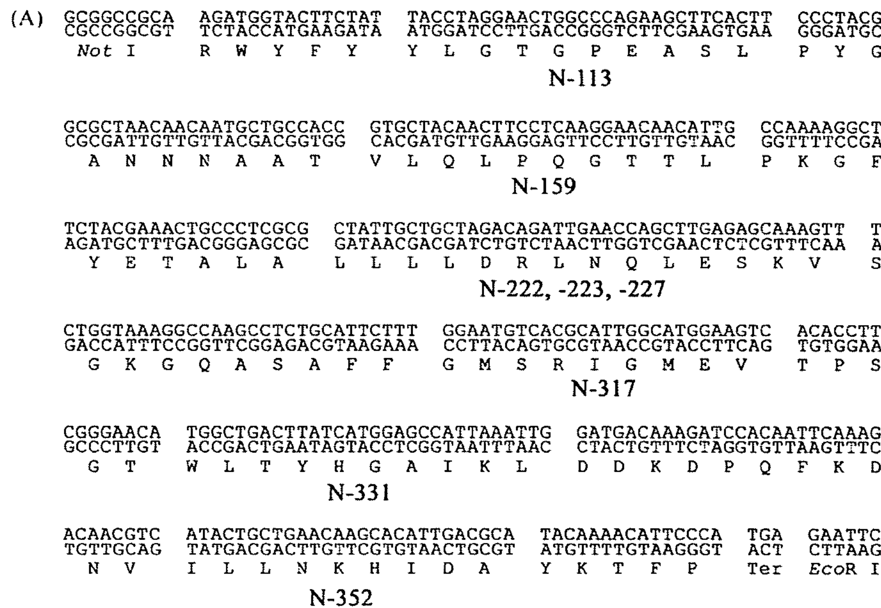
A mouse lymphoma cell line transfected with the HHD gene, RMA-HHD (H-2^b) was previously described [19]. T2 [21] is a TAP-deficient, human lymphoblastoid cell line expressing natural HLA-A*0201. Human kidney cell lines, 293 and 293T cells were obtained from the American Type Culture Collection (Rockville, MD). African green monkey-derived kidney cell lines, CV-1 and BS-C-1, and a human osteosarcoma, thymidine kinase-defective cell line, C143 were kindly provided by Dr. T. Shioda (Osaka University, Japan). The T2 cell line was maintained in RPMI-1640 medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS) (R-10). The 293, 293T, CV-1, BS-C-1 and C143 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich) with 10% FCS (D-10). The RMA-HHD cell line was maintained in D-10 containing G418 (Sigma–Aldrich) at a final concentration of 500 μg/ml.

2.4. Peptide binding assay

Peptide binding assay was performed as described [22]. Briefly, T2 cells were incubated with a synthetic peptide at various concentrations overnight at 37 °C. Cells were stained with the anti-HLA-A*0201 monoclonal antibody (mAb), BB7.2 [23], followed by fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Sigma–Aldrich). The mean fluorescence intensity (MFI) was measured by flow cytometry (FACScan, BD Biosciences, San Jose, CA). The concentration of each peptide that yields the half-maximal MFI of T2 cells pulsed with a control peptide derived from hepatitis C virus (HCV), NS3-1585 [22] was calculated as the half-maximal binding level (BL_{50}). Experiments were performed three times, and data are given as mean values ± SD.

2.5. Construction of a multiepitope minigene

A multiepitope minigene that encodes the eight predicted epitopes with 5–11 natural flanking amino acid residues at both of the N and C termini (Fig. 1) (SARS-N8E) was constructed using



(B)

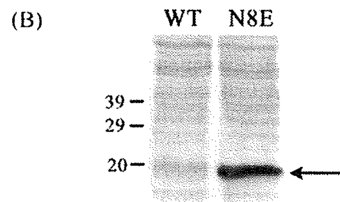


Fig. 1. (A) Nucleotide and amino acid sequences of eight predicted epitopes (N-113, N-159, N-222, N-223, N-227, N-317, N-331 and N-352) with flanking amino acid residues encoded in a minigene, termed SARS-N8E. (B) Expression of the SARS-N8E fusion protein. 293 T cells were infected with either Ad-WT (WT) or Ad-SARS-N8E (N8E). After 2 days' incubation, cells were lysed and separated on by SDS-12% polyacrylamide gel electrophoresis and subjected to Western blotting analysis with the anti-FLAG antibody. The positions of protein molecular mass markers (in kDa) are shown in the figure, and an arrow indicates the band of the SARS-N8E fusion protein.

overlapping long oligonucleotides in PCR-based synthesis [24]. In brief, five long oligos, averaging about 90 nucleotides in length with 20–25 nucleotide overlaps, were synthesized and HPLC-purified by Operon Biotechnologies. The minigene was then assembled by extending the five overlapping oligos. After confirming the nucleotide sequence by DNA sequencing, the multiepitope minigene, SARS-N8E was cloned into the NotI and EcoRI sites of p3xFLAG-CMV-10 expression vector (Sigma–Aldrich) (p3xFLAG-SARS-N8E). This vector encodes the three adjacent FLAG-tag epitopes (amino acid sequence: DYKDHDGDYKDHDIDYKDDDDK) upstream of the multiple cloning region, and hence, expresses an N-terminal 3xFLAG fusion protein under the control of the CMV promoter in mammalian cells.

2.6. Generation of recombinant adenovirus and vaccinia virus expressing multiple CTL epitopes

Recombinant adenovirus expressing the eight predicted epitopes (Ad-SARS-N8E) was generated using the Adenovirus Expression Vector Kit (Takara Bio Inc., Shiga, Japan). Briefly, the SARS-N8E minigene linked to the 3xFLAG-tag sequence was isolated by PCR amplification from p3xFLAG-SARS-N8E, and inserted into the cloning site of the cosmid vector, pAxCAwtit containing the entire adenovirus genome except for the E1 and E3 genes. This recombinant cosmid was co-transfected with DNA-TPC containing the adenovirus terminal protein into 293 cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Since 293 cells express E1A and E1B, replication-defective adenovirus can be produced. After cloning, virus was amplified in 293 cells and titered by standard plaque assays on 293 cells. Wild-type adenovirus (Ad-WT) was used as a negative control.

Recombinant vaccinia virus (WR strain) expressing the eight predicted epitopes (VV-SARS-N8E) was generated as described before [25]. In brief, the SARS-N8E minigene with the 3xFLAG-tag sequence was inserted into the transfer vector, pNZ68K2. VV-SARS-N8E was then generated by homologous recombination between wild-type vaccinia virus (VV-WT) and the transfer vector, purified by three cycles of plaque cloning with C143 cells in the presence of bromodeoxyuridine, and propagated in CV-1 cells. Viral titers were measured by standard plaque assays on BS-C-1 cells.

To detect expression of the SARS-N8E fusion protein, Western blotting was performed as described previously [25]. In brief, 293T cells were infected with Ad-SARS-N8E at a multiplicity of infection (MOI) of 30 or VV-SARS-N8E at an MOI of 3 for 1.5 h. After 2 days' incubation, cells were lysed and the solubilized proteins were separated by electrophoresis on a 12% SDS-PAGE under reducing condition, and blotted onto a nitrocellulose membrane. The blot was stained with 5 μg/ml of the anti-FLAG M2 mAb (Sigma–Aldrich), followed by secondary staining with peroxidase conjugated anti-mouse IgG Ab. The protein bands were developed by the BCIP/NBT Phosphatase Substrate System (KPL Inc., Gaithersburg, MD).

2.7. Surface-linked liposomal peptides

Oleoyl liposomes are composed of dioleoyl phosphatidyl choline, dioleoyl phosphatidyl ethanolamine, dioleoyl phosphatidyl glycerol acid, and cholesterol in a 4:3:2:7 molar ratio [26].

Each of CTL peptides and a helper peptide was then coupled to the surface of liposomes at a same molar concentration via disuccinimidyl suberate (DSS) as described previously [15]. Empty liposomes were used as a negative control.

2.8. Immunization

For identification of CTL epitopes, mice were immunized intraperitoneally (i.p.) with 5×10^8 plaque-forming units (PFU) of either Ad-WT or Ad-SARS-N8E. For the immunization with liposomal peptides, mice were subcutaneously (s.c.) immunized with each surface-linked liposomal CTL peptide (25 μg/mouse) mixed with a liposomal helper peptide (25 μg/mouse) and CpG-ODN (5′-TCCATGACGTTCTGATGTT-3′, Hokkaido System Science, Sapporo, Japan) (5 μg/mouse) in 100 μl PBS in the footpad.

2.9. Intracellular IFN-γ staining

Intracellular cytokine staining (ICS) was performed as described previously [27]. Briefly, after 1 week following immunization, spleen cells of immunized mice were incubated with brefeldin A (GolgiPlug, BD Biosciences) for 5 h at 37 °C in the presence or absence of a relevant peptide at a final concentration of 10 μM. After incubation with the rat anti-mouse CD16/CD32 mAb (Fc Block, BD Biosciences), cells were stained with FITC-conjugated rat anti-mouse CD8α mAb (BD Biosciences) for 30 min at 4 °C. Cells were then fixed, permeabilized, and stained with phycoerythrin (PE)-conjugated rat anti-mouse IFN-γ mAb (BD Biosciences). After washing the cells, flow cytometric analyses were performed.

2.10. ^{51}Cr-release assay

^{51}Cr-release assays were carried out as described before [19]. In brief, after 2 weeks following immunization, spleen cells of immunized mice were cultured for 1 week with irradiated (30 Gy), syngeneic naive spleen cells pre-pulsed with 10 μM of a relevant peptide, and employed as effector cells in standard ^{51}Cr-release assays. RMA-HHD cells were pulsed with or without 10 μM of each peptide for 1 h, labeled with 100 μCi of $Na_2{}^{51}CrO_4$, and used for target cells. After a 4-h incubation, supernatant of each well was harvested and the radioactivity was counted. Results were calculated as the mean of a triplicate assay. Percent specific lysis was calculated according to the formula: % specific lysis = $[(cpm_{sample} - cpm_{spontaneous})/(cpm_{maximum} - cpm_{spontaneous})] \times 100$. Spontaneous release represents the radioactivity released by target cells in the absence of effectors, and maximum release represents the radioactivity released by target cells lysed with 5% Triton X-100.

2.11. In vivo CTL assay

In vivo CTL assay was carried out as reported before [28]. Briefly, spleen cells from naive HHD mice were equally split into two populations. One population was pulsed with a peptide at a final concentration of 10 μM for 1 h at 37 °C, and then labeled with a high concentration (2.5 μM) of carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) for 10 min at 37 °C ($CFSE^{high}$). The other was unpulsed and labeled with a lower concentration (0.25 μM) of CFSE ($CFSE^{low}$). An equal number (1×10^7) of cells from each population was mixed and transferred intravenously (i.v.) into mice that had been immunized 1 week earlier. Twelve hours later, spleen cells were prepared and analyzed by flow cytometry. To calculate specific lysis, the following formula was used: % specific lysis = $[1 - \{(\text{number of } CFSE^{low} \text{ cells in normal mice})/(\text{number of } CFSE^{high} \text{ cells in normal mice})\}/\{(\text{number of } CFSE^{low} \text{ cells in immunized mice})/(\text{number of } CFSE^{high} \text{ cells in immunized mice})\}] \times 100$.

2.12. Viral challenge

Viral challenge experiments were performed as described before [29]. Two weeks after immunization, mice were challenged i.p. with 1×10^6 PFU of either VV-SARS-N8E or VV-WT. Five days later, mice were sacrificed, and two ovaries of each mouse were homogenized, and resuspended in 0.5 ml of PBS containing 1% FCS and 1 mM $MgCl_2$. Virus was released from the cells by three freeze–thaw cycles followed by sonication. Viral titers were measured by plating serial 10-fold dilutions on BS-C-1 indicator cells in 6-well plates. All titrations were performed in duplicates, and the average PFU per mouse was calculated.

2.13. Statistical analyses

Statistical analyses were performed with Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Prediction of CTL epitopes derived from SARS-CoV-N

The amino acid sequence of SARS-CoV-N was searched for potential HLA-A*0201-restricted CTL epitopes by two computer-based programs, SYFPEITHI [16] and BIMAS [17]. According to the scores calculated, eight nonameric and decameric peptides were selected and synthesized (Table 1). To evaluate the binding affinity of these peptides to HLA-A*0201 molecules, the peptide binding assay [22] was performed (Table 1). Five (N-113, N-222, N-223, N-317, and N-331) out of the eight peptides were high binders displaying BL_{50} values less than 100 μM, and two (N-227 and N-352) of them were medium binders displaying BL_{50} values ranging from 100 to 200 μM. These data suggest that prediction of CTL epitopes should be mostly successful. In contrast, one peptide, N-159 showed low affinity binding.

3.2. Induction of SARS-CoV-N-specific CTLs in HHD mice infected with adenovirus

To investigate whether CTLs specific for the predicted peptides were elicited, HHD mice were immunized i.p. once with either Ad-SARS-N8E or Ad-WT. One week after immunization, spleen cells were prepared and stimulated with each of the eight predicted peptides derived from SARS-CoV-N for 5 h. Cells were then stained for their surface expression of CD8 and antigen-induced intracellular expression of IFN-γ. As shown in Fig. 2, considerable numbers of IFN-γ-producing $CD8^+$ T cells were induced by stimulation with peptides including N-222, N-223, N-227 and N-317 in Ad-SARS-N8E-infected mice but not in Ad-WT-injected mice, indicating that CTLs specific for these four peptides were induced in mice by immunization with Ad-SARS-N8E. In contrast, none of the remaining peptides significantly elicited IFN-γ-secreting $CD8^+$ T cells (Fig. 2).

We next examined peptide-specific killing activities in spleen cells of mice that had been immunized with Ad-SARS-N8E. Two weeks after immunization, spleen cells of the mice were harvested and stimulated *in vitro* with each of the peptides. One week later, ^{51}Cr-release assays were performed at various effector:target (E:T) ratios. In agreement with the data of ICS (Fig. 2), the four peptides, N-222 (Fig. 3C), N-223 (Fig. 3D), N-227 (Fig. 3E) and N-317 (Fig. 3F) elicited strong peptide-specific CTL responses in Ad-SARS-N8E-infected mice but not in Ad-WT-infected mice. On the other

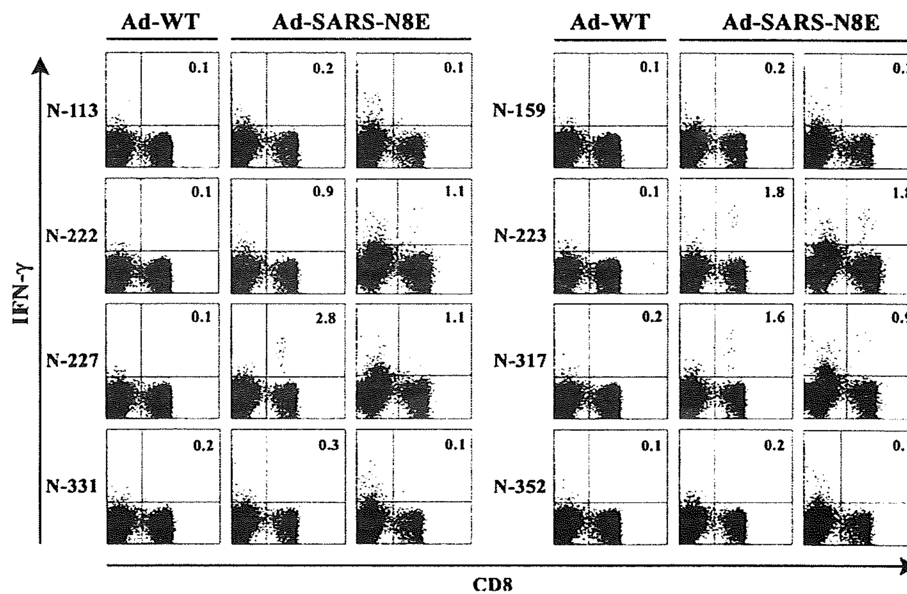


Fig. 2. Intracellular IFN-γ staining of $CD8^+$ T cells specific for SARS-CoV-N-derived peptides in spleen cells of mice immunized with either Ad-SARS-N8E or Ad-WT. HHD mice were immunized i.p. once with either Ad-SARS-N8E or Ad-WT. One week later, spleen cells were prepared and stimulated with each of the eight predicted peptides (N-113, N-159, N-222, N-223, N-227, N-317, N-331, and N-352) for 5 h. Cells were then stained for their surface expression of CD8 (*x*-axis) and their intracellular expression of IFN-γ (*y*-axis). The numbers shown indicate the percentages of intracellular IFN-γ^+ cells within $CD8^+$ T cell. The data shown are representative of three independent experiments.

hand, the remaining peptides, N-113 (Fig. 3A), N-159 (Fig. 3B), N-331 (Fig. 3G), and N-352 (Fig. 3H) failed to induce CTL activities in either Ad-SARS-N8E-injected mice or Ad-WT-injected mice. To further address peptide-specific killing activities in mice, *in vivo* CTL assays were carried out (Fig. 4). After immunization with either Ad-WT or Ad-SARS-N8E, HHD mice received i.v. injection of peptide-pulsed $CFSE^{high}$ targets and unpulsed $CFSE^{low}$ targets. Twelve hours later, spleen cells were prepared and peptide-specific lysis was assessed by flow cytometry. As was expected, N-222-, N-223-, N-227- and N-317-specific CTL killing activities were significantly detected in mice immunized with Ad-SARS-N8E, but not in Ad-WT-injected mice (Fig. 4). Especially, the activity of N-223-specific killing was greatest (Fig. 4), suggesting that N-223 might be an immunodominant epitope. Any of the remaining peptides, N-113, N-159, N-331 and N-352 could not induce peptide-specific killing activities in *in vivo* CTL assays (data not shown).

3.3. Induction of SARS-CoV-N-specific CTLs by immunization with surface-linked liposomal peptides

We next investigated whether surface-linked liposomal peptides could induce peptide-specific CTLs in mice. Since four peptides including N-222, N-223, N-227 and N-317 were expected to be HLA-A*0201-restricted CTL epitopes (Figs. 2–4), these peptides were chemically conjugated on the surface of liposomes. Surface-linked liposomal peptides, Lip-N222, Lip-N223, Lip-N227 and Lip-N317 were then evaluated for their capabilities of CTL induction in HHD mice. One week after immunization with each of the liposomal peptides, spleen cells were prepared, stimulated with a relevant peptide, and stained for their expression of surface CD8 and intracellular IFN-γ. As shown in Fig. 5, significant numbers of IFN-γ-producing $CD8^+$ T cells were elicited in mice that had been immunized once with either Lip-N223 or Lip-N227 in the footpad. In the case of Lip-N317, however, one injection with Lip-N317 did not result in the induction of IFN-γ-producing $CD8^+$ T cells (Fig. 5), and after three injections, a significant expansion of IFN-γ-producing $CD8^+$ T cells in response to N-317 was finally observed (Fig. 5), indicating that Lip-N317 might be less immunogenic than Lip-N223 and Lip-N227. On the other hand, Lip-N222 failed to elicit N-222-specific IFN-γ-producing $CD8^+$ T cells in mice (Fig. 5) even after multiple injections. Since N-222 is a 10-mer peptide composed of an N-223 peptide with one additional leucine at the N-terminus (Table 1), there was a possibility of cross reaction between N-222 and N-223. As shown in Fig. 6, both N-222 and N-223 peptides obviously stimulated IFN-γ-producing $CD8^+$ T cells in mice immunized with Lip-N223, demonstrating that N-223-specific CTLs primed by Lip-N223 was activated by stimulation with N-222 as well as N-223. In contrast, either N-222 or N-223 could not induce IFN-γ-secreting $CD8^+$ T cells in mice primed with Lip-N222.

3.4. Administration of Lip-N223 provided protective immunity in mice against virus challenge

Because Lip-N223 and Lip-N227 effectively primed IFN-γ-producing $CD8^+$ T cells in mice (Fig. 5), we next evaluated whether immunization with either Lip-N223 or Lip-N227 would induce $CD8^+$ CTLs to kill peptide-pulsed target cells *in vivo*. One week after immunization of mice, both peptide-pulsed $CFSE^{high}$ target cells and unpulsed $CFSE^{low}$ target cells were delivered into the mice via i.v. injection. As shown in Fig. 7, the peptide-specific killing activity in Lip-N223-immunized mice was greater than that in Lip-N227-immunized mice, indicating that Lip-N223 was more immunogenic than Lip-N227. Therefore, we next tested whether mice immunized with Lip-N223 were able to clear virus challenged. HHD mice were immunized twice with Lip-N223 at a 2-week interval. Two weeks after the last immunization, the mice were challenged with 1×10^6 PFU of VV-SARS-N8E or VV-WT. After 5 days following the challenge, viral titers were measured in two ovaries of each mouse. As shown in Fig. 8, titers of vaccinia virus in mice challenged with VV-SARS-N8E were 3 logs lower than those in mice challenged with VV-WT. Mice immunized with empty liposomes retained high viral titers after challenge with either VV-SARS-N8E (Fig. 8) or VV-WT (data not shown). Thus, these data indicate that immunization with Lip-N223 is effective in the protection against virus.

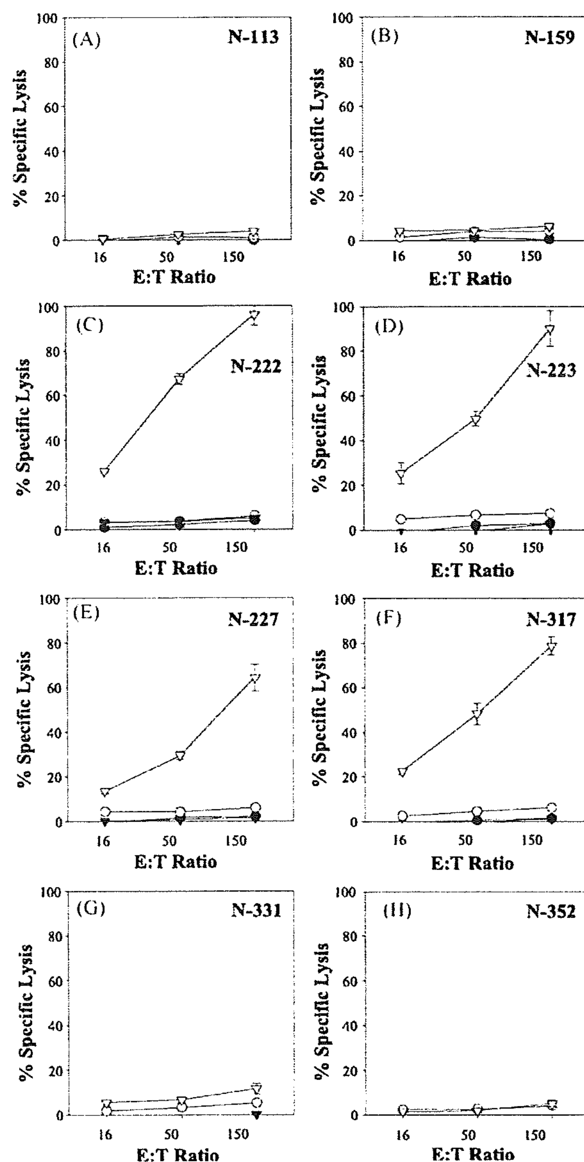


Fig. 3. CTL activities specific for eight predicted epitopes derived from SARS-CoV-N in mice immunized with Ad-SARS-N8E. HHD mice were immunized i.p. with either Ad-SARS-N8E (reverse triangles) or Ad-WT (circles). Two weeks after immunization, spleen cells were prepared and stimulated *in vitro* with each of the eight predicted epitopes (N-113, N-159, N-222, N-223, N-227, N-317, N-331, and N-352) derived from SARS-CoV N protein. After 1 week, ^{51}Cr-release assays were performed at various E:T ratios with RMA-HHD cells pulsed with (open symbols) or without (solid symbols) a relevant peptide as target. Data are shown as the means ± SD of triplicate wells. The experiment was repeated twice with similar results. At least three mice per group were used in each experiment.

4. Discussion

Since S protein is responsible for binding to specific cellular receptors [6], this is a major target for neutralizing antibodies [7]. Moreover, vigorous S-specific CTL responses were generated in SARS-CoV-infected patients [30,31], indicating that S protein is an antigen for CTL as well. In fact, four CTL epitopes derived from S protein have been identified [31–33]. However, since N protein is more conserved and synthesized earlier than S protein, N protein seems preferable to S protein as an antigenic target for T-cell immunity. Therefore, we focused on SARS-CoV N protein for the development of a CTL-based vaccine in the current study.

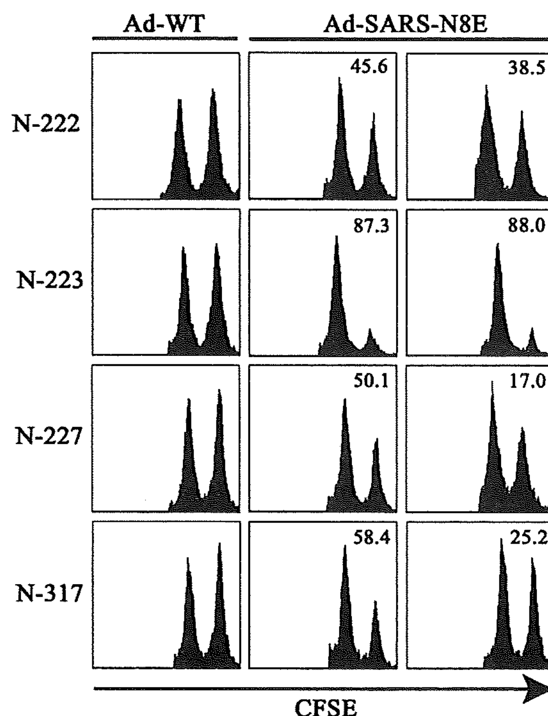


Fig. 4. *In vivo* killing of peptide-pulsed target cells in HHD mice immunized with Ad-SARS-N8E. HHD mice were immunized with either Ad-WT or Ad-SARS-N8E. One week later, an equal number of each peptide (N-222, N-223, N-227, or N-317)-pulsed $CFSE^{high}$ targets and unpulsed $CFSE^{low}$ targets were transferred into the immunized mice by i.v. injection. After 12 h, CFSE-labeled cells were recovered from spleens of recipient mice and analyzed by flow cytometry. The experiment was repeated three times with similar results. The numbers show the percentages of specific lysis.

High-performing computational algorithms have extensively been used for the identification of CTL epitopes [18,31–34]. Chentoufi et al. [34] supported their predictive computational algorithms by multiple immunological screenings. Thus, it is important to use multiple screenings for successful identification of functional CTL epitopes. We also performed multiple immunological screens, including cell surface stabilization of HLA-A*0201 molecules on T2 cells, detection of antigen-driven IFN-γ-producing $CD8^+$ T cells, and functional *in vivo* and *in vitro* CTL assays. Our strategy for the identification of CTL epitopes has several advantages. First, the use of recombinant adenovirus and vaccinia virus allowed us to circumvent the necessity for handling live SARS-CoV. Both of the recombinant viruses, Ad-SARS-N8E and VV-SARS-N8E, carry the multiepitope minigene that encodes eight predicted epitopes with several natural flanking amino acid residues at both of the ends, thereby offering natural antigen processing in the infected cells. The basic idea comes from the observation that flanking sequences proximal to CTL epitopes modulate proteasomal processing of the epitopes [35,36]. Furthermore, we can carry out *in vitro* and *in vivo* experiments using these viruses in BSL-2 facilities. Replication-defective recombinant adenovirus effectively induces CTLs specific for a protein encoded by a gene inserted into the viral genome [29,37,38]. Recombinant vaccinia virus can be employed as a virus challenged in the protection experiment [29,39]. Second, we used highly reactive HLA-A*0201 transgenic mice, termed HHD mice [20]. Because the innate $H\text{-}2D^b$ and mouse β2m genes have been disrupted by homologous recombination in HHD mice, the only MHC class I molecule on the cell surface, HLA-A*0201, is efficiently utilized by HLA- A*0201-restricted CTLs. We used lymphocytes of HHD mice infected with Ad-SARS-N8E as a replacement for PBL of SARS patients. As a consequence, we identified three HLA-

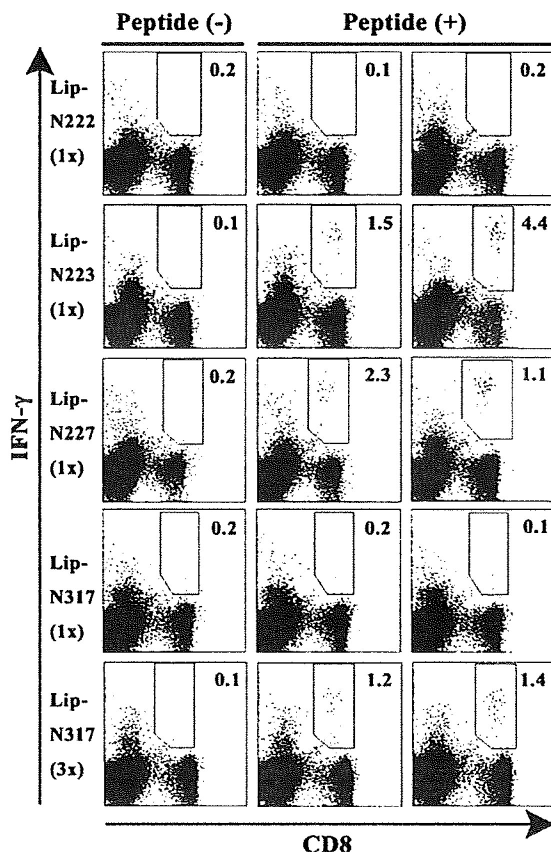


Fig. 5. Intracellular IFN-γ staining of CD8⁺ T cells specific for SARS-CoV-N-derived peptides in spleen cells of mice immunized with surface-linked liposomal peptides. HHD mice received one injection (1×) of either Lip-N222, Lip-N223, Lip-N227 or Lip-N317, or three injections (3×) of Lip-N317 together with a liposomal helper peptide and CpG. After 1 week, spleen cells were prepared and stimulated with a relevant peptide (N-222, N-223, N-227 or N-317) for 5 h. Cells were then stained for their surface expression of CD8 (x-axis) and their intracellular expression of IFN-γ (y-axis). The numbers shown indicate the percentages of intracellular IFN-γ^+ cells within $CD8^+$ T cell. The data shown are representative of three independent experiments.

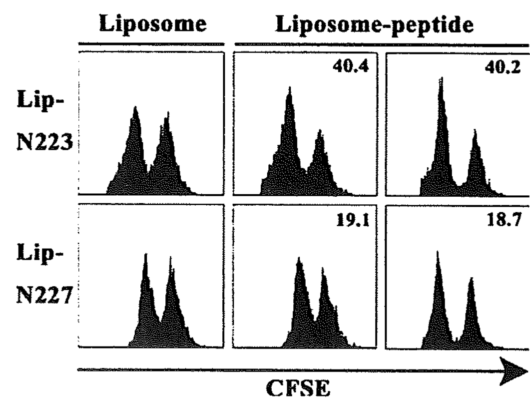


Fig. 7. *In vivo* killing activities specific for N-223 and N-227 in HHD mice immunized with surface-linked liposomal peptides. HHD mice were immunized once with either Lip-N223 or Lip-N227 together with a liposomal helper peptide and CpG in the footpad. One week later, an equal number of a relevant peptide (N-223 or N-227)-pulsed $CFSE^{high}$ targets and unpulsed $CFSE^{low}$ targets were transferred into the immunized mice by i.v. injection. After 12 h, CFSE-labeled cells were recovered from spleens of recipient mice and analyzed by flow cytometry. The numbers show the percentages of specific lysis. The experiment was repeated twice.

A*0201-restricted CTL epitopes, N-223, N-227, and N-317, derived from SARS-CoV-N, which were identical to those reported by Tsao et al. [40]. This indicates that our approach has proved to be practical in the epitope identification for viruses. However, it has to be taken account that there may be differences between the immunogenic variations observed in HLA class I transgenic mice and that in humans primarily because the antigen processing, presentation and ultimately, immunodominance may differ between them. Further, it will be necessary to use SARS-CoV for viral challenge experiments at the final stage.

N-222 peptide is unlikely to be an epitope because N-222-specific CTLs could not be induced by immunization with Lip-N222 (Fig. 5). However, N-223-specific CTLs primed by Lip-N223 were activated by stimulation with N-222 as well as N-223 (Fig. 6). Accordingly, when interpreting the data concerning N-222 in Figs. 2–4, we could propose an explanation that immunization of mice with Ad-SARS-N8E did not induce N-222-specific CTLs but

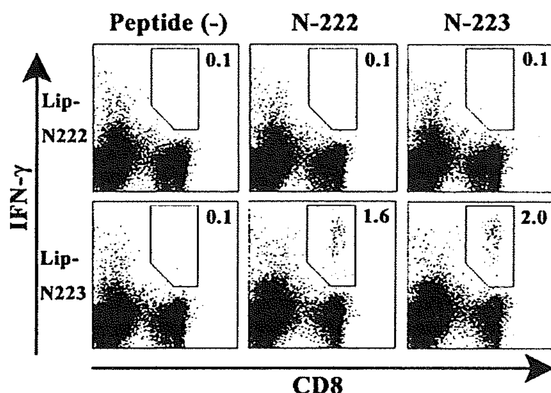


Fig. 6. Cross reactivity between N-222 and N-223 peptides. HHD mice were immunized with either Lip-N222 or Lip-N-223 together with a liposomal helper peptide and CpG in the footpad. One week later, spleen cells were prepared and stimulated *in vitro* with or without either N-222 or N-223 for 5 h. Cells were then stained for their surface expression of CD8 (x-axis) and their intracellular expression of IFN-γ (y-axis). The numbers shown indicate the percentages of intracellular IFN-γ^+ cells within $CD8^+$ T cell. The experiment was repeated twice with similar results.

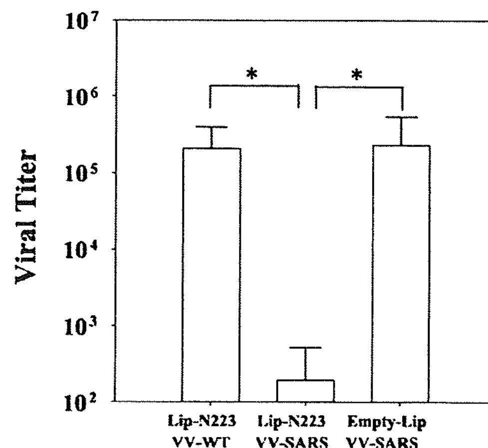


Fig. 8. Resistance to infection with vaccinia virus expressing N-223 in mice immunized with Lip-N223. HHD mice were immunized twice with either Lip-N223 or empty liposomes (Empty-Lip) along with a liposomal helper peptide and CpG at 2-week intervals. Two weeks later, mice were challenged i.p. with 1×10^6 PFU of either VV-SARS-N8E (VV-SARS) or VV-WT. Mice were then sacrificed 5 days after challenge, and viral titers in the ovaries were measured. All titrations were performed in duplicates, and the average PFU per mouse is shown in the figure. Three mice were used in each group, and data are shown as the mean ± SD of three mice. $^*P<0.01$.

did N-223-specific CTLs, which recognized N-222 as well as N-223 (Figs. 2–4).

In the current study, we have shown that surface-linked liposomal peptides such as Lip-N223 and Lip-N227 were very effective for the induction of peptide-specific CTLs in mice as well as recombinant adenovirus, Ad-SARS-N8E (Figs. 2 and 5). Of note, the most immunogenic liposomal peptide, Lip-N223 efficiently induced protection against viral challenge with vaccinia virus expressing N-223 (Fig. 8). These data strongly suggest that the surface-linked liposomal peptide may offer an effective and safe CTL-based vaccine against SARS. However, *in vivo* CTL activity induced by Lip-N223 immunization was half of that induced by Ad-SARS-N8E (Figs. 4 and 7) although the level of IFN-γ-producing CD8$^+$ T cells was similar between the Ad-SARS-N8E and Lip-N223 immunization (Figs. 2 and 5). These data might suggest that liposomes disturb the CTL killing activity in some degree. Furthermore, a number of IFN-γ-producing CD8$^+$ T cells specific for N-317 were elicited in mice that had received one injection of Ad-SARS-N8E (Fig. 2). In contrast, three injections of Lip-N317 were required for the significant induction of N-317-specific IFN-γ-producing CD8$^+$ CTLs in mice (Fig. 5). These data might suggest that an exogenous peptide conjugated on the surface of liposomes may be processed and presented to peptide-specific CTLs in a different way from a naturally processed, endogenous peptide derived from adenovirus.

Our surface-linked liposomal peptide might be similar to the lipopeptide, a form of palmitoyl-lipidated peptide that is currently under intense investigation as human vaccines for many infectious pathogens and cancers [41–43]. Although both effectively induce peptide-specific CTLs, there are several differences between them. Fist of all, self-adjuvanting lipopeptides stimulate peptide-specific CTLs via Toll-like receptor (TLR)-2 without any particular adjuvants [42,43]. In contrast, induction of CTLs by surface-linked liposomal peptides requires external TLR ligands such as CpG [14]. However, it is well known that CpG causes toxicity in humans [44]. Hence, it is essential to find out a safe adjuvant for clinical use of liposomal peptides. Second, lipopeptides administered intranasally, sublingually or intravaginally are able to induce mucosal and systemic immune responses [43]. This application offers the advantage of needle-free delivery. It is, however, still under investigation whether surface-linked liposomal peptides intranasally stimulate peptide-specific CTLs.

In summary, we first tried to identify HLA-A*0201-restricted CTL epitopes derived N protein of SARS-CoV using computational algorithm, recombinant adenovirus and HLA-A*0201 transgenic mice. Four peptides that were expected to be epitopes were then chemically conjugated on the surface of liposomes. It was shown that two of the liposomal peptides were effective for peptide-specific CTL induction, and the most immunogenic liposomal peptide efficiently induced protection against viral challenge with vaccinia virus expressing this peptide. These data suggest that the surface-linked liposomal peptide may be useful for CTL-based immunotherapy against SARS.

Acknowledgments

This work was supported by a grant from The Ministry of Health, Labor and Welfare of Japan. The authors are grateful to Dr. F.A. Lemonnier (Pasteur Institute, Paris, France) for providing HHD mice and the RMA-HHD cell line, and to Dr. T. Shioda for providing CV-1, BS-C-1, C143 cell lines, and vaccinia virus (WR strain).

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Biochemical and Biophysical Research Communications 391 (2010) 1494–1499

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A CTL-based liposomal vaccine capable of inducing protection against heterosubtypic influenza viruses in HLA-A*0201 transgenic mice

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ARTICLE INFO

Article history:
Received 2 December 2009

Keywords:
Influenza
Vaccine
Liposome
CTL

ABSTRACT

The current vaccination strategy against influenza is to induce the production of antibodies directed against surface antigens of viruses. However, the frequent changes in the surface antigens of influenza viruses allow the viruses to avoid antibody-mediated immunity. On the other hand, it is known that cytotoxic T-lymphocyte (CTL) populations directed against internal antigens of influenza A virus are broadly cross-reactive to influenza virus subtypes. In the present study, liposomal conjugates with CTL epitope peptides derived from highly conserved internal antigens of influenza viruses were evaluated for their ability to protect against infection with influenza viruses. Liposomal conjugates with peptide M1 58-66, an HLA-A*0201-binding CTL epitope present within the amino-acid sequence of the M1 coding region, successfully induced antigen-specific $CD8^+$ T-cells and CTLs in HLA-A*0201-transgenic mice. Moreover, after nasal infection with either the H1N1 or H3N2 virus, viral replication in the lung was significantly inhibited in the immunized mice. These protective activities lasted at least 6 months after the immunization. Thus, these results suggest that liposome-coupled CTL epitope peptides derived from highly conserved internal antigens of influenza viruses might be applicable to the development of vaccines that induce protection against infection with heterosubtypic influenza viruses.

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Introduction

A novel swine-origin influenza A (H1N1) virus (S-OIV) emerged in Mexico in March 2009 and continues to spread globally via human-to-human contact. Although S-OIV belongs to the same hemagglutinin (HA) serotype as seasonal human influenza A (H1N1) viruses, vaccination with seasonal influenza vaccine does not result in cross-reactive antibodies [1], possibly because the protein sequence divergence of the S-OIV H1 from human seasonal influenza H1 is wide: around 20–24% [2]. The current vaccination strategy is to elicit neutralizing antibody responses against HA, a surface glycoprotein of influenza viruses. Although antibody-mediated immunity to the surfaces of viruses reduces the probability of infection and morbidity, an antibody against one influenza virus type or subtype can provide limited or no protection against another. Moreover, an antibody to one antigenic variant of an influenza virus might not protect against a new antigenic variant of the same type or subtype, as a consequence of the frequent development of antigenic variants through antigenic drift. Therefore, although vaccines designed to induce antibodies against HA provide reasonable protection against the homologous viruses, it is feared that the vaccines currently being produced may have HA sequences so different from those of any pandemic strain that the vaccines would have little or no efficacy, due to the high rate of viral diversification [3].

Upon natural infection, it is known that the host responds by inducing humoral and cellular immunity against the pathogen. Humoral immune responses are represented by the production of antibodies that bind to the surfaces of bacteria and viruses, whereas cellular immune responses mediate immunity to intracellular pathogens. Since Effros et al. [4] reported in 1977 that influenza A virus-specific cytotoxic T-lymphocytes (CTLs) are broadly cross-reactive for cells of the same MHC class I type infected with serologically distinct H1N1 and H3N2 viruses, it has been known that cytotoxic T-cells specific to internal proteins show high cross-reactivity between strains and between subtypes, reflecting more than 90% conservation of the internal proteins [5]. Lee et al. [6] recently reported that memory T-cells established by seasonal human influenza A infection cross-react with H5N1 in healthy individuals who have not been exposed to H5N1 viruses. $CD8^+$ T-cells of the participants recognized multiple synthesized

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doi:10.1016/j.bbrc.2009.12.100

influenza peptides, including peptides from the H5N1 strain. Thus, vaccine formulas that include heterosubtypic T-cell-mediated immunity might confer broad protection against avian and human influenza A viruses.

In general, extracellular antigens are presented via MHC class II molecules to $CD4^+$ T-cells, whereas intracellular antigens are presented via MHC class I molecules to $CD8^+$ T-cells. To induce influenza-specific CTLs, vaccine antigens must be loaded onto the class I MHC processing pathway in the antigen-presenting cells (APCs) via cross-presentation. In the cross-presentation, exogenous proteins cross over to the endogenous pathway to gain access to MHC class I molecules [7]. However, the currently approved alum adjuvant, which was first described by Glenny and Pope [8] in 1926, is known to be effective only for the induction of humoral immunity, not for the induction of cell-mediated immunity [9]. Consequently, the development of a novel vaccine adjuvant is essential for the production of the $CD8^+$ T-cell vaccine.

In our previous study [10], we produced surface-coupled liposomal antigens that were presented by APCs to $CD8^+$ T-cells via MHC class I molecules. Therefore, this antigen preparation was expected to be applicable to the development of viral vaccines to induce virus-specific CTLs that effectively eliminate virus-infected cells [11]. Since the liposomal conjugates induced CTLs efficiently when CTL epitope peptides were coupled to the surfaces of liposomes [10], the liposomal conjugates were expected to be applicable to the development of peptide vaccines. We recently demonstrated that liposome-coupled peptide $NP_{366-374}$, derived from nucleoprotein (NP) of influenza H3N2 viruses, induced antigen-specific CTLs and successfully suppressed influenza H3N2 viral replication in the lung in C57BL/6 mice [12]. In the present study, we evaluated a possible application of liposome-coupled peptides to the development of an influenza vaccine using HLA-A*0201-restricted CTL epitope peptides and HLA-A*0201-transgenic mice.

Materials and methods

Mice. Mice express a transgenic HLA-A*0201 monochain, designated as HHD, in which human b2-microglobulin (b2m) is covalently linked to a chimeric heavy chain composed of HLA-A*0201 (a1 and a2 domains) and $H-2D^b$ (a3, transmembrane, and cytoplasmic domains) [13]. Eight- to twelve-week-old mice were used for all experiments. The mice were housed in appropriate animal care facilities at Saitama Medical University, Saitama, Japan. Experiments in the present study were approved (No. 214) by the Animal Research Committee of Saitama Medical School and the mice were handled according to international guidelines for experiments with animals.

Reagent. Synthetic CpG ODN (5002: TCCATGACGTTCTTGATGTT) was purchased from Hokkaido System Science (Sapporo, Japan) and was phosphorothioate-protected to avoid nuclease-dependent degradation.

Synthetic peptides. The HLA-A*0201-restricted, influenza virus matrix protein-derived peptide M1 58-66 (GILGFVFTL) [14] and two newly determined peptides—NS1 128-136 (IILKANFSV), which corresponds to residues 128–136 of the nonstructural protein 1 and PB1 410-418 (GMFNMLSTV), which corresponds to residues 410–418 of the polymerase PB1 protein—were synthesized by Operon Biotechnologies (Tokyo, Japan).

Coupling of peptides to liposomes. Liposomal conjugates with peptides derived from internal antigens of influenza virus were prepared essentially in the same way as described previously [10] using disuccinimidyl suberate (DSS). Briefly, a mixture of 10 ml of anhydrous chloroform solution containing 0.136 mM DOPE and 24 ml of TEA was added in drops to 26.6 ml of anhydrous chloroform solution containing 0.681 mM DSS and stirred for 5 h at 40 °C. The solvent was evaporated under reduced pressure, and 18 ml of a 2:1 mixture of ethyl acetate and tetrahydrofuran was added to dissolve the residue. To the solution was added 36 ml of 100 mM sodium phosphate (pH 5.5) and 90 ml of saturated NaCl aqueous solution. The mixture was, shaken for 1 min and allowed to separate. To remove undesirable materials, the upper layer was washed with the same buffer. After evaporation of the solvent, 3 ml of acetone was added to dissolve the residue. Then, 100 ml of ice-cold acetone was added in drops and kept on ice for 30 min to precipitate. Crystals were collected and dissolved in 5 ml of chloroform. After evaporation, 34.4 mg of DOPE-DSS was obtained, then 0.18 mM DOPC, 0.03 mM DOPE-DSS, 0.21 mM cholesterol, and 0.06 mM DOPG were dissolved in 10 ml of chloroform/methanol. The solvent was removed under reduced pressure and 5.8 ml of phosphate buffer (pH 7.2) was added to make a 4.8% lipid suspension. The vesicle dispersion was extruded through a 0.2 mm polycarbonate filter to adjust the liposome size. A 2 ml suspension of DSS-introduced liposome and 0.5 ml of 10 mg/ml peptide solution were mixed and stirred for 3 days at 4 °C. The liposome-coupled and -uncoupled peptides were separated using CL-4B column chromatography. The resulting PBS solution of peptide-liposome conjugates, containing approximately 0.7 mg peptides/ml, was sterile filtered and kept at 4 °C until use.

Cell line. Madin-Darby canine kidney (MDCK) cell line was obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS).

Influenza viruses. Two influenza A virus strains—H1N1 (A/PR/8/34) and H3N2 (A/Aichi/2/68)—were propagated in 10-day-old embryonated hen's eggs at 35 °C for 3 days. Egg allantoic fluid containing either virus was then harvested and stocked at −80 °C. Virus titers were determined by calculating the 50% tissue culture infectious dose ($TCID_{50}$) using MDCK cells [15]. The value of 50% mouse lethal dose (LD_{50}) of the H1N1 (A/PR/8/34) virus was determined in mice that were inoculated intranasally (i.n.) under anesthesia. The A/PR/8/34 (H1N1) virus was purchased from ATCC, and the H3N2 (A/Aichi/2/68) virus was kindly provided by Dr. Hiroshi Kida of Hokkaido University, Japan.

Immunization. Mice were subcutaneously (s.c.) immunized with 100 µl solution of the peptide-liposome conjugates in the presence of 5 µg CpG.

Intracellular cytokine staining. Intracellular cytokine staining (ICS) was performed as described previously [16]. Briefly, after one week following immunization, 2×10^6 spleen cells of immunized mice were incubated with 10 mM of the peptide M1 58-66 for 5 h at 37 °C in the presence of brefeldin A (GolgiPlug™, BD Biosciences, San Jose, CA). After Fc receptors were blocked with the rat anti-mouse CD16/CD32 mAb (Fc Block™, BD Biosciences), cells were stained with FITC-conjugated rat anti-mouse CD8α mAb (BD Biosciences) for 30 min at 4 °C. The cells were then fixed, permeabilized, and stained with phycoerythrin (PE)-conjugated rat anti-mouse interferon-gamma (IFN-γ) mAb (BD Biosciences). After the cells were washed, flow cytometric analyses were performed.

Table 1
Amino acid sequences of CTL epitopes derived from internal antigens of influenza viruses.

Virus strain	M1 58-66	NS1 128-136	PB1 410-418
H1N1 (A/PR/8/34)	GILGFVFTL	IILKANFSV	GMFNMLSTV
H3N2 (A/Aichi/2/68)	GILGFVFTL	VMLKANFSV	GMFNMLSTV
H1N1 (A/New York/4290/2009)	GILGFVFTL	IVLKANFSV	GMFNMLSTV
H5N1 (A/Hong Kong/483/97)	GILGFVFTL	IILKANFSV	GMFNMLSTV

Disagreements are underlined.