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Optimization of Codon Usage of Plasmid DNA Vaccine Is Required for the Effective MHC Class I-Restricted T Cell Responses Against an Intracellular Bacterium

Masato Uchijima, Atsushi Yoshida, Toshi Nagata, and Yukio Koide

In an attempt to study codon usage effects of DNA vaccines on the induction of MHC class I-restricted T cell responses against an intracellular bacterium, Listeria monocytogenes, we designed two plasmid DNA vaccines encoding an H-2Kβ-restricted epitope of listeriolysin O (LLO) of L. monocytogenes, LLO 91–99. One DNA vaccine, p91wt, carries the wild-type DNA sequence encoding LLO 91–99, and the other one, p91mam, possesses the altered DNA sequence in which the codon usage was optimized for murine system. Our read-through analyses with LLO 91–99/luciferase fusion genes confirmed that the optimized 91mam DNA sequence showed extremely higher translation efficiency than the wild-type sequence in murine cells. Consistent with this, i.m. injections of p91mam, but not of p91wt, into BALB/c mice were capable of inducing specific CTL- and IFN-γ-producing CD8+ T cells able to confer partial protection against listerial challenge. Taken together, these observations suggest that optimization of codon should be taken into consideration in the construction of DNA vaccines against nonviral pathogens.


Materials and Methods

Mice

BALB/c mice were maintained in our own animal facilities from a breeding stock obtained from Japan SLC (Hamamatsu, Japan). Mice were used between 6 and 12 wk of age.

Plasmid DNA construction

p91wt and p91mam plasmids. Two different complementary oligonucleotides encoding LLO 91–99 of L. monocytogenes, flanked by ATG start codon and TAG stop codon, were synthesized at Bio-Synthesis (Lewisville, TX) and were cloned into the Smal site located downstream to the

Abbreviations used in this paper: LLO, listeriolysin O; RSCU, relative synonymous codon usage; Tc1, T cytotoxic-1.
CMV immediate-early enhance/promoter region of an expression plasmid pCI (Promega, Madison, WI). p91wt was designed to express wild-type DNA sequence encoding LLO 91–99, whereas the DNA sequence of p91mam was adapted to the most frequently used codon in murine genes (Table I). The sequences designed in the p91wt and p91mam were confirmed by DNA sequencing (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Foster City, CA) and were ATG GGT TAC AAA GAT GGA AAT GAA TAT ATT TAG and ATG GGC TAC AAG GAC GGC AAC GAG AGA in the pCI ATG, respectively.

Table I. Values of RSCU for codons used by p91wt and p91mam sequences in L. monocytogenes and Mus musculus.

<table>
<thead>
<tr>
<th>LLO 91–99</th>
<th>p91wt</th>
<th>RSCU</th>
<th>p91mam</th>
<th>RSCU</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>aa</td>
<td>Triplet</td>
<td>Ln</td>
<td>M.</td>
</tr>
<tr>
<td>91</td>
<td>G</td>
<td>GGU</td>
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<td>0.697</td>
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<td>92</td>
<td>Y</td>
<td>UAC</td>
<td>0.599</td>
<td>1.178</td>
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<tr>
<td>93</td>
<td>K</td>
<td>AAA</td>
<td>1.710</td>
<td>0.767</td>
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<tr>
<td>94</td>
<td>D</td>
<td>GAA</td>
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<td>0.878</td>
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<td>G</td>
<td>GGA</td>
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<td>0.835</td>
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<td>GAA</td>
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<td>99</td>
<td>I</td>
<td>AAU</td>
<td>1.736</td>
<td>0.996</td>
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</tbody>
</table>

* RSCU, relative synonymous codon usage.
* aa, amino acid.
* Ln, L. monocytogenes.

Bacterial infection

L. monocytogenes, strain EGD, was kept virulent by in vivo passage. For inoculations, a seed of L. monocytogenes was cultured overnight in trypticase soy broth (BBL; Becton Dickinson, Cockeysville, MD) at 37°C in a bacterial shaker and suitably diluted with PBS. The exact infection dose was assessed retrospectively by plating. Mice were immunized three times with p91wt or p91mam, as described above, or immunized by single i.v. injection with intact L. monocytogenes (1 × 10⁷ CFU), and 2 wk later were challenged with 3 × 10⁶ CFU of L. monocytogenes. Bacterial titers of the spleen and the liver were determined 72 h after the challenging infection by plating 10-fold dilutions of tissue homogenates on trypticase soy agar (BBL).

Luciferase assay

The p91wt-Luc, p91mam-Luc, or pCI plasmids (0.5 µg) were transfected using SuperFect transfection reagent (Qiagen) into BALB/3T3 cells or COS7 cells cultured in 12-well tissue culture plates. The cells were harvested 48 h after transfection, and cell extracts were prepared by freeze and thaw method as described by Brasier (19). Luciferase reaction was performed using aliquots of cell extracts with PicaGene luciferase assay system (Toyo Ink, Tokyo, Japan), following the manufacturer’s procedure. Luciferase activity was measured with OPTOCOMP II luminometer (MGM Instruments, Hamden, CT). The relative luciferase activities are normalized to the activities of β-galactosidase by the cotransfected pCMVβ (Clontech, Palo Alto, CA).

Statistical analysis

Relative synonymous codon usage (RSCU) values were calculated as described elsewhere (20). An RSCU value for a codon is simply the observed frequency of that codon divided by the frequency expected under the assumption of equal usage of the synonymous codons for an amino acid. Thus,

\[ RSCU_i = \frac{X_i}{n \sum_{j=1}^{n} X_j} \]

where \(X_i\) is the number of occurrences of the \(i\)th codon for the \(i\)th amino acid, and \(n\) is the number (from one to six) of alternative codons for the \(i\)th amino acid.

Values of \(p\) for experiments were analyzed by Student’s \(t\) test using the StatView-J 4.02 statistics program (Abacus Concepts, Berkeley, CA).

Results

Codon usage effects of DNA vaccine on the CTL induction

Our initial attempt to induce CTL by i.m. injection of p91wt encoding LLO 91–99 was unsuccessful. We speculated that one of the reasons for the inability of p91wt to prime CTL was the poor translational efficiency of the plasmid in the murine environment. Therefore, we calculated RSCU values (20) to assess the degree of bias in codon usage in p91wt between L. monocytogenes and the mouse (Mus musculus). To obtain RSCU values, we have taken 2,939,102 codons from murine genes and 28,162 codons from L.
monocytogenes genes (21). The RSCU values for codons in p91wt (LLO 91–99) were presented in Table I. Low RSCU values observed for p91wt (LLO 91–99) in the mice in comparison with those in L. monocytogenes suggest the poor translational efficiency of the plasmid in the murine environment. Therefore, we designed p91mam, in which native codons were substituted with codons frequently found in murine genes, to improve translation efficiency (Table I). As shown in Fig. 1, spleen cells from BALB/c mice immunized three times biweekly with p91mam were found to specifically lyse LLO 91–99-coated but not noncoated J774 cells after in vitro restimulation with LLO 91–99 peptide. However, the same procedure failed to have p91wt induce CTL against LLO 91–99-coated J774 cells. To assess the effectors of p91mam-induced CTL, the immunized mice were in vivo depleted of specific T cell subsets by i.p. injection with a CD4-specific or a CD8-specific mAb before harvest of spleens. Depletion was >95% efficient, as checked by flow cytometry (data not shown). As shown in Fig. 2, depletion of CD4+ T cells did not significantly alter the cytotoxic activity of spleen cells against LLO 91–99-coated J774 cells. In contrast, depletion of CD8+ T cells abolished the cytotoxic activity. These results indicate that CD8+ T cells are responsible for the p91mam-induced CTL activity.

**Codon usage effects of DNA vaccine on the cytokine expression**

Furthermore, we examined the ability of cytokine mRNA expressions in the DNA-immunized spleen cells. Spleen cells from mice immunized with p91mam, but not with p91wt, were capable of inducing mRNA for IFN-γ upon restimulation with LLO 91–99 peptide (Fig. 3). Expression of mRNA for IL-4 was also induced in the nonamer peptide-restimulated spleen cells that had been immunized with p91mam; however, the level of which was substantially lower than that of IFN-γ mRNA. We also evaluated the IFN-γ production of p91wt-immunized and p91mam-immunized spleen cells employing ELISA. Upon in vitro restimulation with the nonamer peptide for 5 days, the p91mam-immunized spleen cells produced 41,552 pg/ml of IFN-γ. However, p91wt-immunized spleen cells produced only background level of IFN-γ (Fig. 4). In vivo depletion experiments with anti-CD4 or anti-CD8 mAb revealed that the IFN-γ-producing cells induced by p91mam were CD8+ T cells (data not shown). These data suggest that the immunization of p91mam may generate T cytotoxic-1 (Tc1)-type responses.

**Comparison of translational efficiency between p91wt and p91mam by a read-through analysis**

As an experimental approach to compare translational efficiencies between p91wt and p91mam, we employed a read-through analysis in which the luciferase cDNA was fused to downstream of the wild-type or adapted LLO 91–99 sequence, resulting in p91wt-Luc and p91mam-Luc, respectively, to express LLO 91–99/luciferase fusion proteins, and the conventional luciferase assay was performed using BALB/3T3 murine fibroblast cells and COS7 monkey kidney cells. In this experiment, luciferase activities are critically dependent on the translational efficiencies of the upstream sequences, LLO 91–99wt and LLO 91–99mam, because the promoter and luciferase DNA sequence that we used in the construction of p91wt-Luc and p91mam-Luc for the read-through analysis.

**FIGURE 1.** Codon usage effects of DNA vaccine on CTL induction. BALB/c mice were immunized i.m. with p91wt (■, □) or p91mam (○, △) three times biweekly. Spleen cells from immunized mice were harvested 2 wk after the last immunization and stimulated in vitro with LLO 91–99-pulsed spleen cells for 5 days. Percentage of specific lysis was determined using J774 cells (H-2d) pulsed with LLO 91–99 peptide (●, ○) or control medium (□, △) as target cells. Results are expressed as mean ± SD for eight independent experiments. *, Statistically significant differences in specific lysis between p91mam against J774 pulsed with LLO 91–99 and the others (p < 0.05).

**FIGURE 2.** p91mam induces CD8+ CTL specific for LLO 91–99. Immunized mice were T cell subset depleted by i.p. injection of 1 mg of ascites mAbs against CD4 (∗) or CD8 (▲) 2 days before removal of spleens. The T cell subset-depleted and control (●) spleen cells were restimulated in vitro, and percentage of specific lysis was determined, as described in Fig. 1. Results are expressed as mean ± SD for eight independent experiments.

**FIGURE 3.** Cytokine mRNA expressions by LLO 91–99-stimulated spleen cells from p91wt- or p91mam-immunized mice. BALB/c mice were immunized i.m. with p91wt or p91mam three times biweekly. Spleen cells from immunized mice were harvested 2 wk after the last immunization and stimulated in culture in the presence or absence of LLO 91–99 peptide for 48 h. IFN-γ, IL-4, and β-actin mRNA expressions were detected using semiquantitative reverse-transcriptase PCR.
are the same. As shown in Fig. 5, the relative luciferase activities of p91mam-Luc were remarkably higher than those of p91wt-Luc in both BALB/3T3 and COS7 cells, suggesting that p91mam expresses a much higher level of LLO 91–99 in the immunized murine cells than p91wt. The possible differences of transfection efficiencies were normalized to the activities of β-galactosidase by the cotransfected pCMVβ.

**Bacterial clearance in p91wt- and p91mam-immunized mice**

Given that p91mam was capable of inducing specific CTL- and IFN-γ-producing CD8⁺ cells, we wished to determine the biologic effect of p91mam and p91wt in combating bacterial challenge. Seventy-two hours after sublethal bacterial challenge, mice from each group were sacrificed, and the CFUs from the spleen and liver were counted. As shown in Fig. 6, mice immunized with p91wt showed the number of *L. monocytogenes* in the spleen and liver comparable with those in nonimmunized control mice. However, mice immunized with p91mam have 1 to 2 logs fewer CFUs in the spleen and liver than those immunized with p91wt (p < 0.001).

**FIGURE 4.** IFN-γ secretion by LLO 91–99-stimulated spleen cells from p91wt- or p91mam-immunized mice. BALB/c mice were immunized i.m. with p91wt or p91mam three times biweekly. Spleen cells from immunized mice were harvested 2 wk after the last immunization and stimulated by culturing in the presence or absence of LLO 91–99 peptide for 5 days. Concentration of IFN-γ in the culture supernatants was determined by capture ELISA. Results are expressed as mean ± SD for six experiments. Statistically significant difference was observed in IFN-γ secretion between spleen cells immunized with p91wt and those with p91mam (p < 0.001).

**FIGURE 5.** Translational efficiencies of p91wt and p91mam were determined by measurement of luciferase activity in cultured cells transiently transfected with p91-luciferase fusion reporter plasmids. A, A schematic diagram and the DNA sequences of the 5’ region of the LLO 91–99-luciferase fusion reporter genes. The LLO 91–99 and luciferase-encoding regions are connected by the connecting region (CT; Lys-Leu-Glu). The nucleotides in p91mam-Luc identical to that in p91wt-Luc are shown as hyphens (–). The corresponding amino acid sequences are shown over the nucleotide sequences. The LLO 91–99 region is underlined, and the luciferase region is italicized. B, Relative luciferase activities assayed by transient transfections of p91-luciferase fusion reporter plasmids, p91wt-Luc and p91mam-Luc in BALB/3T3 mouse fibroblast cells (left), and COS7 monkey kidney cells (right). The relative luciferase activities are normalized to β-galactosidase activities by the cotransfected pCMVβ. Data are expressed as the mean value from triplicate samples. The value of the background without sample has been subtracted. Error bars indicate SD.

**FIGURE 6.** Immunization with p91mam, but not with p91wt, leads to accelerated clearance of *L. monocytogenes*. Mice were immunized with p91wt, p91mam, or intact *L. monocytogenes* (LM) and 2 wk later were challenged with 3 × 10⁴ CFU of *L. monocytogenes*. Bacterial titers of spleens and livers were determined 72 h after the challenging infection by plating 10-fold dilutions of tissue homogenates on trypticase soy agar plates. Results are expressed as mean ± SE for four independent experiments. *, p < 0.025 compared with control.
spleen and liver than control and p91wt-immunized animals. As expected, mice immunized with intact *L. monocytogenes* (1 × 10^5 CFU) most efficiently cleared the infected bacteria. These data provide the evidence that immunization with p91mam is effective in controlling the organism replication.

**Discussion**

From the data described above, we were able to draw the following conclusions about plasmid DNA vaccine encoding a CTL epitope against *L. monocytogenes*: 1) DNA vaccine encoding a single epitope, LLO 91–99, is capable of inducing specific CTL- and IFN-γ-producing CD8⁺ T cells in BALB/c mice as long as codon usage of the DNA vaccine is optimized; 2) the optimized 91mam DNA sequence shows substantially higher translational efficiency than the wild-type sequence in mammalian cells; and 3) the p91mam vaccine confers partial protection against listerial infection.

Attempts have been made to demonstrate efficacy of a single epitope to induce antilisterial CTL. However, synthetic LLO 91–99 peptide does not elicit CD8⁺ T cell response unless administrated as a liposome peptide complex (22) or expressed from a *Salmonella* recombinant as a minigene embedded in the *Salmonella* flagellin protein (23), suggesting that an efficient delivery system is required for the peptide immunization. On the contrary, plasmid DNA immunization offers the potential to prime for CTL responses as a result of nonself gene products being expressed within the cytoplasm of cells transfected in vivo. Furthermore, it is suggested that the immunostimulatory CpG motifs that stimulate IL-12 result in promoting the generation of CTL (24, 25). Thus, DNA immunization has potential advantage to induce CTL against intracellular pathogens.

In our initial experiments, i.m. injection of p91wt into BALB/c mice failed to induce either CTL against LLO 91–99-coated J774 cells or IFN-γ mRNA and protein expressions in response to the nonamer peptide. It is well known that codon bias has been observed in many species and that the usage of selective codons in a given gene is positively correlated with its expression efficiency (26, 27). Therefore, we constructed another plasmid-expressing LLO 91–99, p91mam, in which native codons were substituted with codons frequently found in highly expressed murine genes. We found that i.m. injection of p91mam successfully induces not only CD8⁺ CTL against the nonamer peptide-coated J774 cells, but also IFN-γ mRNA and protein productions in response to the nonamer peptide, suggesting that the immunization generates Tc1 responses.

Consistent with the capability of p91mam to induce the CD8⁺ T cell response, our read-through analyses revealed that p91mam-Luc showed significantly higher luciferase activities than p91wt-Luc in mammalian cells, including a murine cell line, showing that p91mam expresses much higher level of LLO 91–99 in murine cells than p91wt. Because the promoter and luciferase DNA sequence that we used in the construction of p91wt-Luc and p91mam-Luc for the read-through analysis are the same, we consider that the varied expression levels are due to the different translational efficiencies between a wild-type DNA sequence and a codon-optimized DNA sequence encoding LLO 91–99. In fact, these codon usage optimizations have been employed in heterologous gene expression systems to produce efficient protein expressions (28, 29). Several other methods are possibly employed to demonstrate the difference in the translational efficiency. However, Western blotting was not an effective measure to detect such a short nonamer peptide. Furthermore, transfection of H-2d target cell lines with p91wt or p91mam, followed by detection by LLO 91–99-specific CD8⁺ T cells, seems not to be fully quantitative because the cell lines transfected with wild-type LLO gene were reported to be successfully used as target cells for the CTL (15, 30).

The present data by no means indicate that the codon optimization is required for all antibacterial DNA vaccines. For example, the difference in codon usage bias between *M. tuberculosis* and mice is much less than that between *L. monocytogenes* and mice (21). This is in line with previous studies that provided evidence for successful vaccination of mice against tuberculosis by the injection with the plasmid containing the wild-type DNA sequence encoding a *Mycobacterium* Ag (65-kDa heat-shock protein) (10, 11).

One can envisage that codon optimization resulted in the introduction of immunostimulatory CpG motifs in the p91mam, leading to the increased in vivo priming of CD8⁺ T cell responses. However, two lines of evidence suggest that the possibility seems unlikely. First, the immunostimulatory CpG motifs consisting of unmethylated CpG dinucleotides flanked by two 5’ purines and two 3’ pyrimidines (31) are not found in the 91mam sequence. Second, although immunostimulatory CpG motifs were proved to trigger IL-6, IL-12, and IFN-γ production of spleen cells in vitro (25), stimulation of BALB/c spleen cells with p91mam showed no increase in levels of IFN-γ and IL-12 compared with that with p91wt (data not shown).

Although our study demonstrated that immunization with p91mam, which induces CD8⁺ T cell responses, accelerated clearance of *L. monocytogenes* from the spleen and the liver, the mechanism by which p91mam leads to bacterial eradication is less certain. As CD8⁺ T cells are known to exert their antilisterial effect in a perforin-dependent manner (32), CTL activity induced by p91mam is most likely involved in the clearance. In addition, IFN-γ derived from the CD8⁺ T cells may play a pivotal role in protection against the infection via activation of macrophages, although IFN-γ has been reported to be only marginally important in CD8⁺ T cell-mediated protection against *L. monocytogenes* (33).

Because CD4⁺ Th1 cells as well as CTL play critical roles in clearance of infection with *L. monocytogenes* (34), development of DNA vaccine against MHC class II-binding epitopes of *L. monocytogenes* seems to provide useful information for DNA vaccine primed for Th1 cells against other intracellular bacteria. A peptide region, LLO 215–226, was shown to have high binding affinity for MHC class II molecules, I-Eβ, to induce strong Th cell responses (35). Another peptide region, LLO 203–226, is known to bind to several MHC class II alleles and elicit Th cell responses (35). Therefore, we are currently engaged in investigating whether codon-optimized DNA vaccines encoding these peptides are capable of inducing protective Th1 responses.

One potential problem in applying this approach to vaccination of outbred human population is that large numbers of CTL epitopes of a given Ag such as LLO should be prepared to span the HLA polymorphism. For this purpose, it is conceivable to synthesize the optimized DNA sequences encoding whole Ag proteins. However, the preparation of such long DNA sequences seems to be almost impossible. The problem can be solved by DNA vaccine coding for an artificial polypeptide containing multiple T cell epitopes (36) that are able to be deduced from the known HLA ligand motifs (37). This approach also has the advantage of avoiding the potential hazards of immunizing with DNA vaccine expressing a full-length of Ag that is toxic.

Taken together, our results suggest that optimization of codon usage is an important consideration in constructing DNA vaccines against nonviral pathogens.

Note: Recently, André et al. (38) also reported that DNA immunization with a synthetic HIV gp120 sequence with optimized
codon usage resulted in enhanced humoral and cellular immune responses.

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References


