

Modification of the Anchor Residue to MHC Class I Augments CTL-Inducing Ability of Epitope Peptides Derived from Dengue Virus NS3 Protein

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Abstract

The residue (M) at the C-terminus of the original cytotoxic T lymphocyte (CTL) epitope peptide Den2.4 (GYISTRVEM) spanning the amino acid residues 298-306 of NS3 of dengue virus types 2 and 4 was substituted for L to prepare the peptide Den2.4-9L with a complete H-2K^d-binding motif. Similarly, the peptide Den1.3-9L with the binding motif was prepared from dengue virus types 1 and 3. In the present study, we investigated whether immunization with the corresponding CTL epitope peptide of the different serotype dengue virus induces specific cytotoxic T lymphocytes (CTLs). Subcutaneous immunization of BALB/c mice with Den1.3-9L emulsified with complete Freund adjuvant (CFA) induced the CTLs which lysed the target cells (P815) pulsed with peptide Den1.3-9L as well as those pulsed with peptide Den1.3 corresponding to the amino acid residues 299-307 (GYISTRVGM) of NS3 of dengue virus serotypes 1 and 3. Immunization with peptide Den1.3 did not induce CTLs *in vivo*. Furthermore, immunization with the peptide Den1.3-9L emulsified with incomplete Freund adjuvant (IFA) induced CTLs with less non-specific cytotoxicity. These results indicate that modification of dengue virus-derived CTL epitope peptide for providing the complete MHC class I binding motif augments the immunogenicity to induce specific CTLs.

Keywords: Dengue virus, cytotoxic T lymphocyte, epitope peptide, MHC class I, binding motif.

Introduction

MHC class I – restricted, CD8⁺ cytotoxic T lymphocytes (CTLs) play an important role in the clearance of virus-infected cells.^[1] They recognize specific structures on the surface of target cells as their antigens, which are composed of MHC class I molecules and the peptides of 8

to 11 amino acids (a.a.) in length. The peptides are derived from the protein synthesized in cytoplasm, fitting to the groove of the MHC class I molecule.^[2] MHC molecule binds to a peptide with a binding motif, which is determined by peptide length, and a.a. residues called anchor residues.^[3] Recently, there has been a great deal of interest in the CTL epitope-based

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immunomodulation therapy, including peptide vaccines, mainly for treatment of malignancies. It has been reported that the immunogenicity of antigen peptide correlates with its binding affinity to MHC class I molecules.^{14,51}

Dengue viruses, of which there are four serotypes, cause dengue fever and dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS).¹⁶¹ DHF/DSS occurs more commonly in the secondary infections, suggesting that pre-existing immunity to different serotypes of the dengue virus contributes to the pathogenesis of DHF/DSS.¹⁷¹ The ideal strategy for the prevention of dengue virus infection is vaccination. However, the vaccine development has not been accomplished yet. Peptide vaccine can be one of the candidate strategies.

In the present study, we addressed the induction of specific CTLs by epitope peptide immunization. We examined whether modified CTL epitope peptides with a complete binding motif induced CTLs more efficiently than the original ones of dengue viruses, which did not possess complete binding motif. The immunogenicity of modified epitope peptides and original ones for CTL induction was investigated.

Materials and methods

Mice

Female BALB/cAJcl mice were purchased from Clea Japan, and were maintained in the Animal Facility, Kinki University School of Medicine, under conventional conditions. Mice were used at the ages of 6 to 12 weeks.

Cells

Murine mastocytoma line, P815 (H-2^d), was used as target cells in CTL assays. The cells were maintained in RPMI 1640 medium (Sigma, St.

Louis, MO) with 5x10⁻⁵M 2-mercaptoethanol (2-ME), 100U penicillin, 100 µg/ml streptomycin, 10mM HEPES, and 10% heat-inactivated fetal calf serum (Complete medium) at 37 °C in 5% CO₂.

Peptides

The sequences and derivation of peptides, Den2.4 (GYISTRVEM), Den2.4-9L (GYISTRVEL), Den1.3 (GYISTRVGM), and Den1.3-9L (GYISTRVGL), are shown in Table 1. They were synthesized with 9-fluorenylmethoxycarbonyl chemistry by Sigma Genosis, Japan. The purity of these peptides was determined to be more than 95.0% by reverse phase HPLC.

Immunization and CTL induction

Mice were immunized by subcutaneous injection with 1 n mole of the peptide emulsified with complete Freund adjuvant (CFA) (Wako Pure Chemical Industries, Osaka, Japan) or incomplete Freund adjuvant (IFA)

Table 1: Synthetic peptides used in the study

Peptide	Sequence	Virus derivation	
Den2·4	GYISTRVEM	Dengue virus types 2·4	NS3 298-306
Den2·4-9L	GYISTRVEL	*1	
Den1·3	GYISTRVGM	Dengue virus types 1·3	NS3 299-307
Den1·3-9L	GYISTRVGL	*2	

Note that the difference between Den2.4 and Den1.3 is only the residue at position 8, that is “E” in Den2.4 or “G” in Den1.3.

*1 The sequence corresponds to the residues NS3 298-306 of dengue virus types 2 and 4 except the residue of C-terminus substituted for “L”, and to the residues NS3 299-307 of kunjin virus.

*2 The sequence corresponds to the residues NS3 299-307 of dengue virus types 1 and 3 except the residue of C-terminus substituted for “L”.



(Wako Pure Chemical Industries) into two foot pads. Four weeks later, draining lymph nodes (popliteal lymph nodes) were collected, minced into single cell suspension, and treated with anti-CD4 antibody (BD PharMingen) at 1 µg/1x10⁷ cells and 10% baby rabbit complement (Cederlane, Hornby, Ont., Canada) to deplete CD4-positive cells. Five million cells were then co-cultured with 5x10⁶ of 33Gy X-ray-irradiated syngeneic spleen cells, pulsed with homologous peptide at the concentration of 15 µM and then extensively washed, in 2 ml of EHAA medium (Sigma) supplemented with 100 µg/ml nucleic acid precursors, 2mM L-glutamine, 5x10⁻⁵M 2-ME, 100U penicillin, 100 µg/ml streptomycin, 10 mM HEPES, and 10% fetal calf serum in 24-well plate at 37 °C in 5% CO₂. On day 4, half volume of the medium was replaced with fresh one, and 10 U/ml recombinant mouse IL-2 was added. On day 7, cells were harvested, and used as CTL (cytotoxic T lymphocyte) effector cells.

Cytotoxicity assays

P815 cells (1x10⁶) were pulsed with the peptide at a concentration of 10 mM in complete medium at 37 °C for 3 hours. Cells were labelled with 100 µCi of Na₂⁵¹CrO₄ (NEN Life Science Products, Boston, MA) for last 1 hour, then washed 3 times, and suspended in complete medium. Peptide-pulsed, ⁵¹Cr-labelled cells were seeded in 96-well V-bottom plate at 5x10³ cells in 100 µl of complete medium per well. Effector cells were added to the plate to make effector : target ratio (E/T ratio) of 5:1 or 20:1 in a total volume of 0.2 ml per well, and the plate was incubated at 37 °C in 5% CO₂ for 4 hours. The supernatant fluids were harvested with a Supernatant Collecting System (Skatoron, Lier, Norway), and ⁵¹Cr content was measured by a gamma counter (Aloka model ARC-300). Maximum ⁵¹Cr release was determined by adding 0.1% Triton X, and spontaneous ⁵¹Cr release was determined with the wells that contained target cells and medium only. Assays

were performed in triplicate, and percent-specific lysis was calculated by the formula: % specific lysis = 100 x [(release with effector cells – spontaneous release) / (maximum release – spontaneous release)]. Spontaneous release did not exceed 21.0% of the maximum release. The data were expressed as the mean value of percent-specific lysis.

Results and discussion

We previously reported that intravenous immunization with bone marrow-derived syngeneic dendritic cells pulsed with the modified epitope peptide Den2.4-9L (GYISTRVEL) induced specific CTLs.^{18,91} The last amino acid residue M of the original epitope peptide Den2.4 (GYISTRVEM), which corresponds to the residues 298-306 of NS3 of dengue virus types 2 and 4, was substituted for L in order to provide the complete H-2K^d-binding motif (i.e. Y at position 2 and hydrophobic L or I at C-terminus of 9-mer peptide). We also demonstrated that two foot pad immunization with peptide Den2.4-9L emulsified with CFA induced CTLs which lysed the target cells pulsed with peptide Den2.4 of original epitope as well as those pulsed with peptide Den2.4-9L to similar levels, although two foot pad immunizations with the peptide Den2.4 did not.⁹¹ Thus, we speculated that modification of the epitope peptide by providing a complete binding motif for MHC class I molecule may augment immunogenicity for CTL induction without affecting the specificity.

In the present study, we examined whether a similar result is obtained with peptide Den1.3 (GYISTRVGM), which corresponds to a.a residues 299-307 of NS3 of dengue virus types 1 and 3, as is the case with Den2.4 (GYISTRVEM). We prepared a modified epitope peptide Den1.3-9L (GYISTRVGL), in which the last residue M was substituted for L, and compared the abilities of Den1.3-9L and Den1.3 to induce specific CTLs. Immunization with peptide



Den1.3-9L induced the CTLs which lysed target cells pulsed with the modified epitope peptide Den1.3-9L and those pulsed with original epitope peptide Den1.3 to similar levels, as was the case with Den2.4-9L. Immunization with the original epitope peptide Den1.3 with only one anchor residue did not induce specific CTLs, as was similar to Den2.4 (Table 2). These results indicate that the presence of a complete MHC class I-binding motif is important and probably essential for induction of specific CTLs *in vivo*.

We next examined whether immunization with a single modified epitope peptide Den2.4-9L or Den1.3-9L induced CTLs cross-reactive to another epitope peptide. Immunization with peptide Den2.4-9L induced the CTLs which lysed target cells pulsed with peptide Den2.4-9L as well as those pulsed with peptide Den1.3-9L. Reciprocally, immunization with peptide Den1.3-9L induced the CTLs which lysed Den1.3-9L-pulsed target cells as well as Den2.4-9L-pulsed ones (Table 3). These results indicate that immunization with a single modified epitope peptide Den2.4-9L or Den1.3-9L emulsified with CFA induced the CTLs cross-reactive to another epitope peptide.

We also examined whether immunization with modified epitope peptide Den1.3-9L emulsified with IFA, which is mycobacteria component-free and hence applicable for human use, induced the CTLs. Immunization with peptide Den1.3-9L using IFA induced the CTLs which lysed the target cells pulsed with peptide Den1.3 as well as those pulsed with peptide Den1.3-9L (Exp.2, Table 2). These results demonstrate that modification of CTL epitope peptide derived from dengue viruses by amino acid residue replacement to provide with a complete MHC class I-binding motif augmented the immunogenicity for CTL induction to such a degree that Th1 responses through Toll-like receptor-triggering elicited by mycobacteria component in CFA may not be required.^[10]

Thus, it may be theoretically possible to apply synthetic peptides as a candidate peptide vaccine for human use by anchor residue substitution. As a matter of fact, Ennis et al. defined HLA-B35- or HLA-B07-restricted human CTL epitopes of dengue virus, which comprise of dengue type 4 virus NS3 a.a. residues 500-508 or NS3 a.a. residues 221-232, respectively.^[11,12] Enhanced immunogenicity of

Table 2: Induction of specific CTLs by immunization with the modified dengue virus CTL epitope peptide Den2.4-9L or Den1.3-9L emulsified with complete Freund adjuvant (CFA) or incomplete Freund adjuvant (IFA)

Experiment No.	Immunization	% Specific lysis				
		Non-pulsed	Den2.4-pulsed	Den2.4-9L-pulsed	Den1.3-pulsed	Den1.3-9L-pulsed
1.	PBS/CFA	3.2	4.9	3.2	ND	ND
	Den2.4/CFA	0	0.3	1.2	ND	ND
	Den2.4-9L/CFA	15.9	50.5	60.5	ND	ND
2.	PBS/CFA	11.4	ND	ND	13.9	11.7
	Den1.3/CFA	32.8	ND	ND	30.7	33.1
	Den1.3-9L/CFA	31.8	ND	ND	56.9	58.6
	Den1.3-9L/IFA	11	ND	ND	55.3	66.4

Cytotoxicity assay was carried out for 4 hours at effector / target ratio of 5, using ⁵¹Cr-labeled P815 mastocytoma (H-2^d) pulsed with the peptide (10 mM, 3 hours) as target cells.

PBS: phosphate buffered saline.

ND: not done.



Table 3: Induction of cross-reactive CTLs by immunization with the modified dengue virus CTL epitope peptide

Experiment No.	Immunization	% Specific lysis		
		Non-pulsed	Den2.4-9L-pulsed	Den1.3-9L-pulsed
3.	Den2.4-9L/CFA	32	87.8	84.2
4.	Den1.3-9L/CFA	27.4	81	88.6

Cytotoxicity assay was carried out for 4 hours at effector / target ratio of 20, using ⁵¹Cr-labeled P815 mastocytoma (H-2^d) pulsed with the peptide (10 μM, 3 hours) as target cells.

antigenic peptide by means of anchor residue substitution was reported with tumor-associated antigen of human melanoma.^[4]

There are some problems to be addressed for the application of modified dengue virus CTL epitope peptide as vaccine. First, the immunogenicity of a defined CTL epitope peptide is dependent not only on binding affinity to MHC molecule but also on T cell repertoire which is different from individual to individual. Thus, the CTL epitope, which is most commonly shared with majority of population of a certain MHC allele, needs to be defined for the peptide vaccine application. Second, the CTLs cross-reactive to different serotype dengue virus are induced by immunization with a single epitope peptide of dengue virus (Table 3). Presence of these cross-reactive CTLs may induce DHF/DSS after infection with dengue viruses. We have already analysed amino acid residues of peptide

Den2.4 or Den1.3 responsible for recognition by serotype - cross-reactive CTLs, and will determine whether immunization with the other modified epitope peptides may only induce the serotype-specific CTLs. Development of candidate peptide vaccine is an attractive idea. In conclusion, the modification of dengue virus CTL epitope peptide by replacing anchor residue augmented the immunogenicity for CTL induction. It is important to determine whether immunization with these modified epitope peptides really induces protective immunity *in vivo* against dengue virus infection.

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