Enhancement of MHC class I binding and immunogenic properties of the CTL epitope peptides derived from dengue virus NS3 protein by anchor residue replacement

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Abstract

The immunogenecity of the defined H-2Kd-restricted, murine cytotoxic T lymphocyte (CTL) epitopes of dengue viruses were examined for CTL induction in epitope peptide / H-2Kd tetramer assays. The peptides used in the study included those corresponding to amino acid (a.a.) residues 298-306 (GYISTRVEM) of NS3 of dengue virus types 2 and 4 (named DENV-2/4), and to a.a. residues 299-307 (GYISTRVGVM) of NS3 of dengue virus types 1 and 3 (named DENV-1/3), and their respective modified epitope peptides, DENV-2/4-9L (GYISTRVE L) and DENV-1/3-9L (GYISTRVGM), in which the C-terminal residue M of the original epitope peptide was replaced by L, in order to provide the complete H-2Kd-binding motif. Immunization of BALB/c mice with the original epitope peptide, DENV-2/4 or DENV-1/3, did not induce specific CTLs, while that with the modified epitope peptide, DENV-2/4-9L or DENV-1/3-9L, induced epitope peptide/H-2Kd tetramer-binding CD8+ cells indicating specific CTLs. Competition-based binding assay with biotinylated epitope-related reference peptides (DENV-2/4-9L-Biotin and DENV-1/3-9L-Biotin) demonstrated that the modified epitope peptide, DENV-2/4-9L and DENV-1/3-9L, had higher avidity to H-2Kd than the respective original epitope peptides. These results indicate that modification of dengue virus-derived CTL epitope peptide by replacing a.a. residue at the position of anchor residue increases the binding avidity to MHC class I, resulting in the induction of specific CTLs. The strategy to enhance the immunogenecity of CTL epitope peptide may contribute to investigation of CTL biology in dengue virus infection.

Keywords: Dengue virus; CTL epitope; Binding motif; Anchor residue; MHC class I; Affinity; Immunogenicity.

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Avidity and immunogenecity of dengue virus CTL epitope

Introduction

MHC class I – restricted, CD8+ cytotoxic T lymphocytes (CTLs) play an important role in the elimination of virus-infected and tumor cells by antigen-specific lysis\(^1,2,3\). They recognize specific structures on the surface of target cells as their antigens, which are composed of self MHC class I molecules and the peptides of 8 to 11 a.a. in length. The peptides are derived from the endogenous protein, fitting to the groove of the MHC class I molecule\(^4,5,6\). Recently, there has been a great deal of interest in the CTL epitope-based immunomodulation therapy, including peptide vaccines, mainly for the treatment of malignancies\(^7,8,9,10,11,12\).

Dengue viruses, of which there are four serotypes, cause dengue fever and dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS), the severe manifestation of infection, which is often fatal\(^13\). They are of a great global health importance, particularly in the tropical regions, causing up to 100 million infections including a couple of thousands of deaths each year\(^14\). Protective immunity against the same serotype virus is life-long, while re-infection with a different serotype can occur. The secondary infections are often complicated with DHF/DSS, suggesting that pre-existing immunity to a different serotype virus may contribute to the pathogenesis of DHF/DSS\(^15\). One of the strategies for the prevention of dengue virus infection is vaccination. However, a vaccine has not been developed yet. Immunization with dengue vaccine may have the potential risk of inducing DHF/DSS manifestation. In this context, peptide vaccine based on CTL epitopes that binds to MHC class I molecule is thought to be a candidate, because it is anticipated that this vaccine induces the least cross-reaction due to its minimal component. Furthermore, immune response elicited by immunization with a single epitope peptide is thought to be much simpler than those elicited by virus infection, which evokes multiple immune responses against various epitopes on viruses. Establishment of a strategy by immunization with a single dengue virus-derived epitope peptide, thus, is anticipated to facilitate dissection of immunobiology of dengue virus infection. This strategy is expected to contribute to investigation of the immunopathogenesis (DHF/DSS may be involved).

Rothman et al.\(^16\) elucidated CTL responses to an immunodominant epitope on the dengue virus NS3 protein in BALB/c mice after primary infection. They mapped the minimal CTL epitopes consisting of nine amino acids. By using CTL clones, they defined the H-2K\(^d\)-restricted CTL epitopes, which corresponded to the amino acid (a.a.) residues 298-306 (GYISTRVEM) of NS3 of dengue virus types 2 and 4, or a.a. residues 299-307 (GYISTRVGM) of NS3 of dengue virus types 1 and 3\(^16,17\). Immunodominant epitopes for human CD8+ CTLs have been also defined on dengue virus NS3 protein\(^18,19,20,21\) suggesting that NS3 is the main target for the CTL response in humans as well.

Previously, by using cytotoxicity assay, we have demonstrated that immunization with the modified epitope peptide, DENV-1/3-9L (GYISTRVGL) or DENV-2/4-9L (GYISTRVEL), in which the original epitope peptide C-terminal residue M was replaced by residue L to provide a complete H-2K\(^d\)-binding motif\(^22,23\), induced specific CTLs with little affection to antigen specificity, while immunization with original one, DENV-1/3 or DENV-2/4, which corresponded to the defined CTL epitope spanning a.a. residues 299-307 (GYISTRVGM) of NS3 of dengue virus types 1 and 3 or which corresponded to that spanning a.a. residues 298-306 (GYISTRVEM) of NS3 of dengue virus types 2 and 4, respectively, did not\(^24\).
In the present study, we analysed immunogenic properties of the modified epitope peptides for CTL induction more in detail. We first examined whether immunization with the modified epitope peptide induces epitope peptide/H-2K\(^d\)-tetramer-binding CD8\(^+\) cells, the specific CTLs more significantly than that with the original peptide, as was observed in cytotoxicity assays. We also analysed the avidity of the original epitope peptides and modified peptides to H-2K\(^d\) molecule by competition-based binding assay, using the biotinylated epitope peptide-related reference peptides. We demonstrated that the modification of the original epitope peptides by substitution of the C-terminal a.a. residue increased the binding avidity to H-2K\(^d\), and that this modification enhanced the immunogenicity of the epitope peptides in CTL induction.

**Materials and methods**

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

**Cells:** Murine mastcytoma line P815 (H-2\(^d\)), fibroblast cell line L929 (H-2\(^b\)), and cell line L-K\(^d\)-172 (kindly provided by Dr Jack R. Bennink, NIAID, NIH), which is H-2K\(^d\)-gene transfectant cell line derived from L929, were maintained in RPMI 1640 medium (Sigma, St. Louis, MO) with 5x10\(^{-5}\)M 2-mercaptoethanol (2-ME), 100 U penicillin, 100 mg/ml streptomycin, 10 mM HEPES, and 10% heat-inactivated fetal calf serum at 37 °C in 5% CO\(_2\).

**Peptides:** The sequences and derivation of peptides DENV-2/4 (GYISTRVEM), DENV-2/4-9L (GYISTRVEL), DENV-2/4-9L-Biotin (GYISTRVELGEAC-Biotin), DENV-1/3 (GYISTRVGM), DENV-1/3-9L (GYISTRVGL), and DENV-1/3-9L-Biotin (GYISTRVGLGEAC-Biotin) are shown in the Table. They were synthesized with 9-fluorenylmethoxycarbonyl chemistry by Sigma Genosis Japan (Ishikari, Hokkaido, Japan). Peptides were purified by reverse phase HPLC in the conditions of 5% to 80% gradient elution with acetonitrile in 0.1% Table: Synthetic peptides used in the study

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Virus derivation</th>
</tr>
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<tbody>
<tr>
<td>DENV-2/4</td>
<td>GYISTRVEM</td>
<td>Dengue virus types2/4</td>
</tr>
<tr>
<td>DENV-2/4-9L</td>
<td>GYISTRVEL</td>
<td>*1</td>
</tr>
<tr>
<td>DENV-2/4-9L-Biotin</td>
<td>GYISTRVELGEAC-Biotin</td>
<td>*2</td>
</tr>
<tr>
<td>DENV-1/3</td>
<td>GYISTRVGM</td>
<td>Dengue virus types1/3</td>
</tr>
<tr>
<td>DENV-1/3-9L</td>
<td>GYISTRVGL</td>
<td>*3</td>
</tr>
<tr>
<td>DENV-1/3-9L-Biotin</td>
<td>GYISTRVGLGEAC-Biotin</td>
<td></td>
</tr>
</tbody>
</table>

Peptide sequence is expressed by Dayhoff's one-letter code of amino acid except “-Biotin”.

Note that the difference between DENV-2/4 and DENV-1/3 is only the residue at position 8, that is “E” in DENV-2/4 or “G” in DENV-1/3.

*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 298-310 of dengue virus type 4 except the substituted residue “L” and the biotinylated residue “C”.

*3 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”.

**Table:** Synthetic peptides used in the study
trifluoroacetic acid using TSKgel ODS-80Ts QA column (Toso, Tokyo, Japan). The purity of peptides was determined to be more than 95.0%, and mass-spectrometry (Applied Biosystems Voyager System 1162) analysis proved every peptide molecular weight to be the anticipated one.

Immunization and CTL induction: Immunization and CTL induction were carried out as described before. Briefly, mice were immunized by subcutaneous injection with peptide DENV-2/4-9L or peptide DENV-1/3-9L with complete Freund adjuvant (CFA). Then, more than three weeks later, lymph node cells prepared from the draining lymph nodes were co-cultured with irradiated syngeneic spleen cells pulsed with the same peptide in EHAA medium (Sigma), supplemented with 100 µg/ml nucleic acid precursors, 2mM L-glutamine, 5x10⁻⁴M 2-ME, 100U penicillin, 100 µg/ml streptomycin, 10mM HEPES, and 10% fetal calf serum (FCS) at 37 °C in 5% CO₂. On day 4, half volume of the medium was replaced with a fresh one, and 10 U recombinant mouse IL-2 was added. On day 7, viable cells were harvested and used as CTL effector cells.

Detection of MHC tetramer-binding cells: The phycoerythrin (PE)-labelled MHC tetramer composed of four monomeric complexes which consisted of the modified H-2Kd heavy chain, β₂-microglobulin, and peptide DENV-2/4-9L (named DENV-2/4-9L-tetramer) or peptide DENV-1/3-9L (named DENV-1/3-9L-tetramer) were prepared by MBL Co., Ltd. (Nagoya, Aichi, Japan). The CTL effector cells (1x10⁶) suspended in 100 µl of phosphate buffered saline (PBS) containing 0.02% NaN₃ (PBS/Na₃) were incubated with 5 µl of FITC-conjugated anti-mouse CD8 antibody (Immunotech, Marseille, France) and 5 µl of DENV-2/4-9L-tetramer or DENV-1/3-9L-tetramer at room temperature for 30 minutes. Cells were washed three times with PBS/Na₃, at 4 °C, then fixed with 1 ml of PBS containing 1% paraformaldehyde, and analysed by a FACS Calibur (Becton Dickinson, San Jose, CA) and CELL Quest™ version 3.3 software.

Analysis of the avidity between MHC molecule and peptide: Peptide binding competition assay was carried out to determine the relative avidity of the original epitope peptides (DENV-2/4 and DENV-1/3) and that of the modified epitope peptides (DENV-2/4-9L and DENV-1/3-9L) to H-2Kd molecule, using H-2Kd-gene transfectant L-Kd-172 cells and the biotinylated, epitope-related reference peptide. Briefly, 1x10⁶ of L-Kd-172 cells suspended in complete medium were incubated with various concentration of non-biotinylated, original or modified epitope peptide in the presence of 1 µM of biotynilated, epitope-related reference peptide (DENV-2/4-9L-Biotin or DENV-1/3-9L-Biotin, respectively) at 37 °C for two hours. Cells were washed three times with PBS/Na₃ at 4 °C, and then incubated with 0.5 µg of streptavidin-conjugated Cy-Chrome™ (SA-CyC) (BD PharMingen) at 4 °C for 30 minutes. Cells were washed three times, fixed with 1 ml of PBS containing 1% paraformaldehyde, and subjected to FACS analysis.

Geometric mean fluorescence intensity (MFI) was measured, and we defined delta MFI (ΔMFI) by subtracting the background MFI (i.e. MFI in the case stained with SA-CyC only). Per cent fluorescence intensities were calculated by the formula: % fluorescence intensity = 100 x (ΔMFI with the non-biotinylated epitope peptide/ΔMFI without the non-biotinylated epitope peptide), and plotted against non-biotinylated epitope peptide concentrations in logarithmic scale. Concentration of the non-biotinylated epitope peptide that correspond to the per cent fluorescence intensity fifty (IC₅₀: 50% inhibitory concentration) was obtained by the chart, and used as an index of the relative avidity of each peptide to H-2 Kd molecule.
Results and discussion

Because of incomplete set of anchor residues (only one anchor residue Y at position 2) in the original epitope peptide for preparation of peptide/H-2K\textsuperscript{d} tetramers, and because of less affection to the specificity recognized by CTLs with replacement of C-terminal residue M by L\textsuperscript{24}, we prepared phycoerythrin-labelled DENV-1/3-9L/H-2K\textsuperscript{d} and DENV-2/4-9L/H-2K\textsuperscript{d} tetramers. We then examined whether the modified epitope peptides, DENV-2/4-9L and DENV-1/3-9L, induce DENV-2/4-9L/H-2K\textsuperscript{d} tetramer-binding cells and DENV-1/3-9L/H-2K\textsuperscript{d} tetramer-binding cells, respectively, more efficiently than the original peptides, DENV-2/4 and DENV-1/3, as was observed in cytotoxicity assays.

Immunization with the modified peptide DENV-2/4-9L followed by in vitro stimulation with same peptide-pulsed APC induced 5.59% of CD8-positive DENV-2/4-9L/H-2K\textsuperscript{d} tetramer-binding cells, while that with the original peptide DENV-2/4 did 1.25% (Fig. 1A). Similarly, immunization with the modified peptide DENV-1/3-9L followed by in vitro stimulation with same peptide-pulsed APC induced 5.81% of CD8-positive DENV-1/3-9L/H-2K\textsuperscript{d} tetramer-binding cells, while that with the original peptide DENV-1/3 did 0.34% (Fig. 1B). In addition, immunization with PBS emulsified with CFA followed by in vitro stimulation with non-pulsed spleen cells (i.e., pulsed with PBS only) induced 0.15% of CD8-positive DENV-1/3-9L/H-2K\textsuperscript{d} tetramer-binding cells (data not shown). The results indicate that immunization and in vitro stimulation with the modified epitope peptides, which possessed a complete binding motif to H-2K\textsuperscript{d} (i.e. Y at position 2 and hydrophobic L or I at C-terminus of 9-mer peptide), efficiently induced CD8-positive, epitope peptide/H-2K\textsuperscript{d} tetramer-binding cells implying CTLs, as was observed in cytotoxicity assays.

It has been reported that the affinity of a peptide for MHC binding is an important parameter determining the immunogenicity of an MHC-presented epitope peptide\textsuperscript{25,26}. Indeed, only peptides derived from tumour-associated antigens, hepatitis B virus or influenza A virus with a high binding affinity for MHC class I molecules, have been demonstrated to be immunogenic enough for inducing CTL response\textsuperscript{7,9,11,26,27,28,29,30}. However, so far, there had been no report regarding the correlation of binding affinity to MHC class I molecules and immunogenicity of dengue virus-derived CTL epitope peptides for CTL induction. Thus, we assessed the avidity of the modified epitope peptides, DENV-2/4-9L and DENV-1/3-9L, to H-2K\textsuperscript{d} molecules in comparison with the original epitope peptides, DENV-2/4 and DENV-1/3, respectively. Rothman et al.\textsuperscript{16} demonstrated that elongation of the epitope peptide at the C-terminus did not affect the specific lysis of the target cells\textsuperscript{17}. We, thus, prepared the biotinylated epitope peptides of 13-mer elongated at the C-terminal side with 4 a.a. residues spanning NS3 307-310 of dengue virus types 2 and 4, except for C-terminal residue A replaced with C to conjugate biotin, DENV-2/4-9L-Biotin (GYISTRVELGEAC-Biotin) and DENV-1/3-9L-Biotin (GYISTRVGLGEAC-Biotin) (Table), and examined whether they bound to H-2K\textsuperscript{d} molecules. L-K\textsuperscript{d}-172 cells, which are H-2K\textsuperscript{d}-gene transfectant cells derived from cell line L929, gained increased fluorescence intensity in dose-dependent manner after incubation with various concentration of the biotinylated-modified epitope peptide (Fig. 2). In contrast, no specific staining with SA-CyC was observed in L929 cells incubated with the biotinylated epitope peptide. The results indicate that the biotinylated epitope peptides, DENV-2/4-9L-Biotin (GYISTRVELGEAC-Biotin) and DENV-1/3-9L-Biotin (GYISTRVGLGEAC-Biotin), bound to H-2K\textsuperscript{d} molecule specifically, suggesting that they could be used as epitope-related reference peptides for binding competition.
**Figure 1:** Induction of the tetramer-binding cells by immunization with the modified epitope peptides

[Source of the CTLs was pooled lymph node cells (5 mice each).
(A) CD8-positive, DENV-2/4-9L/H-2Kd-tetramer-binding cells (right upper quadrant) accounted for 5.59% of the analysed cells after immunization and in vitro stimulation with modified epitope peptide DENV-2/4-9L, while 1.25% after those with the original epitope peptide DENV-2/4.
(B) CD8-positive, DENV-1/3-9L/H-2Kd-tetramer-binding cells (right upper quadrant) accounted for 5.81% of the analysed cells after immunization and in vitro stimulation with the modified peptide DENV-1/3-9L, while 0.34% after those with the original epitope peptide DENV-1/3.]
Based on the findings mentioned above, we carried out competitive binding inhibition assays with non-biotinylated peptide (i.e., the original epitope peptide or the modified epitope peptide), and compared the relative avidity to H-2K^d molecule. L-K^d-172 cells were incubated with various concentrations of the non-biotinylated peptides in the presence of 1 mM biotinylated reference peptide, and stained with SA-CyC. The geometric mean fluorescence intensity was measured by FACS analysis. Percent fluorescence intensities was plotted against non-biotinylated peptide concentrations, and 50% inhibitory concentrations (IC_{50}) were estimated to evaluate the avidities, meaning that the lower the IC_{50} value is, the higher the avidity of the peptide to H-2K^d is. Percent fluorescence intensity decreased in a dose-dependent manner.
manner as concentration of the non-biotinylated peptide increased (Fig. 3). The IC\textsubscript{50} of DENV-2/4-9L (4.5 µM) was 10.7 times lower than that of DENV-2/4 (48.0 µM). Similarly, the IC\textsubscript{50} of DENV-1/3-9L (7.3 µM) was 2.4 times lower than that of DENV-1/3 (17.8 µM). These results indicate that the modified epitope peptides (DENV-2/4-9L and DENV-1/3-9L) demonstrated higher avidity to H-2K\textsuperscript{d} molecule than the original epitope peptides (DENV-2/4 and DENV-1/3), respectively. These findings, taken together, indicate that modification of dengue virus-derived CTL epitope peptide by replacing a.a. residue at the position of anchor residue to provide a complete binding motif for MHC class I increases the binding avidity to MHC class I, resulting in immunogenicity augmentation for CTL induction by its immunization as has been reported about the CTL epitope peptides derived from tumour-associated antigens or other viruses, and that this strategy may be applicable for induction of dengue virus-specific CTLs by immunization with other CTL epitope peptides of relatively poor immunogenicity.

**Figure 3:** Increase in the avidity of the peptides to H-2K\textsuperscript{d} molecule by substitution of amino acid residue to provide a complete H-2K\textsuperscript{d}-binding motif

[L-K\textsuperscript{d}-172 cells were incubated with various concentrations of non-biotinylated peptides in the presence of 1 µM of the biotinylated peptide. Percent fluorescence intensity (See Materials and methods.) was plotted against logarithmic scale of non-biotinylated peptide concentration, and lines were drawn by using Microsoft Excel 2003\textsuperscript{®} software (DENV-2/4-9L : y = – 44.237x + 78.87 , DENV-2/4 : y = – 94.446x + 208.76 , DENV-1/3-9L : y = – 57.164x + 99.239 , DENV-1/3 : y = – 47.947x + 109.9). The similar experiments were repeated more than three times, and the representative data are shown. The fluorescence intensity of the biotinylated peptide decreased in a dose-dependent manner as concentration of the non-biotinylated peptides increased.

(A) *A : The 50 % inhibitory concentration (IC\textsubscript{50}) of peptide DENV-2/4-9L was 4.5 µM.
   *B : The IC\textsubscript{50} of peptide DENV-2/4 was 48.0 µM.
(B) *C : The IC\textsubscript{50} of peptide DENV-1/3-9L was 7.3 µM.
   *D : The IC\textsubscript{50} of peptide DENV-1/3 was 17.8 µM.

Note that X-axis (peptide concentration) is expressed as logarithmic scale. The modified epitope peptides (DENV-2/4-9L and DENV-1/3-9L) with the substitution of C-terminal residue L for M, that possessed a complete H-2K\textsuperscript{d}-binding motif, demonstrated higher avidity to H-2K\textsuperscript{d} molecule than the original epitope peptides (DENV-2/4 and DENV-1/3).]
There was no significant difference in the avidity to H-2K\(^d\) between DENV-2/4-9L (IC\(_{50}\): 4.5 µM) and DENV-1/3-9L (IC\(_{50}\): 7.3 µM). However, there was an apparent difference between DENV-2/4 (IC\(_{50}\): 48.0 µM) and DENV-1/3 (IC\(_{50}\): 17.8 µM). It was reported that the immunogenicity of MHC class I-restricted peptide is determined not only by binding affinity to MHC molecule but also by T cell repertoire\(^{31}\). Because immunization with the modified epitope peptide, DENV-1/3-9L, induced the CTLs that lysed the target cells pulsed with the original epitope peptide, DENV-1/3, and that these CTLs are cross-reactive to the other original epitope peptide, DENV-2/4 (data not shown), it is not plausible that low immunogenicity of DENV-1/3 is attributed to poor T cell repertoire. We, thus, think of other two possibilities. One possibility is that the avidity to H-2K\(^d\) needs to be between 7.3 µM and 17.8 µM of IC\(_{50}\) to induce CTLs. The other possibility is that not only anchor residues but also the non-anchor residues at secondary position contribute to increased MHC class I avidity and peptide-MHC complex stability\(^{32,33}\). In the present study, we only evaluated the binding avidity. Thus, a detailed study including analysis of the stability of H-2K\(^d\)/DENV-1/3 complex and H-2K\(^d\)/DENV-2/4 complex will be a future subject.

In this paper, we present the first report that modification of dengue virus-derived CTL epitope peptide increasing the binding avidity to MHC class I augmented immunogenicity for CTL induction. The strategy to augment the immunogenicity of dengue virus-derived CTL epitope peptide, established here, is expected to contribute to the immunobiology analysis of dengue virus infection, such as investigation of the immunopathogenesis, protection against infection, and vaccine development.

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References


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