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Note

Two Distinct Epitopes on the Ovalbumin 323-339 Peptide Differentiating CD4⁺T Cells into the Th2 or Th1 Phenotype

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The epitopes for OVA323-339-specific CD4⁺T cells from OVA23-3 food allergy model and DO11.10 tolerant induction model mice were analyzed. We found that OVA23-3 CD4⁺T cells recognized the N-terminal region, showing strong proliferation and the Th2-phenotype, and that DO11.10 CD4⁺T cells recognized the C-terminal region, showing milder proliferation and a Th1-skewed response. These differences may regulate the responses of those mice to OVA-feeding, inflammation and tolerance.

Key words: OVA323-339 amino acid residue; food allergy; tolerance; oral immunotherapy; epitope

T-cell receptor (TCR) transgenic (TCR-Tg) mice have been often used for examining the immune responses to orally administered antigens, because antigen-specific T-cell responses can be visualized. TCR-Tg mice play important roles for this characteristic in clarifying the mechanism or developing oral immunotherapy (OIT) in respect of the antigen-specific T-cell response with food allergy. There are two strains of OVA-specific TCR-Tg mice that are transgenic for OVA323-339-specific and I-A^d-restricted TCR genes, DO11.10 (D10) mice¹⁾ and OVA23-3 mice.²⁾ OVA23-3 mice have been reported as an effective model for food allergy inflammation by feeding an egg white diet (EW-diet) alone.³⁾ It was suggested that IL-4 and the subsequent Th2 cytokine responses would play critical roles in inducing food allergy inflammation in EW-diet-fed OVA23-3 mice.³⁾ On the other hand, D10 mice have been often used as an oral tolerance induction model.⁴⁾ CD4⁺T cells, by recognizing the epitope peptides presented by MHC class II molecules, critically regulate the antigen-specific immune responses in food allergy⁵⁾ or tolerance induction. We have therefore tried to determine CD4⁺T cell epitopes in mice or food allergic patients⁶⁾ to

develop effective induction of tolerance or a treatment for food allergy. We were also interested in the regions of OVA323-339 recognized by naïve CD4⁺T cells from the two strains which exhibited different characteristics by an oral administration of OVA. Since OVA323-339 was a dominant determinant of CD4⁺T cells from egg allergy patients,⁷⁾ examining the epitopes of the two strains was thought to present important information for the development of OIT.

It has been reported that OVA323-339 contained at least two epitopes binding to the I-A^d molecule by using distinct registers.⁸⁾ The crystal structure of the I-A^d-restricted OVA323-339 peptide complex was determined and the core epitope of OVA323-339 was clearly identified in the N-terminal region of the peptide.⁹⁾ This suggested that the N-terminal region might contain the OVA-specific CD4⁺T cell epitope for OVA23-3 and D10 mice.¹⁰⁾ However, some reports have indicated that an epitope for OVA-specific D10 CD4⁺T cells was in the C-terminal region of OVA323-339.¹¹⁾ We therefore examined in this study whether each epitope would be different between CD4⁺T cells of OVA23-3 and that of D10 mice.

To effectively obtain OVA-specific CD4⁺T cells, we used RAG-2-deficient OVA23-3 and D10 (ROVA23-3 and RD10) mice which respectively were kindly given by Dr. S. Habu of Tokai University and by Dr. Y. Wakatuki of Kyoto University.¹²⁾ Both strains were maintained in a specific-pathogen-free room at the University of Tokyo. ROVA23-3 mice have also developed severe inflammation by feeding with an EW-diet.³⁾ The N-terminal 323-336 peptide and C-terminal 326-339 peptide derived from the original OVA323-339 peptide were designed according to previous reports (Fig. 1).^{8,11)} These peptides were synthesized by Laboratory Center for Proteomics Research at The University of Tokyo. The CD4⁺T cells were purified by MACS CD4 beads with the Auto

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Abbreviations: OVA, ovalbumin; RAG, RAG-2-deficient; SP, spleen; EW-diet, egg-white-diet; TCR, T-cell receptor; Tg-mice, transgenic mice

MACS cell separation system (Miltenyi Biotec, Bergish Gladbach, Germany). CD4⁺T cells (1×10^5 cells/well), whose purity was more than 95%, were cultured with different concentrations of three synthetic peptides (0.00005 μ M–5 μ M) and mitomycin-C-treated splenocytes from BALB/c mice (4×10^5 cells/well). Proliferation was analyzed by a tritiated thymidine (37 kBq/well) incorporation assay.³⁾ The cytokine concentration in the 24-h culture supernatant was measured by ELISA (R&D Systems, MN, USA).

The proliferative response and cytokine production of the OVA-specific naïve CD4⁺T cells are indicated in Fig. 2. In response to OVA323-339, ROVA23-3 CD4⁺T cells showed significantly higher response than RD10 CD4⁺T cells at concentrations of 0.05 μ M and 0.5 μ M. RD10 CD4⁺T cells had relatively low potential to produce IL-4 at any concentration of OVA323-339, but showed a biased Th1 response producing IFN- γ . ROVA23-3 CD4⁺T cells produced the highest level of IL-4 at 0.05 μ M. However, the IL-4 production decreased with increasing peptide concentration,¹³⁾ suggesting that the expression of Th2 cytokine transcription factors was inhibited by signaling *via* the IL-4-receptor and TCR signals in the naïve CD4⁺T cells stimulated with the peptide at high concentrations. ROVA23-3 CD4⁺T cells produced lower levels of IFN- γ at 0.5 and 5 μ M, in contrast with the higher IFN- γ production of RD10 CD4⁺T cells.

We further evaluated the OVA-specific naïve CD4⁺T-cell responses by using C-terminal peptide

OVA326-339 and N-terminal peptide OVA323-336. The proliferative response at 0.05 μ M of OVA323-336 was significantly higher in the ROVA23-3 CD4⁺T cells than in the RD10 CD4⁺T cells. The response to OVA326-339 at 0.05 μ M was significantly higher in the RD10 CD4⁺T cells than in the ROVA23-3 CD4⁺T cells. These results indicate OVA323-336 to be a more strictly defined epitope than OVA323-339 for ROVA23-3 CD4⁺T cells, while OVA326-339 was the case for RD10 CD4⁺T-cells. The proliferation level of ROVA23-3 CD4⁺T-cells in response to OVA323-336 at a concentration of 0.05 μ M was significantly higher than that of RD10 CD4⁺T cells in response to OVA326-339. These results demonstrate that, even in response to their respective strictly defined epitope peptides, the response of ROVA CD4⁺T cells was stronger than that of RD10 CD4⁺T cells. In respect of the cytokine profile, ROVA23-3 CD4⁺T cells showed marked IL-4 production when stimulated with N-terminal OVA323-336. The amount of IL-4 decreased at concentrations of 0.5 and 5 μ M, reflecting the inhibited expression of Th2 cytokine transcription factors as already described. Stimulated by OVA326-339, IL-4 production by ROVA23-3 CD4⁺T cells was detected at 5 μ M, but IFN- γ production was suppressed. RD10 CD4⁺T cells, however, maintained marked IFN- γ production in response to OVA323-336 as well as to OVA326-339 at 0.5 μ M. These results clearly indicate that, in response to epitopes as well as original peptide OVA323-339, ROVA23-3 CD4⁺T cells showed the Th2 phenotype, while RD10 CD4⁺T cells showed the Th1 phenotype. OVA323-339 therefore contained two epitopes for OVA-specific CD4⁺T-cells, each epitope-specific CD4⁺T-cell respectively exhibiting the Th2 or Th1 response. The present results indicate that OVA23-3 mice, which have naïve CD4⁺T cells preferentially differentiating to Th2-type CD4⁺T cells, had intestinal inflammation induced by the oral administration of OVA, while D10 mice with naïve CD4⁺T cells

	323	326	336	339	amino acid
Original OVA323-339	ISQAVHAAHAEINEAGR				
N-terminal OVA323-336	ISQAVHAAHAEINE				
C-terminal OVA326-339		VHAAHAEINEAGR			

Fig. 1. Amino Acid Sequences of the OVA Peptides.

OVA323-339 peptide, N-terminal OVA323-336 peptide and C-terminal OVA326-339 peptide were synthesized by the f-moc system and purified by HPLC.

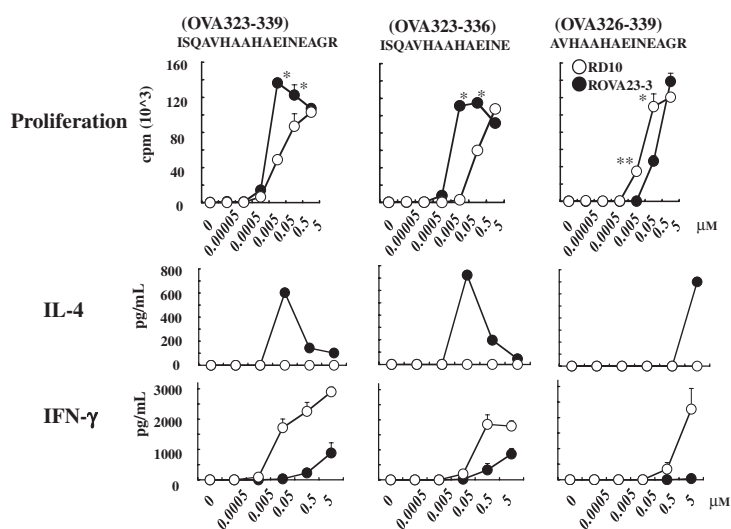


Fig. 2. Proliferation and Functions of Naïve OVA-Specific CD4⁺T Cells from ROVA23-3 Mice and Those from RD10 Mice in the Immediate Response to OVA323-339, OVA326-339 and OVA323-336 Peptides.

CD4⁺T cells were cultured in the presence of OVA peptides and mitomycin-C-treated splenocytes from BALB/c mice to examine the proliferation and cytokine production. Proliferation was analyzed by a tritiated thymidine incorporation assay. Culture supernatants were collected and the cytokine production was determined by ELISA. The dose-response curves were confirmed by three repeated experiments. Each point is presented as the mean $\pm$ SD. The Mann-Whitney *U* test was used for comparisons between 2 groups, and differences are considered statistically significant when *p* values were <0.05 . **p* < 0.01 , ***p* < 0.05 .

Table 1. Comparison of OVA23-3 and DO11.10 Mice Immune Responses

Mouse	TCR	Epitope	CD4 ⁺ T-cell responses	Characteristics of the mice with oral administered OVA
OVA23-3	V α 3, V β 15	OVA 323-336	Th2	Intestinal inflammation
DO11.10	V α 13, V β 8.2	OVA 326-339	Th1	Tolerance induction

preferentially differentiating to the Th1 type showed immediate tolerance induction. The continuous oral administration of OVA to D10 mice has been reported to induce a Th2 response.¹⁴⁾ The tolerance to orally administered OVA being immediately acquired in D10 mice without facing a risk of severe inflammation might have been due to excess Th2-skewing being inhibited by the immediate naïve Th1 CD4⁺T cell responses. It has been suggested that an excess production of Th2 cytokines might affect Foxp3 molecule expression in the intestinal tissues,¹⁵⁾ so that activating C-terminal epitope-specific naïve Th1 CD4⁺T cells may provide an appropriate environment to induce regulatory T cells as well as to inhibit inflammatory responses.

Table 1 shows a summary of this study. CD4⁺T cells recognizing different epitopes exhibited contrasting functions. The affinity between each epitope and each TCR on CD4⁺T cells may be higher in ROVA23-3 mice than in RD10 mice. Some molecules associated with TCR signaling influence the function of CD4⁺T cells. This strong affinity between TCR and the epitope bound to the I-A^d molecule may therefore affect TCR signaling, subsequently inducing significant Th2 responses, in CD4⁺T cells from ROVA23-3 mice. OIT has recently been recognized as one of the effective treatments for food allergy inflammation,¹⁶⁾ although many people fear the side effects of this treatment.¹⁷⁾ One idea for developing effective OIT may be using immunodominant T-cell epitope peptides to avoid this risk.¹⁸⁾ However, it has been reported that an intranasal treatment did not succeed when using OVA323-339 in a food allergy model.¹⁹⁾ Our results may suggest the necessity of using suitable and strictly determined epitopes to effectively induce tolerance and avoid inflammation. We should examine the effect of each N-terminal and C-terminal epitope by using the food allergy model of BALB/c mice to confirm this hypothesis.

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