

Localization of T Cell Epitope Regions of Chicken Ovomucoid Recognized by Mice

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We localized the T cell epitope regions of chicken ovomucoid (OVM), a potent egg allergen, with the overlapping pin-peptides covering the entire sequence of OVM and three strains of mice with different haplotypes. In C3H/He (H-2^k) mice, the T cells recognized relatively broad regions on OVM; the dominant regions were 49–93 and 97–114 residues, and the subdominant regions were 7–21, 37–48, 94–96, 115–123 and 145–177 residues. In contrast, a more limited number of T cell epitope regions were localized in BALB/c (H-2^d) and C57BL/6 (H-2^b) mice. The T cells from BALB/c mice recognized 100–114 and 157–171 residues, and the T cells from C57BL/6 mice recognized only 157–180 residues. These results were confirmed by using peptides separately synthesized and purified on the putative epitope regions. The roles of the carbohydrate moieties and cysteine residues involved in the disulfide bridges of OVM were also examined, and we found that they were not important in recognition by the T cell/antigen presenting cell.

Key words: ovomucoid; T cell epitope; synthetic peptide

Chicken egg is one of the most common causes of food allergic reactions in infants and children. Among the egg white proteins, ovalbumin, ovomucoid (OVM), ovotransferrin and lysozyme have been identified as major allergens, and OVM is known to be the most potent allergen.^{1–3)} Chicken OVM has a molecular weight of 28,000 containing 20–25% carbohydrate. It is composed of 186 amino acids arranged tandemly in three homologous domains (domains I, II and III). Each domain is cross-linked by three intradomain disulfide bridges.⁴⁾

Substantial knowledge has been accumulated on the antigenic properties of OVM by using patients'

sera or animal models; the B cell epitopes,^{5–10)} allergenic activities of heated or denatured OVM^{11,12)} and antigenic properties of OVM carbohydrate chains.^{13–15)} In terms of T cell recognition on OVM, several studies using peripheral blood mononuclear cells (PBMC) from allergic patients^{7,9,16)} and OVM-primed T cells from mice¹⁷⁾ have been reported, but the detailed T cell epitope regions on the entire OVM sequence are still unclear.

In the present study, we systematically investigated the T cell epitopes of chicken OVM recognized by the three strains of mice with different haplotypes by using a set of 15-mer overlapping pin-peptides covering the entire sequence of OVM. We also studied the roles of cysteine residues involved in the disulfide bridges of OVM in the recognition by the T cell/antigen presenting cell (APC).

Materials and Methods

Mice. Female BALB/c, C3H/He and C57BL/6 mice were purchased from Charles River Japan (Tokyo). The mice were used at 6–8 weeks of age. All experimental animals received humane care as outlined in the guide for the care and use of experimental animals (Animal Care Committee, National Institute of Livestock and Grassland Science).

Preparation of ovomucoid. Chicken OVM was prepared from fresh egg white of White Leghorn hens according to the method of Lineweaver and Murray.¹⁸⁾ Briefly, the egg white was vigorously stirred with a Waring blender. Two volumes of a cold precipitation solution (1 volume of 0.5 M trichloroacetic acid and 2 volumes of acetone) were added, and the suspension was gently mixed overnight at 4°C, before the precipitate was removed by centrifugation.

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Abbreviations: OVM, ovomucoid; PBMC, peripheral blood mononuclear cells; APC, antigen presenting cell; LN, lymph node; TFMS, trifluoromethanesulfonic acid; CFA, complete Freund's adjuvant; RCM-OVM, reduced carboxymethylated OVM; MHC, major histocompatibility complex

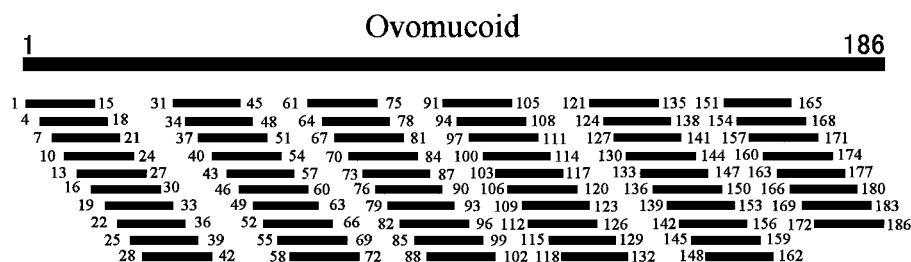


Fig. 1. Schematic Representation of the Synthetic Pin-peptides Used for the T Cell Epitope-scanning of OVM.

Fifteen-mer peptides offset by 3 amino acids encompassing the entire OVM sequence were synthesized on cleavable-linker derivatized polyethylene pins. The residue numbers in the primary structure of OVM are shown at the N- and C-termini of the peptides.

gation. Two to 2.5 volumes of cold acetone were added to the supernatant while vigorously stirring, and the suspension was kept overnight at 4°C. The precipitate recovered by centrifugation was dissolved in distilled water, and the resulting solution was dialyzed against distilled water and lyophilized. Further purification of crude OVM was carried out by ion-exchange chromatography on DEAE-Sephacel (Amersham Pharmacia Biotech, Uppsala, Sweden) with a Waters W650 Protein System (Millipore, Milford, MA, U.S.A.). The DEAE column (26 × 240 mm) was equilibrated with a 0.05 M imidazole-HCl buffer at pH 6.4, and the protein was eluted by a linear gradient of NaCl concentration from zero to 0.2 M. The main peak was pooled, dialyzed against distilled water and lyophilized. The purity of OVM obtained was confirmed by SDS-PAGE.

Preparation of deglycosylated ovomucoid. Deglycosylation was carried out according to the method of Gu *et al.*¹⁵⁾ Briefly, 1 ml of anisole and 2 ml of trifluoromethanesulfonic acid (TFMS) were mixed and cooled to 0°C. Twenty-five milligrams of dry OVM was dissolved in the anisole-TFMS mixture, and nitrogen was bubbled through the solution for 30 s. The solution in a sealed tube was mixed by magnetic stirring in an ice cooled water bath for 60 min, and then diluted with a two-fold excess of diethyl ether that had been cooled to -40°C. After mixing, the ethereal phase was removed, and the aqueous phase was dialyzed against distilled water and lyophilized. The product was used as deglycosylated OVM. The carbohydrate content in the glycoprotein was determined by a commercial kit (Pierce, Rockford, IL, U.S.A.), the carbohydrate content being 3.2% of the total weight.

Synthetic peptides. Fifty-eight overlapping 15-mer peptides offset by 3 amino acids encompassing the entire OVM sequence were synthesized on cleavable-linker derivatized polyethylene pins (Cleavable peptide kit, Chiron Mimotopes, San Diego, CA, U.S.A) according to the synthesis strategy described

by Maeji *et al.*¹⁹⁾ The locations of the synthesized peptides are presented in Fig. 1. The peptides were cleaved from the pins by exposing to a 0.1 M HEPES buffer (pH 7.8) for 4 h. The resulting peptide solutions were stored at -20°C until needed. The average concentration of the peptides was assumed to be 0.75 mg/ml according to the manufacturer's instructions.

The peptides corresponding to the partial sequences of OVM, p4-18, p52-66, p73-87, p100-114 and p157-171, were synthesized with an automatic peptide synthesizer (model 433A, Applied Biosystems, Foster City, CA, U.S.A.) by solid-phase peptide synthesis with F-moc chemistry. The amino acid sequences of the peptides are shown in Table 1. The peptides with serine residues substituted for cysteine residues in regions 52-66, 73-87, 100-114 and 157-171, and a control peptide corresponding to 139-154 residues of bovine β -lactoglobulin were also synthesized. These are designated as p52-66(S), p73-87(S), p100-114(S), p157-171(S) and BLGp139-154, respectively (Table 1). The peptides were purified by preparative HPLC (Waters) in a reversed-phase column (μ -Bondasphere, 19 × 150 mm, Nihon Waters, Tokyo, Japan). Elution was performed with an acetonitrile/water gradient (5-75%) containing 0.1% trifluoroacetic acid. The peptides were purified to >95%, and their sequences were confirmed by a gas-phase protein sequencer (LF3400DT, Beckman Instruments, Fullerton, CA, U.S.A.).

Immunization and T cell proliferation assay. The T cell proliferation assay was performed as previously described.²⁰⁾ Briefly, the mice were immunized with 10 μ g (C3H/He) or 50 μ g (BALB/c and C57BL/6) of OVM emulsified in complete Freund's adjuvant containing *Mycobacterium tuberculosis* H37Ra (CFA, Difco, Detroit, MI, U.S.A.) at the base of tail and in the hind footpads. Seven days after the immunization, the draining lymph nodes (LNs) were aseptically harvested, and a single-cell suspension was prepared by filtering the LN cells through a nylon mesh. These cells (2 × 10⁵ cells/well) were

Table 1. OVM Peptides and the Control Peptide Synthesized and Purified for Confirmation Studies on the T Cell Epitopes Regions of OVM

Peptide	Sequence
p4-18	DCSRFPNATDKEGKD
p52-66	TNISKEHDGECKETV
p52-66(S)	TNISKEHDGESKETV
p73-87	YANTTSEDGKVMVLC
p73-87(S)	YANTTSEDGKVMVLS
p100-114	VTYDNECLLCAHKVE
p100-114(S)	VTYDNESLLSAHKVE
p157-171	DNKTYGNKCNFCNAV
p157-171(S)	DNKTYGNKSNFNAV
BLGp139-154	ALKALPMHRLSFNPT

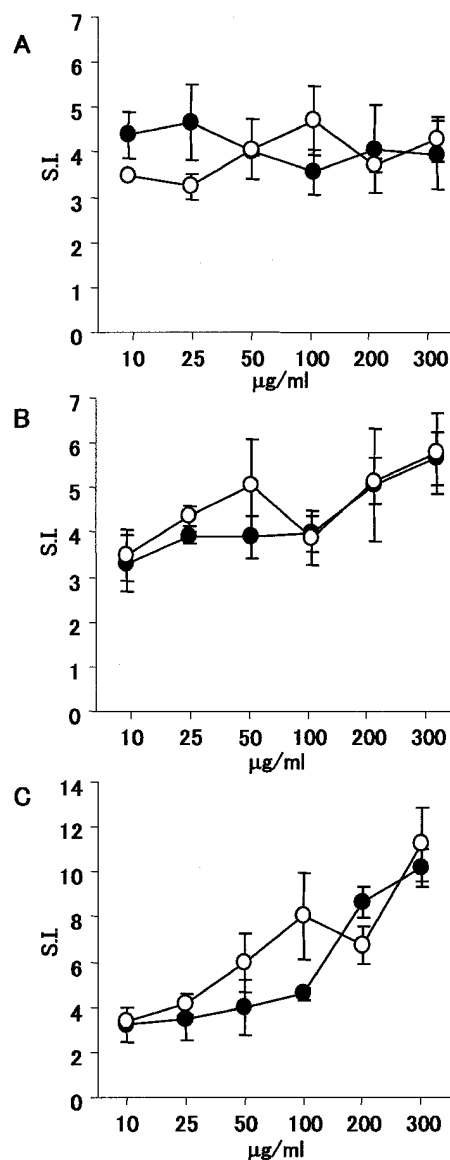
The peptides were synthesized by an automatic peptide synthesizer with solid-phase peptide synthesis and F-moc chemistry and then purified by reverse-phase HPLC. Cysteine residues were replaced by serine residues at the positions shown by underlines.

cultured in 96-well round-bottom plates (Falcon 3077, Becton Dickinson, Franklin Lakes, NJ, U.S.A.) with RPMI-1640 (Gibco, Grand Island, NY, U.S.A.) containing 50 μ M 2-mercaptoethanol, 10 mM HEPES, 100 U/ml of penicillin, 100 μ g/ml of streptomycin and 1% syngeneic normal mouse serum. The cells were cultured in the presence of the protein antigens (10–300 μ g/ml), the pin-peptides (approximately 3.75 μ g/ml) or the purified peptides (12.5–100 μ g/ml) for 4 days and pulsed with 37 kBq of [^{3} H]-thymidine (Moravek Biochemicals, Brea, CA, U.S.A.) during the last 16–20 h. The cells were harvested, and the amount of [^{3} H]-thymidine incorporation was measured by a liquid scintillation counter (Packard Tri-Carb 1600, Packard Instruments, Meriden, CT, U.S.A.).

Results

Effects of deglycosylating OVM on the stimulative activity in T cell proliferation

It has been reported that, in some cases, T cells recognize carbohydrates on protein antigens and that the sugar chains block the action of proteases to escape from recognition by T cells.²¹⁾ To investigate the effects of the carbohydrate moieties of OVM on the stimulative activity in T cell proliferation, LN cells from C3H/He, BALB/c and C57BL/6 mice immunized with OVM were challenged *in vitro* with intact OVM or deglycosylated OVM. The results are shown in Fig. 2. In all strains, the proliferative response of the T cells to intact OVM and to deglycosylated OVM was almost the same. These results show that the carbohydrate moieties of OVM made no significant contribution to the stimulative activity. Therefore, T cell epitope-scanning with the overlapping peptides taking no account of the carbohydrate moieties should be effective on OVM.

**Fig. 2.** T Cell Proliferative Response to Intact and Deglycosylated OVM.

The lymph node (LN) cells from C3H/He (A), BALB/c (B) and C57BL/6 (C) mice immunized with OVM were cultured with varied concentrations of intact OVM (○) and deglycosylated OVM (●). The proliferative responses shown as the stimulation index (S.I.) in the ordinate are averages of triplicate cultures $\pm$ SD. S.I. was calculated by the following formula: incorporated radioactivity in the presence of antigen/incorporated radioactivity in medium alone. The background responses of LN cells in the medium alone were 20,378 cpm (C3H/He), 4,382 cpm (BALB/c) and 1,818 cpm (C57BL/6).

T cell epitope-scanning of OVM with pin-peptides

The LN cells from the three strains of OVM-primed mice were analyzed for T cell proliferation against the 58 overlapping peptides representing the primary structure of OVM. Each strain of different H-2 haplotype showed a different profile of T cell proliferative response to the peptides (Fig. 3). In the case of C3H/He mice, strong proliferative responses (stimulation index: S.I. > 4) to peptides #17 to #27,

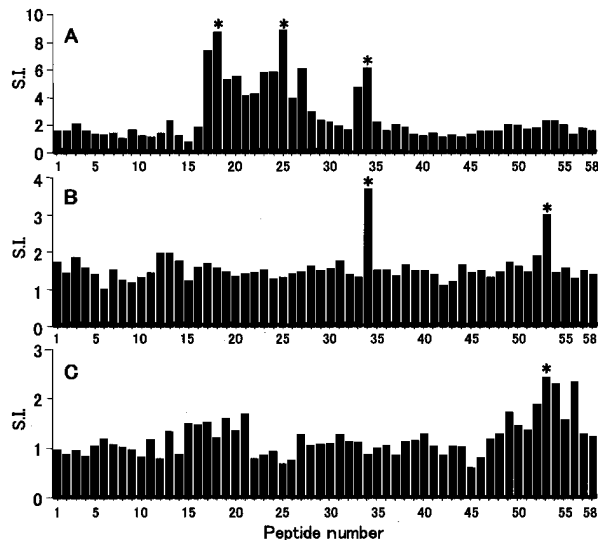


Fig. 3. T Cell Epitope-scanning of OVM with the Pin-peptides.

The lymph node (LN) cells from C3H/He(A), BALB/c (B) and C57BL/6 (C) mice immunized with OVM were cultured with OVM pin-peptides. The abscissa shows the peptide number. The N-terminal position of each peptide is calculated by the following formula: $3 \times (\text{peptide number} - 1) + 1$. The proliferative responses shown as S.I. in the ordinate are averages of triplicate cultures. The background responses of LN cells in the medium alone were 20,378 cpm (C3H/He), 4,382 cpm (BALB/c) and 7,942 cpm (C57BL/6). Confirmation studies were performed on the peptides marked with asterisks.

#33 and #34 corresponding to residues 49–93 and 97–114 were observed (Fig. 3A). In particular, peptides #17, #18, #25, #27 and #34 induced the most significant T cell proliferative response ($S.I. > 6$). In addition, peptides #3, #13, #28 to #30, #35, #37, #49, #50 and #53 to #55 were also stimulative ($S.I. 2$ to 4). The regions encompassed by these peptides were residues 7–21, 37–51, 82–102, 103–123 and 145–177. In BALB/c mice, only peptides #34 and #53, corresponding to residues 100–114 and 157–171, were stimulative (Fig. 3B). C57BL/6 mice responded less than the other two strains, but peptides #53, #54 and #56 corresponding to residues 157–171, 160–174 and 166–180 showed stimulative activity ($S.I. > 2$) (Fig. 3C). We infer from these results that the dominant T cell epitope regions of OVM were located in residues 49–93 and 97–114 in C3H/He, residues 100–114 and 157–171 in BALB/c and residues 157–180 in C57BL/6 mice. The subdominant regions in C3H/He mice are presumed to have been residues 7–21, 37–48, 94–96, 115–123 and 145–177, excluding the dominant regions.

T cell proliferative response to the peptides synthesized on the putative epitope regions

In order to reconfirm the results obtained by epitope scanning with the pin-peptides, the peptides corresponding to such representative stimulative peptides as #18, #25, #34 and #53 were synthesized by

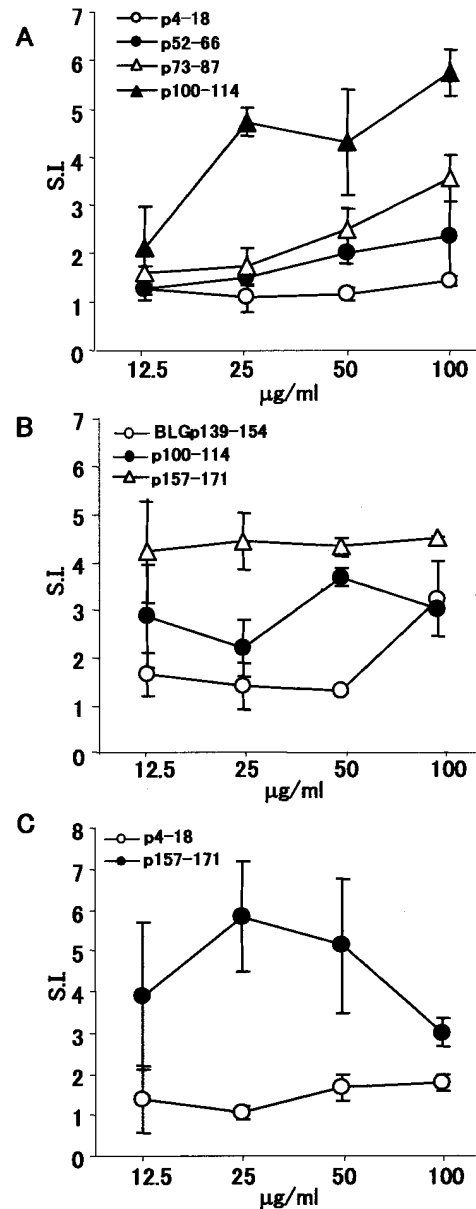


Fig. 4. T Cell Proliferative Response to the Peptides Synthesized on the Putative Epitope Regions.

The lymph node (LN) cells from C3H/He (A), BALB/c (B) and C57BL/6 (C) mice immunized with OVM were cultured with varied concentrations of the purified peptides. The proliferative responses shown as S.I. in the ordinate are averages of triplicate cultures $\pm$ SD. The background responses of LN cells in the medium alone were 893 cpm (C3H/He), 1,399 cpm (BALB/c) and 1,011 cpm (C57BL/6).

the conventional method for a T cell proliferative assay. The T cell proliferative responses to the purified peptides were examined in the three strains of mice (Fig. 4). The putative epitope peptides in each strain showed T cell proliferation with S.I. of more than 2. The LN cells from C3H/He mice showed a strong concentration-dependent response to p100–114 and a medium response to p52–66 and p73–87 of up to 100 $\mu\text{g/ml}$ (Fig. 4A). In BALB/c

mice, the LN cells showed a significant proliferative response to p157–171 even at 12.5 $\mu\text{g/ml}$ and a medium response to p100–114 (Fig. 4B). In C57BL/6 mice, p157–171 was confirmed to be stimulative and gave a maximum response at 25 $\mu\text{g/ml}$ (Fig. 4C). The response to p4–18 or BLGp139–154 as a negative control resulted in low proliferation with the tested concentrations of the peptide, except for 100 $\mu\text{g/ml}$ of BLGp139–154 in BALB/c mice. We thus confirmed the results obtained by epitope-scanning with the pin-peptides and also revealed the dose responses and optimal doses of the peptides in T cell proliferation.

Role of the cysteine residues in the T cell stimulative activities of the epitope peptides

Enomoto *et al.* have reported that the T cell epitopes on OVM were dependent on the 3D structure because of the diminished T cell response to reduced carboxymethylated OVM (RCM-OVM).¹⁷⁾ Their results also suggest the involvement of cysteine residues in the recognition by the T cell/APC. We therefore examined the effects of cysteine residues in the disulfide bridges of OVM on the stimulative activity in T cell proliferation. For that purpose, we determined the T cell stimulative activities of the peptide derivatives with the substitution of serine for cysteine residues, p52–66(S), p73–87(S), p100–114(S) and p157–171(S). The parent peptides of these derivatives are representative of the significantly stimulative peptides revealed by the pin-peptide assay (peptides #18, 25, 34 and 53) and were confirmed by the experiments already described.

All these peptides with the substitution showed stimulative activity in T cell proliferation (Fig. 5). In C3H/He mice, the stimulative activity of p52–66(S) was not significantly different from that of p52–66 at doses of 12.5 and 25 $\mu\text{g/ml}$, but the peptide derivative was slightly less stimulative than the parent peptide (Fig. 5A). The stimulative activity of p73–87(S) was comparable to that of p73–87 at doses of 12.5 and 25 $\mu\text{g/ml}$, but p73–87(S) gave a significantly lower response at higher doses (Fig. 5B). However, p100–114(S) exhibited 2.5- to 8.8-fold higher activity than the parent peptide (Fig. 5C). In BALB/c mice, the stimulative activities of p100–114 and p100–114(S) were similar (Fig. 5D), but p157–171(S) showed 3.7- to 4.8-fold higher stimulative activity than p157–171 in the tested dose range (Fig. 5E).

Discussion

OVM is a highly glycosylated protein, and it has been reported that the carbohydrate moieties were involved in the binding of OVM to the antibodies from humans^{13,14)} and mice.^{13,22)} However, no studies on their role in T cell stimulation have been performed, except for the investigation on the T cell

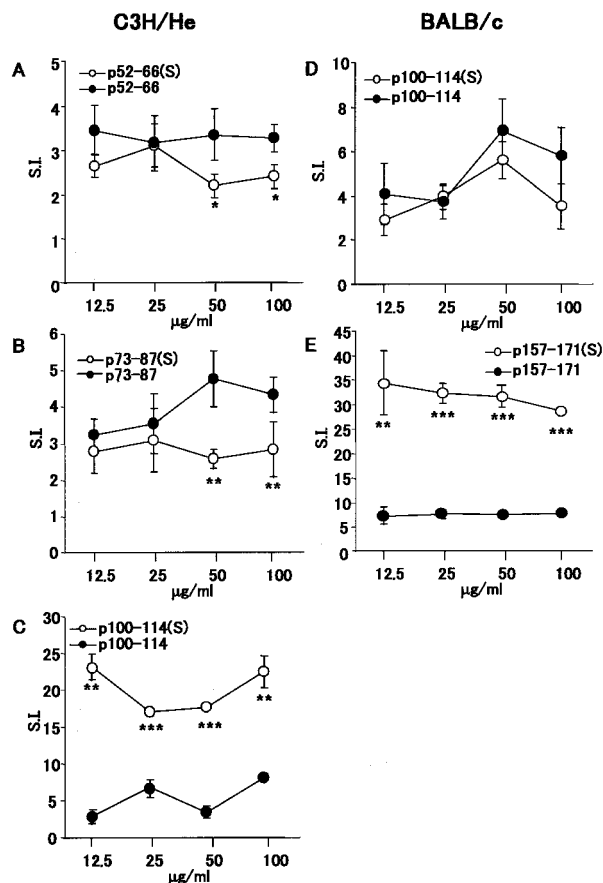


Fig. 5. T Cell Proliferative Responses to the Epitope Peptide Derivatives with the Substitution of Serine for Cysteine Residues.

The lymph node (LN) cells from C3H/He (A, B and C) and BALB/c (D and E) mice immunized with OVM were cultured with varied concentrations of the purified peptides (Table 1). The proliferative response shown as S.I. in the ordinate are averages of triplicate cultures $\pm$ SD. The background responses of LN cells in the medium alone were 1,907 cpm (C3H/He) and 832 cpm (BALB/c). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the parent peptide at the same challenge dose.

stimulative activity of domain III with or without carbohydrate by Enomoto *et al.*¹⁷⁾ They have pointed out that the sugar chains of domain III were not recognized by OVM-primed T cells from mice. Since the carbohydrate moieties are conjugated with the other two domains, we examined the T cell stimulative activity of chemically deglycosylated OVM. There was no significant difference between deglycosylated OVM and intact OVM in their stimulative activity in all three strains of mice (Fig. 2). These findings suggest that the carbohydrates in OVM had no significant effect on the T cell stimulative activity.

The insignificance of the carbohydrate moieties in murine T cell recognition that has just been described made it possible to take advantage of the strategy of epitope-scanning with pin-peptides. This method was successfully applied to OVM, and the epitope regions

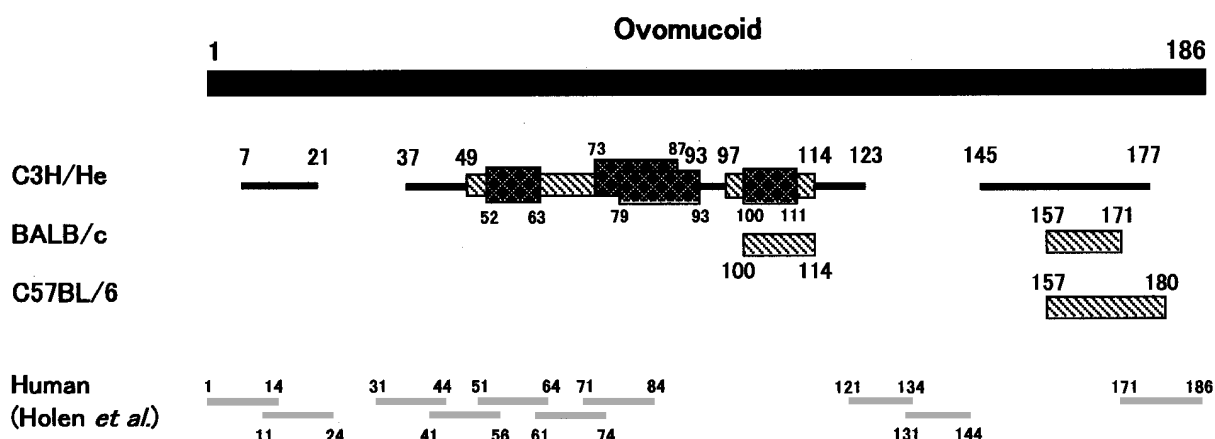


Fig. 6. Schematic Diagram Summarizing the Locations of the T Cell Epitope Regions on OVM.

Hatched and black bars respectively show the dominant and subdominant T cell epitope regions of OVM in mice. Cross-hatched bars show the regions that were most effectively recognized by C3H/He mice. Gray lines show the T cell epitope regions recognized by OVM-specific T cell lines derived from egg-allergic patients according to Holen *et al.*⁹⁾

were localized in the three haplotypes of mice. Figure 6 shows a summary of the locations of the T cell epitope regions on OVM. In C3H/He (H-2^k) mice, the T cells recognized relatively broad regions on OVM; the dominant regions were 49–93 and 97–114 residues, and the subdominant regions were 7–21, 37–48, 115–123 and 145–177 residues, and the regions including 94–96 residues. However, 52–63, 73–87, 79–93 and 100–111 residues in the dominant regions were most effectively recognized by C3H/He mice. In contrast, a limited number of T cell epitope regions were localized in BALB/c (H-2^d) and C57BL/6 (H-2^b) mice. The T cells from BALB/c mice recognized 100–114 and 157–171 residues and the T cells from C57BL/6 mice recognized only 157–180 residues. Thus, multiple epitope regions extended over the OVM molecule in the high-responder strain, C3H/He mice, but the regions were more limited in the low- and medium-responder strains, C57BL/6 and BALB/c mice. Enomoto *et al.* have reported that the LN cells from C3H/He and BALB/c responded to each domain of OVM, but that domain I was inferior to the other domains in stimulative activity, while the T cells from C57BL/6 responded mainly to domain III.¹⁷⁾ Our results are in good agreement with their study, except for C3H/He mice in our work, in which the dominant regions were localized in domains I and II. It has been reported in a human study that the T cell lines and clones derived from PBMC of egg-allergic patients recognized all three domains,¹⁶⁾ or 10 regions consisting of the 14 to 16 residue range of the OVM molecule.⁹⁾ Interestingly, the T cell epitope regions on OVM in the patients shown by Holen *et al.*⁹⁾ extended over 60% of the molecule, as was seen in C3H/He mice, a high-responder strain to OVM (Fig. 6).

According to the prediction method for T cell epitope regions by Rothbard and Taylor,²³⁾ five

regions, 7–10, 89–92, 99–103, 140–143 and 159–162 residues, are assumed in OVM. Of these, four regions, excepting 140–143 residues, are included in the epitope regions revealed in the present study. Although no regions corresponding to the major histocompatibility complex (MHC) class II motif for H-2^d proposed by Sette *et al.*²⁴⁾ and Rammensee *et al.*²⁵⁾ were found on OVM, the T cells from BALB/c mice actually proliferated in the presence of p100–114 and p157–171.

The pin-peptide method is quite efficient for the simultaneous synthesis of many short peptides. In addition, T cell epitope-scanning with the pin-peptide method has been successfully applied to protein antigens.^{26–28)} However, it would be difficult to purify each synthesized peptide and check the quality, and the results obtained by epitope-scanning need to be reconfirmed by another set of experiments in order to exclude the possibility of non-specific reactions to the pin-peptides. Therefore, we examined the T cell stimulative activities of the peptides synthesized separately by an automatic peptide synthesizer according to the putative sequence revealed by epitope-scanning with the pin-peptides. We finally found that the purified peptides were also stimulative in T cell proliferation and confirmed the location of the dominant T cell epitope regions on OVM. In addition, we tested the stimulative activities of those peptide derivatives with the substitution of serine for cysteine residues in order to examine the participation of the disulfide bridges of OVM in immune recognition. The results suggest that cysteine residues were not important for T cell/APC recognition in both C3H/He and BALB/c mice. It is to be noted, however, that the T cell stimulative activities of the peptide derivatives, p100–114(S) and p157–171(S), were respectively at least 2.5- and 3.7-fold higher than those of the parent peptides in C3H/He and

BALB/c mice (Fig. 5C, E). It has been reported that modification of the native peptide sequence sometimes induced enhanced immunogenicity;^{29,30)} this would also have been the case with p100–114(S) and p157–171(S).

Enomoto *et al.* have reported that RCM-OVM showed much lower T cell stimulative activity than native OVM.¹⁷⁾ However, some of the overlapping peptides and the epitope peptide derivatives were actually confirmed to be stimulative in the present study, in spite of the lack of an intact 3D structure of OVM. In order to rationalize these rather contradictory results, the method for processing RCM-OVM by APCs should be taken into consideration. It is possible that the processing of RCM-OVM and the presentation of their fragments would not have been efficient, while the synthetic peptides were almost directly presented on APC without processing.

This is the first work detailing the immunodominant T cell epitope regions of OVM recognized by mice. Some of the antigen-derived T cell epitope peptides, when orally administered, have recently been shown to suppress the T cell response to the parent protein.^{31,32)} Such peptides are clinically quite useful for preventing an allergy, because the intact molecules induce harmful reactions. However, the mechanism for the phenomenon and the characteristics of the tolerogenic epitopes are still unclear, even in mice. Therefore, the information gained on the T cell epitopes in the present study would be useful to clarify the mechanism for oral tolerance and to design effective peptides to induce tolerance.

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