Fine mapping of T-cell determinants of bovine β -lactoglobulin

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Abstract

T-cell recognition sites, i.e. T-cell determinants, of bovine β -lactoglobulin, a major allergen in milk, were analyzed in detail. For this purpose, we prepared primary cultures of lymph node cells from three strains of mice, C57BL/6 (H-2^b), C3H/HeN (H-2^k), and BALB/c (H-2^d), and examined the proliferative response of these cells to a complete set of overlapping 15-mer peptides which covered the entire sequence of β -lactoglobulin by shifting in single amino acid steps. We were able to determine the putative core sequence of each T-cell determinant and estimate its relative importance. In the case of C57BL/6 mice, dominant, subdominant, and minor determinants were identified as residues 122–130, 16–26, and 108–122, respectively, as represented by their core sequences. Each determinant peptide induced the production of interferon- γ , the amount of which showed a correlation with the intensity of the proliferative response induced by each determinant. In the case of C3H/HeN mice, a dominant determinant comprised of residues 140–148 was identified together with three subdominant and two minor determinants. Dominant T-cell determinants recognized in BALB/c mice were identified as residues 67–75, 71–79, and 80–88, and six other regions were identified as subdominant determinants. Comparisons between our results and the determinants predicted from relevant MHC-binding motifs reported to date revealed the inadequacy of the motifs in predicting even the dominant determinants. The information obtained by complete mapping of T-cell determinants as done in this study is expected to be helpful in establishment and evaluation of new prediction methods and also may contribute to the development of a new approach to control immune responses by manipulation of the T-cell determinants of allergens.

Abbreviations: β -Lg – β -lactoglobulin; ELISA – enzyme-linked immunosorbent assay; IFN- γ – interferon- γ ; mAb – monoclonal antibody; MHC – major histocompatibility complex; TCR – T-cell receptor

Introduction

T cells recognize peptide fragments of a protein antigen in the form of a complex with a major histocompatibility complex (MHC) molecule via T-cell receptors (TCR). The three dimensional structures of MHC/peptide complexes have been determined for several MHC class I (Silver *et al.*, 1992; Fremont *et al.*, 1992) and class II molecules (Stern *et al.*, 1994; Fremont *et al.*, 1996). The characteristics of the MHC binding peptides have been depicted as MHC binding motifs (Rammensee *et al.*, 1995). Recently, the tertiary structures of tri-molecular complexes of

TCR/peptide/MHC have been reported (Garcia *et al.*, 1996; Garboczi *et al.*, 1996). In spite of these lines of structural information, it is still difficult to predict the peptide regions recognized by T cells in a protein antigen, i.e. the T-cell determinants, based on amino acid sequence data alone. That is principally because the molecular mechanism of induction of the T-cell response by a protein antigen, including enzymatic digestion of the protein, selection of MHC binding peptides, and interaction between the TCR and the peptide/MHC complex, is not yet fully understood (Germain, 1994). Therefore, the only method available at present to determine precisely the location of

T-cell determinants within a protein antigen is to examine in vitro the ability of defined peptide fragments of the antigen to induce a response in assays of T cells derived from immunized animals. This method cannot be easily applied to identifying T-cell determinants in the case of humans, thus efficient prediction methods are sought. To establish and evaluate such prediction methods, the accumulation of pertinent information through complete mapping of the T-cell determinants in protein antigens is required.

Focusing on T cells as the target is considered to be the most efficient approach in efforts to control immune responses, because T cells play a crucial role in regulating other cells involved in immune reactions. The importance of identifying the T-cell determinants in allergens or self proteins relevant to autoimmune diseases lies in the probability that such studies will lead to the development of a method for the antigen-specific control of immune responses. The treatment of allergy or autoimmune disease by antigen-specific inhibition of immune responses is expected to be a safer and more efficient means of therapy than treatment by nonspecific and general inhibition methods, which often cause side effects due to total impairment of the immune system (Kaminogawa, 1996; Tisch and McDevitt, 1994; Fairchild *et al.*, 1994). Recent studies have revealed that T-cell responses can be modified in an antigen-specific manner using analogs of T-cell determinant peptides with a single substitution at TCR contact residues (Evavold and Allen, 1991). Some of these analogs inhibit T-cell responses, and such peptides are designated as TCR antagonists (De Magistris *et al.*, 1992). On the other hand, oral administration of an antigen can induce immunological tolerance for the specific antigen (Weiner *et al.*, 1994). However, in allergic patients, the administration of a whole protein molecule may elicit an adverse effect because it can cross-link immunoglobulin E (IgE) molecules bound to their receptors on mast cells leading to the release of chemical mediators. The use of a T-cell determinant peptide instead of the whole protein molecule is less likely to elicit such an adverse effect. Thus, TCR antagonism and oral tolerance provide great potential for development of new therapies with antigen specificity, if the T-cell determinants of the relevant antigen are identified (Kaminogawa, 1996).

β -lactoglobulin (β -Lg) is known as one of the major allergens in cow's milk (Taylor, 1986). Although its physiological role is not yet clarified, β -Lg together with retinol-binding protein and others constitute a protein superfamily named lipocalin, each member of

which has the ability to bind small hydrophobic molecules (Flower, 1994). The antigenic structure of β -Lg has been extensively analyzed using anti- β -Lg monoclonal antibodies (Kaminogawa *et al.*, 1989; Hattori *et al.*, 1993; Katakura *et al.*, 1994). In addition, we have already established a yeast expression system for bovine β -Lg (Totsuka *et al.*, 1990; Katakura *et al.*, 1994), which has enabled us to produce mutant forms of β -Lg with amino acid substitutions that may be useful for the study of immune modulation with antigen analogs.

The T-cell determinants in several protein antigens, including those in β -Lg, have been determined by using a panel of synthetic peptides which covers the entire sequence of the protein, shifting in multi-amino acid steps (Adorini *et al.*, 1988; Bellone *et al.*, 1991; Robinson *et al.*, 1991; Tsuji *et al.*, 1993). However, when such a panel of peptides is employed, some T-cell determinants extending across the junction of two adjacent peptides could be overlooked. Therefore, in the present study, we used a complete set of overlapping 15-mer peptides (total of 148 peptides) which covers the entire sequence of bovine β -Lg, shifting in single amino acid steps. Using this approach, we were able to determine the putative core sequence of each T-cell determinant (i.e. the sequence of residues considered to be essential for the determinant), and estimate the dominance of each T-cell determinant. Furthermore, we were able to newly identify a dominant determinant and several subdominant determinants that were overlooked in previous studies (Takahashi *et al.*, 1990; Tsuji *et al.*, 1993).

Materials and methods

Animals

Female C57BL/6 (H-2^b), C3H/HeN (H-2^k), and BALB/c (H-2^d) mice were purchased from Charles River Japan Inc. (Yokohama, Japan). The mice were immunized at 8 weeks of age for the purposes of the lymph node cell proliferation assay.

Protein

Bovine β -lactoglobulin (β -Lg) was purified from fresh milk of a cow with the genotype AA as described previously (Kaminogawa *et al.*, 1989).

Figure 1. Amino acid sequence of bovine β -lactoglobulin and peptide design for T-cell determinant mapping. The sequence of bovine β -lactoglobulin (β -Lg; genetic variant A) is cited from the report of Kolde and Braunitzer (Kolde and Braunitzer, 1983). In total, 148 peptides encompassing the entire β -Lg sequence were synthesized by the method of Maeji *et al.* (Maeji *et al.*, 1990). Each peptide is referred to by indicating the positions of the first and last residues (ex. peptide 3–17).

Peptides

A set of 15-mer overlapping peptides (148 peptides) which covered the entire sequence of bovine β -Lg (genetic variant A) (Kolde and Braunitzer, 1983) by shifting in single amino acid steps (Fig. 1) was synthesized on polyethylene rods with a Cleavable Peptides kit (Chiron Mimotopes, Clayton, Australia) by the multi-pin peptide synthesis strategy (Maeji *et al.*, 1990). Non- β -Lg peptide (PLRQG₁₁) was also synthesized as a control peptide. Each peptide was cleaved into 150 μ l of sterilized PBS (phosphate-buffered saline; 0.11 M phosphate buffer at pH 7.2 with 0.04 M NaCl), resulting in an approximate amount of 30 nmol in 150 μ l (200 μ M solution) as estimated by amino acid analysis. Without further purification, aliquots of each peptide solution were used for the assays. All of the above-mentioned peptides had a diketopiperazine moiety at the C-terminus (Maeji *et al.*, 1990) and were acetylated at the amino terminus. A peptide encompassing amino acids 107–122 (p107–122; MENSAEPEQSLVCQCL) was purchased from Chiron Mimotopes. Peptides encompassing amino acids

11–28 (p11–28; DIQKVAGTWYSLAMAASD) and 119–133 (p119–133; CQCLVRTPEVDDEAL) were synthesized with a peptide synthesizer (Model 430A; Perkin Elmer, Norwalk, CT, U.S.A.) with F-moc chemistry. These peptides were purified by reversed-phase HPLC on a C₁₈ column, Lichrosorb RP-18 (Merck, Darmstadt, Germany).

Lymph node cell proliferation assay

Mice were immunized subcutaneously in the hind footpads with 6 nmol of β -Lg emulsified in Complete H37Ra Adjuvant (CFA-H37Ra; Difco, Detroit, MI, U.S.A.). After seven days, the inguinal and popliteal lymph nodes were removed, and the cells were cultured in 96-well plates at 4×10^5 cells/well with 5 μ l of the multi-pin peptide solution in 0.2 ml of RPMI1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 50 μ M 2-mercaptoethanol, 100 U/ml of penicillin, 100 μ g/ml of streptomycin and 1% autologous normal mouse serum (RPMI-NMS). The set of peptides was tested using one well for each peptide. One micro-Curie of 3 H-thymidine was added to each

culture for the last 18–22 hr of a four-day culture, and the uptake was determined by liquid scintillation counting. To determine whether the observed response was significant or not, the average cpm value in the presence of the non- β -Lg peptide (PLRQG₁₁) plus 3 x SD was used as the criterion, similar to the study of Gammon *et al.* (Gammon *et al.*, 1991) who analyzed the T-cell determinant structures by the same strategy as that used in this study.

Interferon- γ production assay

Mice were immunized subcutaneously in the hind footpads with 6 nmol of β -Lg emulsified in CFA-H37Ra. After seven days, the inguinal and popliteal lymph nodes were removed, and the cells were cultured in 96-well plates at 8×10^5 cells/well with various concentrations of antigen in 0.2 ml of RPMI-NMS. After a 72 hr culture period, the culture supernatant was collected and the amount of interferon- γ (IFN- γ) was determined by enzyme-linked immunosorbent assay (ELISA) as described previously (Hisatsune *et al.*, 1994). Briefly, 96-well plate wells were coated with an anti-IFN- γ monoclonal antibody (mAb), R4-6A2 (Spitalny and Havell, 1984), and appropriately diluted samples were added to the wells. The wells were then incubated with a biotinylated anti-IFN- γ mAb, XMG1.2 (Cherwinski *et al.*, 1987), and subsequently with a streptavidin-alkaline phosphatase conjugate. An enzyme substrate solution was added to the wells and the absorbance was measured. The amount of IFN- γ in the sample was determined from a standard curve. The limit of detection of IFN- γ by ELISA was 0.27 U/ml. The mAbs used were purified from ascites by affinity chromatography with a MAb Trap GII kit (Pharmacia Biotech, Uppsala, Sweden).

Results

T-cell determinants of bovine β -lactoglobulin in C57BL/6 mice

We first examined the T-cell determinants recognized in C57BL/6 mice using a complete set of 15-mer peptides covering the entire sequence of bovine β -lactoglobulin (β -Lg) (Fig. 1). C57BL/6 mice were immunized with β -Lg emulsified with adjuvants, and the proliferative response of the lymph node cells to each of the peptides in the series was analyzed. A region was identified as a T-cell determinant only if

more than two adjacent peptides covering the region induced a reproducible positive response, since the minimal essential residues of most T-cell determinants are considered to comprise a region of less than 15 residues (Nelson *et al.*, 1992). The results of two experiments using the same peptides are shown in Fig. 2. Lymph node cells responded most intensely to peptides 115–129 to 122–136 in both experiments, this intensity being followed by that of peptides 11–25 to 16–30 and peptides 107–121 to 108–122. These results demonstrate the existence of a dominant T-cell determinant within region 115–136, and a subdominant determinant and a minor one in regions 11–30 and 107–122, respectively.

Although some other peptides induced a positive response ($\geq$ background + 3 x SD), the regions covered by these peptides were not deemed to include T-cell determinants according to the criteria described in the Materials and methods section. For example, the sets of peptides 4–18 and 5–19, 54–68 and 55–69, 61–75 and 62–76, 75–89 and 76–90, 99–113 to 103–117, 127–141 to 129–143, and 145–159 and 146–160 were stimulatory for T-cell proliferation in experiment 1 alone (Fig. 2A), and peptides 85–99 to 89–103 in experiment 2 alone (Fig. 2B). These are regarded as not including T-cell determinants due to the lack of reproducibility of their effects.

Interferon- γ production by lymph node cells from C57BL/6 mice immunized with β -lactoglobulin

The intensity of the T-cell response induced by each T-cell determinant peptide was examined by means of a cytokine production assay. Lymph node cells derived from β -Lg-immunized C57BL/6 mice were stimulated with each of the peptides corresponding to a T-cell determinant, as identified by the proliferation assay, and the cytokine levels in the culture supernatant were measured by ELISA. p119–133 containing a dominant determinant induced a high level of IFN- γ (Fig. 3). p11–28 containing a subdominant determinant induced a low level of IFN- γ production only at 50 μ M, while IFN- γ was not detected in the supernatant of cells stimulated with p107–122 containing a minor determinant. Interleukin 4 (IL-4) was not detected in any of the culture supernatants (data not shown). These findings support the relative importance of the T-cell determinants as estimated from the proliferation assay results, although induction of cytokine production by the minor determinant peptide could not be detected probably because of the lower sensitivity of the cytokine assay

Figure 2. Proliferative response of lymph node cells from β -lactoglobulin-primed C57BL/6 mice against a β -Lg peptide series. Five C57BL/6 (H-2^b) mice were immunized with 6 nmol of β -Lg emulsified with CFA-H37Ra, and the pool of lymph node cells was used for subsequent assays. The results of two experiments (A for Exp. 1 and B for Exp. 2) using the same peptide series are shown. The peptides were added to the culture at an approximate final concentration of 5 μM . The peptide number shown at the bottom corresponds to the position of the N-terminal residue of the overlapping 15-mer peptide in the β -Lg sequence. The average non- β -Lg peptide background response of three wells $\pm$ SD was 7.9 ± 0.6 ($\text{cpm} \times 10^{-3}$) for Exp. 1 and 5.4 ± 0.9 for Exp. 2. Solid and open bars represent a response which was greater or less than, respectively, the background + 3 x SD value that was used as the criterion for determining the positive responses. The non- β -Lg background response has been subtracted in each case. The response to 10 μM β -Lg $\pm$ SD was 40.9 ± 10.1 ($\text{cpm} \times 10^{-3}$) for Exp. 1 and 89.8 ± 10.4 for Exp. 2.

as compared to the proliferation assay.

T-cell determinants of bovine β -lactoglobulin in C3H/HeN mice

We next examined the T-cell determinant structure

recognized in C3H/HeN mice. As shown in Fig. 4, peptides 131–145 to 140–154 elicited the most intensive response of lymph node T cells. The intensity of the response to these peptides was followed by that to peptides 86–100 to 91–105 and 73–87 to 79–93, in the order of response magnitude. Lymph node

Figure 3. Interferon- γ production by lymph node cells from β -lactoglobulin-primed C57BL/6 mice in response to T-cell determinant peptides. C57BL/6 mice were immunized with 6 nmol of β -Lg emulsified with CFA-H37Ra, and the pool of lymph node cells was used for subsequent assays. Lymph node cells (8×10^5 cells/well) were cultured with the indicated antigen at 5 or 50 μ M for 72 hr. The amount of IFN- γ in the culture supernatant was determined by ELISA. The limit of detection of IFN- γ by ELISA was 0.27 U/ml. *: >700 U/ml. **: Not detected.

cells showed weak proliferation in response to peptides 98–112 and 99–113, and 127–141 to 129–143. From these results, two regions 131–154 and 86–105 were identified as containing dominant T-cell determinants, and region 73–93 was found to include a subdominant determinant. Two other regions 98–113 and 127–143 were found to contain minor T-cell recognition sites.

Peptides 16–30 and 17–31, 25–39 to 30–44, 33–47 and 34–48, 63–77 and 64–78, and 68–82 and 69–83 each induced the proliferation of lymph node cells only in experiment 1 (Fig. 4A), and were not deemed to contain T-cell determinants. Peptides 12–26, 81–95 and 113–127 were not identified as T-cell determinants, although each of these peptides induced positive responses reproducibly, because adjacent peptides of the series did not.

T-cell determinants of bovine β -lactoglobulin in BALB/c mice

T cells from β -Lg-immunized BALB/c mice showed the strongest responses to the series of peptides 60–74 to 71–85, next followed in magnitude by the responses to peptides 73–86 to 80–94 (Fig. 5). Intense but some-

what weaker proliferative responses than those to the above-mentioned peptides were elicited by five sets of peptides; 1–15 to 8–22, 23–37 to 30–44, 32–46 to 39–54, 102–116 to 107–121, and 127–141 to 140–154. In addition, weak but significant proliferative responses of T cells were induced by sets of peptides 11–25 and 12–26, 41–55 to 44–58, 48–62 to 49–63, and 109–123 to 110–124. These results indicate that there are eleven sites recognized by T cells of H-2^d mice, and these were ranked according to the magnitude of the response induced; regions 60–85 > 73–94 > 1–22, 23–44, 32–54, 102–121, and 127–154 > 11–26, 41–58, 48–63, and 109–124. As shown for the other strains of mice, some peptides induced positive responses only in one experiment. For example, peptides 14–28 to 16–30, and peptides 143–157 and 144–158 induced a proliferative response only in experiment 2 (Fig. 5B). Such peptides were not deemed to contain T-cell determinants due to the lack of reproducibility of their effects. Peptide 46–60 and peptide 112–126 induced positive responses reproducibly, but adjacent peptides of the series did not.

Figure 4. Proliferative response of lymph node cells from β -lactoglobulin-primed C3H/HeN mice against a β -Lg peptide series. The experimental conditions are the same as described in the legend to Fig. 2, except for the use of C3H/HeN ($H-2^k$) mice. The results of two experiments (A for Exp. 1 and B for Exp. 2) using the same peptide series are shown. The average non- β -Lg peptide background response of three wells $\pm$ SD was 12.0 ± 1.8 ($\text{cpm} \times 10^{-3}$) for Exp. 1 and 20.6 ± 2.5 for Exp. 2. The non- β -Lg background response has been subtracted in each case. The response to $10 \mu\text{M}$ β -Lg $\pm$ SD was 88.3 ± 7.6 ($\text{cpm} \times 10^{-3}$) for Exp. 1 and 106.7 ± 4.0 for Exp. 2.

Discussion

In the present study, we accomplished the fine mapping of T-cell determinants of bovine β -Lg recognized in three strains of mice, using a primary cultures of lymph node cells and a set of overlapping 15-mer peptides which covered the entire sequence of β -Lg by shifting in single amino acid steps (Fig. 1). By means

of the strategy employed, we were able to determine the putative core sequence of each T-cell determinant and estimate the relative importance of the determinants in each strain of mice. The results obtained in this study, summarized in Fig. 6, demonstrate the potential of using such a complete set of peptides to identify not only the regions of a protein antigen which include T-cell determinants without omission but also

Figure 5. Proliferative response of lymph node cells from β -lactoglobulin-primed BALB/c mice against a β -Lg peptide series. The experimental conditions are the same as described in the legend to Fig. 2, except for the use of BALB/c (H-2^d) mice. The results of two experiments (A for Exp. 1 and B for Exp. 2) using the same peptide series are shown. The average non- β -Lg peptide background response of three wells $\pm$ SD was 11.5 ± 2.0 ($\text{cpm} \times 10^{-3}$) for Exp. 1 and 14.8 ± 1.9 for Exp. 2. The non- β -Lg background response has been subtracted in each case. The response to $10 \mu\text{M}$ β -Lg $\pm$ SD was 170.1 ± 16.3 ($\text{cpm} \times 10^{-3}$) for Exp. 2 (not determined for Exp. 1).

the essential region at the amino-acid-residue level for each determinant.

The core sequence of T-cell determinants of β -lactoglobulin

The core sequence of a T-cell determinant restricted to a MHC class II molecule has been defined by Gammon

et al. as the residues shared by all peptides inducing a positive response of polyclonal T cells specific for the determinant (Gammon *et al.*, 1991). The multi-pin peptides used in this study have the uncharged diketopiperazine moiety at the C-terminus (Maeji *et al.*, 1990). This moiety may function as an additional C-terminal residue, since it has been reported that I-E^k molecules bind to peptides 82–102 and 82–103 of moth

Figure 6. The core sequences of T-cell determinants of β -lactoglobulin in C57BL/6, C3H/HeN, and BALB/c mice. The location of each T-cell determinant of β -Lg in C57BL/6, C3H/HeN, and BALB/c mice is indicated as the combination of a region identified as containing T-cell determinant(s) (thin line) and a putative core sequence (solid box) defined as described in the Discussion section. The thick, intermediate and thin boxes show the core sequences of dominant, subdominant, and minor determinants, respectively. The numbers at both ends of each core sequence show the positions of the first and the last residues of the core sequence. Regions determined to contain two determinants are indicated as two separate lines, which are bonded with vertical broken lines, each with a box indicating the core sequence. In these regions, the N-terminal boundary of the upstream core and the C-terminal boundary of the downstream core possibly shift towards the N-terminus and C-terminus, respectively. It is also possible that additional core sequences exist within these regions.

cytochrome c synthesized by the multi-pin technology and to conventionally synthesized peptide 82–103 but not to conventional peptide 82–102 (Reay *et al.*, 1994). Therefore, to avoid excluding any possible essential residues when determining the core sequences, we considered each peptide to have an additional residue at the C-terminus.

In the case of C57BL/6 mice, the core sequences of the determinants in regions 115–136, 11–30 and 107–122 were identified as residues 122–130, 16–26 and 108–122 (Fig. 6). All of these determinants are restricted to I-A^b molecules, because C57BL/6 mice, unlike BALB/c and C3H/HeN mice, have only I-A molecules as MHC class II molecules due to a deficiency in the promoter region of the I-E α -chain gene which leads to the lack of I-E molecule expression. The core sequence should contain the minimum of 9 (or occasionally 8) residues required for a peptide to bind to a MHC class II molecule (Rammensee *et al.*, 1995), thus the identified core sequences of not less than 9 residues can be considered to include all residues essential for I-A^b binding. The involvement of residue ¹³⁰Asp in

I-A^b binding is implied by the result that peptide 115–129 induced only marginal positive responses in both experiments (Fig. 2).

If the essential regions of two determinants are located side by side or partially overlap each other, the core sequence determined simply as a shared region of positive peptides would not represent the essential region for any of the determinants. We can assume that sequential positive peptides sharing less than the minimum of 9 residues contain two (or more) determinants. In such case, we defined the core sequences as the last 9 residues of the first peptide in the series of positive peptides and the first 9 residues of the last peptide. According to this definition, the core sequences of the T-cell determinants recognized in C3H/HeN and BALB/c mice were determined (Fig. 6). In the case of C3H/HeN mice, the series of peptides encompassing region 131–154 that induced the most intense proliferation of T cells shared a region of 7 residues. Therefore, the core sequences of the dominant determinants in region 131–154 were determined to be residues 138–146 and 140–148. Considering the proliferation

induced by each peptide relating to this region, we suggest that the determinant containing 140–148 as a core is the dominant determinant and that the other is the subdominant one. Similarly, the core sequences of the subdominant determinants in regions 86–105 and 73–93 were identified as residues 91–101 and 79–88. The core sequences of the minor determinants in regions 98–113 and 127–143 were residues 99–113 and 129–142, respectively.

As for the T-cell determinants recognized in BALB/c mice, region 60–85 was identified as containing two co-dominant T-cell determinants, the core sequences of which were determined to be residues 67–75 and 71–79. Another dominant determinant in region 73–94 contains 80–88 as a core sequence. Region 127–154 was also considered to contain two subdominant determinants, the cores of which were identified as 134–142 and 140–148. The core sequences of other subdominant determinants were identified as 8–16, 30–38, 39–47, and 107–117, and those of minor determinants were 12–26, 44–56, 49–63, and 110–124, respectively.

Correlation between interferon- γ production and proliferative response

The relative importance of each T-cell determinant was estimated by the intensity of the T-cell response induced by the determinant and this is shown as the thickness of the box indicating the core sequence (Fig. 6). The intensity of T-cell response to each determinant was also evaluated by measuring cytokine production, and we found a positive correlation between IFN- γ production and proliferation (Fig. 3). The helper T-cell response can be classified on the basis of cytokine production profiles into two types; the Th1-type response characterized by high levels of IFN- γ and IL-2 production and the Th2-type response characterized by production of IL-4, IL-5, IL-6, and IL-10 (Abbas *et al.*, 1996). The observed positive correlation between the intensity of proliferation and the amount of IFN- γ produced suggests that the T-cell determinants identified and ranked according to the intensity of the proliferative response may represent determinants which preferentially induce the Th1-type response. Therefore, screening by means of a Th2-type cytokine production assay, such as an assay for IL-4, may identify distinct T-cell determinants which preferentially induce a Th2-type response.

Comparison with the previously identified T-cell determinants of β -lactoglobulin

T-cell determinants of β -Lg recognized in BALB/c mice have been identified in previous studies using peptides derived from an enzymatic digest of β -Lg (Takahashi *et al.*, 1990) or a series of 16 synthetic peptides with an overlap of five residues encompassing the entire sequence of β -Lg (Tsuji *et al.*, 1993). All of the determinants identified in these previous studies were also identified in this study. However, both of the previous studies failed to identify the dominant determinant with a core of 80–88, although Tsuji *et al.* suggested that peptide 72–86 may contain a minor determinant (Tsuji *et al.*, 1993). In addition, a subdominant determinant with a core of 39–47 and a minor determinant with a core of 49–63 were also overlooked. These results clearly demonstrate the limitations of using a small number of peptides in identifying the T-cell determinants in a protein antigen.

Tsuji *et al.* have also identified T-cell determinants of β -Lg recognized in C3H/HeN and C57BL/6 mice (Tsuji *et al.*, 1993). In the case of C3H/HeN mice, the minor determinant with a core of 99–113 was not identified by them. In the case of C57BL/6 mice, the dominant and subdominant determinants containing 122–130 or 16–26 as core sequences, respectively, were also identified in the previous study. However, the minor determinant with a core sequence of 108–122 was not identified in Tsuji's report. We did not identify determinant regions corresponding to the determinants in peptides 72–86 and 100–113 identified by Tsuji *et al.* Peptides 75–89 and 76–90, and 99–113 to 103–117, which were not identified as containing determinants in this study due to lack of reproducibility of their effects, may include subdominant or minor determinants for individual mice, since it has been reported that T cells from an individual mouse can show different responses to subdominant determinants (Gammon *et al.*, 1990).

Prediction of T-cell determinants based on MHC class II binding motifs

The method of choice at present for predicting T-cell determinants in a protein antigen from its amino acid sequence is that based on MHC binding motifs, which are defined as the combinations of amino acid residues at relevant positions required for binding (Rammensee *et al.*, 1995). The potential of predictions based on binding motifs for MHC class I molecules has been

demonstrated (DiBrino *et al.*, 1993; Pamer *et al.*, 1991; Rotzschke *et al.*, 1991). To examine the validity of the putative binding motifs for relevant MHC class II molecules in prediction of T-cell determinants, we compared our results with the determinants predicted on the basis of the binding motifs reported previously.

Three motifs have been reported for I-A^b, which differ from one another. Rudensky *et al.* have deduced the binding motif of Asn, Gln or Asp at position 1 (P1) and Pro, Ile or Ser at P7 (Rudensky *et al.*, 1992). The second I-A^b binding motif proposed by Hobohm and Meyerhans was defined as Phe, Tyr or Met at P1 followed by Pro or Ala with a 2-4-residue spacing where Phe and Pro were dominant at each position (Hobohm and Meyerhans, 1993). Wall and colleagues have defined the I-A^b motif as an aromatic or hydrophobic residue at P1 and an uncharged residue at P6 (Wall *et al.*, 1994). The ratio of the number of combinations of residues conforming to each motif in the whole β -Lg sequence to the number of these combinations existing in the T-cell determinants identified in the case of C57BL/6 mice was 1 to 1 for the motif of Rudensky *et al.* (Rudensky *et al.*, 1992), 5 to 2 for that of Hobohm and Meyerhans (Hobohm and Meyerhans, 1993), and 33 to 8 for that of Wall *et al.* (Wall *et al.*, 1994). Although the motif of Wall *et al.* predicted all of the three determinants (Table 1), this motif does not seem to be effective for prediction, because β -Lg has as many as 33 combinations of amino acid residues conforming to this motif. The motif provided by Rudensky *et al.* could successfully predict only the dominant determinant. However, this successful prediction may be by chance, since one of the two motif-composing residues, ¹²⁰Gln, is not involved in the core sequence of this determinant. Therefore, none of the I-A^b motifs previously reported proved effective to predict the T-cell determinants.

Nelson *et al.* have reported that the A^k molecule preferentially binds peptides containing negatively charged amino acids at the P1 position (Nelson *et al.*, 1996). They proposed an A^k binding motif as Asp or Glu (Cys, Ser, Asn, Gln, and Thr allowed) at P1, a large aliphatic amino acid residue, Ile, Val, or Leu, at P4, and a medium to large polar amino acid residue, Glu or Gln, at P6. Residues 115–120 alone agreed with this motif, but no determinant was identified around this region in the case of C3H/HeN mice. Another six regions displaying such a motif with a disagreement at either P4 or P6 were found, and among them residues 129–134 alone correspond to the determinant identified. Binding motifs for I-E molecules (d, k, b and s haplotypes) have

Table 1. I-A^b-restricted T-cell determinants of β -lactoglobulin and residues corresponding to the putative I-A^b binding motifs previously reported

Region containing a T-cell determinant	Core sequence	Motif residues	Motif
115–136	122–130	¹²⁰ E . . . ¹²⁶ P	a)
		¹¹⁷ L . . . ¹²² L	b)
		¹¹⁸ V . . . ¹²³ V	b)
		¹²³ V . . . ¹²⁸ V	b)
		¹²⁸ V . . . ¹³³ L	b)
11–30	16–26	²⁰ Y . . . ²³ A	c)
		²⁰ Y . . . ²⁵ A	c)
		¹⁵ V . . . ²⁰ A	b)
		¹⁹ W . . . ²⁴ A	b)
		²⁰ Y . . . ²⁵ A	b)
		²² L . . . ²⁷ A	b)
107–122	108–122	¹⁰⁷ M . . . ¹¹¹ A	c)
		¹¹⁷ L . . . ¹²² L	b)

a) Motif of Rudensky *et al.* (Rudensky *et al.*, 1992).

b) Motif of Wall *et al.* (Wall *et al.*, 1994).

c) Motif of Hobohm and Meyerhans (Hobohm and Meyerhans, 1993).

been proposed by Schild *et al.* (Schild *et al.*, 1995), who defined the E^k motif as Ile, Leu or Val (Trp, Tyr, and Phe allowed) at P1, Ile, Leu, Phe, Ser or Ala (Arg, Lys, Asp and Glu allowed) at P4, Gln or Asn (Ser, Thr, Ala, His, Arg, and Glu allowed) at P6, and Lys or Arg (Gly allowed) at P9. The region of residues 92–100 was the only region agreeing entirely with the E^k motif, which corresponds to the core sequence of residues 91–101 in a subdominant determinant. Regions 140–148 and 133–141 each corresponding to the E^k motif with a disagreement at P6, agreed with the core sequence of the dominant determinant (residues 140–148) and that of the subdominant determinant (residues 129–142), respectively. Three other motif regions similar to this motif, with disagreeing at residues P4 or P6, were not identified as containing T-cell determinants recognized in C3H/HeN mice. The rest of the determinants that we identified were not predicted on the basis of the A^k and E^k motifs.

Peptide regions corresponding to the I-A^d motif reported by Sette *et al.* (Sette *et al.*, 1989) are found in residues 1–6, 22–27, 29–34 and 145–151. These motif residues are included in regions 1–22, 23–44 and 127–154 which contain subdominant determinants recognized in BALB/c mice, but do not correspond to their

core sequences. The E^d motif of Schild *et al.* (Schild *et al.*, 1995) was defined as Trp, Tyr, or Phe (Ile, Leu, and Val allowed) at P1, Arg or Lys (Ile and Val allowed and no negative charge) at P4, Ile, Leu, or Val (Gly allowed) at P6, and Lys or Arg at P9. Although no region completely agreed with the I-E^d motif, residues 67–75 agreed with the motif except for ⁶⁷Ala at P1 and residues 140–148 agreed except for ¹⁴³Leu at P4 and ¹⁴⁵Met at P6. These motif residues correspond to the core sequence of the dominant determinant (residues 67–75) and that of the subdominant determinant (residues 140–148), respectively. The dominant determinants of regions 71–79 and 80–88 could not be predicted by the A^d and E^d motifs.

Collectively, the binding motifs for relevant MHC class II molecules proved not to be sufficient for the precise prediction of T-cell determinants. The binding motifs for I-E molecules seem more effective for predicting T-cell determinants and also their essential residues than those for I-A molecules. The different efficiency between I-A and I-E motifs may represent the difference in accuracy of the motifs, because the information available for the I-E molecule and its human homolog HLA-DR is much more abundant than that for I-A and HLA-DQ molecules. For example, the tertiary structures of the I-A and HLA-DQ molecules have not yet been elucidated. Therefore, more extensive studies may be required especially focusing on I-A/DQ molecules in order to establish an efficient method of predicting T-cell determinants.

Concluding remarks

In conclusion, we have elucidated the T-cell determinant structures of β -Lg at the amino-acid-residue level. The binding motifs for relevant murine MHC class II alleles reported to date were found to be insufficiently effective to predict T-cell determinants precisely. Methods for predicting T-cell determinants based on amino acid sequence data are attracting considerable research attention, because for a protein antigen it is difficult to determine the T-cell determinants recognized in humans. Complete mapping of the T-cell determinants recognized in mice, which can be determined more easily than those recognized in humans, will provide information useful for developing effective prediction methods.

The next step to further analyze the T-cell recognition of β -Lg is to distinguish the MHC binding residues and TCR binding residues in the core sequences. This can be achieved by analyzing whether analogs of a

determinant-containing peptide with a substitution at each core residue can bind to the relevant MHC molecules and induce T-cell responses specific for the determinant. Using this strategy, we were able to determine the MHC and TCR binding residues within residues 122–130, the core sequence of the dominant determinant in C57BL/6 mice (M. Totsuka, unpublished). The T-cell determinant structures at the amino-acid-residue level will provide information required for study of the manipulation of immune responses by changing the structure of the allergen.

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