

Three T-cell determinants of Cry j 1 and Cry j 2, the major Japanese cedar pollen antigens, retain their immunogenicity and tolerogenicity in a linked peptide

TOMOMI YOSHITOMI,* KAZUKI HIRAHARA,* JUNKO KAWAGUCHI,* NOBUFUSA SERIZAWA,* YOSHIFUMI TANIGUCHI,† SABURO SAITO,‡ MASAHIRO SAKAGUCHI,§ SAKAE INOUE,§ & AKIO SHIRAISHI*
**Sankyo Co., Ltd, Tokyo, Japan, †Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, ‡The Jikei University School of Medicine, Tokyo, Japan, §National Institute of Infectious Diseases, Tokyo, Japan*

SUMMARY

It has been demonstrated in detail that administration of a dominant T-cell determinant to animals induces activation or immunological tolerance of T cells. However, it has not been determined whether multiple T-cell determinants, when integrated into a single peptide, retain their potential to induce T-cell activation and tolerance. We prepared a synthetic peptide comprising three T-cell determinants of Cry j 1 and Cry j 2, the major Japanese cedar pollen antigens, and investigated the immunogenicity and tolerogenicity of each T-cell determinant in the linked peptide by means of lymph node cell proliferation assays using mice. Lymph node cells from mice immunized with each of the three T-cell determinants proliferated against the linked peptide in a dose-dependent manner, similar to that of the immunized peptide. Lymph node cells from mice immunized with the linked peptide proliferated against all of the three T-cell determinants. In addition, the degree of proliferation against the three T-cell determinants occurred according to their original immunogenicity, as observed in the native protein antigens. Oral administration of the linked peptide to mice before they were immunized with Cry j 1 and Cry j 2 inhibited lymph node cell proliferation against the three T-cell determinants, depending on the dose of the linked peptide administered. In conclusion, it was demonstrated that three T-cell determinants retain their original immunogenicity and tolerogenicity in a linked peptide comprising them.

INTRODUCTION

It has been demonstrated in detail that administration, to animals, of a peptide (with or without adjuvant), corresponding to a dominant T-cell determinant of a protein antigen, induces activation or immunological tolerance of specific T cells.^{1–5} The peptide-specific T-cell response is critically controlled by molecular interaction between T-cell receptors on T cells and major histocompatibility complex (MHC) class II molecules on antigen-presenting cells (APCs). Theoretically, a sequentially

linked peptide comprising multiple T-cell determinants is able to react on many peptide-specific T cells, as long as each component T-cell determinant has sufficient length and interaction with the MHC class II molecules. T-cell reactivity of T-cell determinants in a linked peptide has been progressively investigated.^{6–10} However, it has not been experimentally determined whether multiple (at least three) T-cell determinants, when integrated into a single peptide, retain their original immunogenicity and tolerogenicity, namely, the potential to induce activation or inactivation of specific T cells.

In this study, we prepared a synthetic peptide of 42 amino acid residues in length, which comprised three 14-mer murine T-cell determinants of Cry j 1 and Cry j 2, the major Japanese cedar (*Cryptomeria japonica*) pollen antigens. In a previous study, we searched for T-cell determinants of Cry j 1 and Cry j 2 in BALB/c mice by using overlapping peptides. We identified three peptides corresponding to a dominant T-cell determinant of Cry j 1 (P1-277–290) and a dominant (P2-246–259) and a subdominant (P2-70–83) T-cell determinant of Cry j 2. We previously reported that a dominant peptide, P2-246–259, induces T-cell tolerance against the peptide itself and Cry j 2

Received 3 April 2002; revised 14 August 2002; accepted 30 August 2002.

Abbreviations: APC, antigen-presenting cell; CFA, complete Freund's adjuvant; i.n., intranasally; LNC, lymph node cell; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; s.c., subcutaneously.

Correspondence: Tomomi Yoshitomi, Biological Research Laboratories, Sankyo Co., Ltd, 2–58 Hiromachi 1-chome, Shinagawa-ku, Tokyo 140-8710, Japan. E-mail: yositomi@shina.sankyo.co.jp

when orally administered to mice. In the present study, we investigated whether three T-cell determinants in a linked peptide would retain their immunogenicity and tolerogenicity, even after they were integrated into a single peptide.

MATERIALS AND METHODS

Mice

Six-week-old female BALB/c mice were purchased from Charles River Japan (Tokyo, Japan) and housed under conventional conditions. The Institutional Animal Care and Use Committee of Sankyo approved all the experiments in this study. Three mice were used for each group in each experiment.

Antigens and peptides

Cry j 1 (353 amino acids, 45 kDa) and Cry j 2 (388 amino acids, 38 kDa) were purified using monoclonal antibodies (mAbs), as described previously.^{11,12} Three peptides corresponding to the three T-cell determinants (P1-277–290, P2-70–83 and P2-246–259) were synthesized using a solid-phase peptide synthesizer (Symphony; Protein Technologies, Tucson, AZ), which uses standard Fmoc chemistry. A linked peptide comprising the three T-cell determinants (hereafter called '3TD peptide') without spacer amino acids between them was synthesized using a 430-A multipetide synthesizer (Applied Biosystems, Foster City, CA). The amino acid sequences of these peptides are shown in Table 1. These peptides were purified by high-performance liquid chromatography (HPLC) and used after verifying the purity and validating the amino acid sequences.

Immunization

Mice were injected subcutaneously (s.c.) at the base of the tail and into the footpads with 1 µg of Cry j 1 and 1 µg of Cry j 2, 1 µg of each T-cell determinant, or 1 µg of the 3TD peptide in emulsion. The emulsion was prepared by mixing the antigens in phosphate-buffered saline (PBS) and complete Freund's adjuvant (CFA) containing *Mycobacterium tuberculosis* H37 Ra (Difco Laboratories, Detroit, MI) (W : O = 1 : 1). Ten days later, popliteal and inguinal lymph node cells (LNCs) were removed and used in the proliferation assay.

In experiments evaluating tolerogenicity of the 3TD peptide, mice were injected intranasally (i.n.) with 1 µg of Cry j 1 and 1 µg of Cry j 2. The antigens were mixed with 1 µg of cholera toxin B subunit (Research Biochemicals Inc., Natick, MA) and 5 ng of cholera toxin (Sigma Chemical Co., St Louis, MO) as a mucosal adjuvant, and all antigens were dissolved in 7.3 µl of PBS. Two weeks later, the mice were boosted i.n. with the same

antigens. One week after the booster, cervical LNCs were removed and used for the proliferation assay.

Induction of oral tolerance

Mice were administered, orally (using a plastic animal-feeding needle), 2.5, 25, or 250 µg of the 3TD peptide dissolved in 0.2 ml of PBS. Mice were administered the peptide four times at intervals of 3 or 4 days within 2 weeks, and 3 days after the last administration the mice were subsequently immunized i.n. with Cry j 1 and Cry j 2, as described above.

T-cell proliferation assay

Single-cell suspensions were prepared from LNCs. In the experiments evaluating tolerogenicity of the 3TD peptide, an erythrocyte-depleted, X-ray-irradiated spleen-cell suspension was also prepared from autologous normal mice and used as APCs. The pooled LNCs from the popliteal and inguinal lymph nodes were cultured at 4×10^5 cells/well, and the cervical LNCs were cultured at 3×10^5 cells/well together with the APCs at 4×10^5 cells/well, in 0.2 ml of culture medium in 96-well flat bottom plates (Corning Inc., Corning, NY). The cells were cultured in RPMI-1640 supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin (all from Life Technologies, Inc., Grand Island, NY) and 1% autologous normal mouse serum. The cells were cultured with or without antigens in triplicate wells for 3 days at 37°. In some experiments, concanavalin A (Sigma Chemical Co.) was used as a positive control antigen. Each well then received 0.5 µCi [³H]thymidine (Amersham Pharmacia Biotech. Ltd, Tokyo, Japan) and the cells were incubated for a further 18 hr. The cells were harvested and the incorporated radioactivity was measured by using a liquid scintillation counter. The results are shown as mean counts per minute (c.p.m.) ± standard deviation (SD).

Statistics

Significant differences were determined by the parametric Williams' test¹³ using the computer software SAS, Version 4.0 (SAS Institute Inc., Cary, NC).

RESULTS

Preparation of the 3TD peptide comprising three T-cell determinants of Cry j 1 and Cry j 2

In order to investigate the immunogenic and tolerogenic potential of the T-cell determinants in a linked peptide, we prepared a synthetic peptide comprising three T-cell determinants of Cry j 1 and Cry j 2 (Table 1). Peptides P1-277–290, P2-70–83 and P2-246–259 represent a dominant T-cell determinant of Cry j 1, a subdominant T-cell determinant of Cry j 2 and a dominant T-cell determinant of Cry j 2, respectively. We first investigated whether LNCs from mice immunized with Cry j 1 and Cry j 2 would proliferate against the 3TD peptide as well as against the three T-cell determinants when the cells were stimulated *in vitro* with each of these peptides separately. Mice were immunized s.c. with Cry j 1 and Cry j 2 together with CFA. Ten days later, popliteal and inguinal LNCs were removed from the mice and stimulated *in vitro* with P1-277–290, P2-70–83, P2-246–259 and the 3TD peptide. LNCs proliferated against the three

Table 1. Three T-cell determinants of Cry j 1 and Cry j 2, and 3TD peptide

Peptide	Residue	Length	Amino acid sequence
P1-277–290	Cry j 1/277–290	14-mer	KQVTIRIGCKTSSS
P2-70–83	Cry j 2/70–83	14-mer	HFTFKVDGIIAAYQ
P2-246–259	Cry j 2/246–259	14-mer	RAEVSYPHVNGAKF
3TD peptide		42-mer	RAEVSYPHVNGAKF-KQVTIRIGCKTSSSHF-TFKVDGIIAAYQ

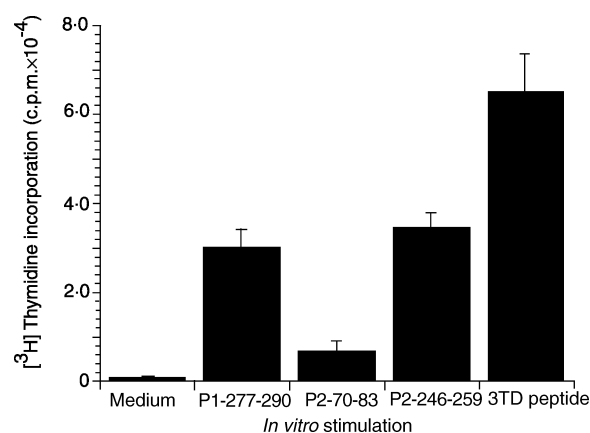


Figure 1. Overall immunogenicity of the 3TD peptide. Three mice were immunized subcutaneously (s.c.) with 1 µg of Cry j 1 and 1 µg of Cry j 2 in complete Freund's adjuvant (CFA), and 10 days later lymph node cells were stimulated *in vitro* with 10 µM of the three T-cell determinants P1-277-290, P2-70-83 and P2-246-259, and with 10 µM of the 3TD peptide comprising them. Cell proliferation was measured as described in the Materials and methods. Results are expressed as mean counts per minute (c.p.m.) ± standard deviation (SD) and are representative of three identical experiments.

T-cell determinants, P1-277-290, P2-70-83 and P2-246-259, according to their immunodominance (Fig. 1). The LNCs also proliferated against the 3TD peptide (Fig. 1). Overall T-cell immunogenicity of the 3TD peptide *in vitro* was confirmed and thus we used it for further investigation.

Immunogenicity of T-cell determinants in the 3TD peptide *in vitro*

We next investigated whether each T-cell determinant in the 3TD peptide had its own immunogenicity *in vitro*. Mice were

immunized s.c. with P1-277-290, P2-70-83 or P2-246-259. LNCs were removed from the mice and stimulated *in vitro* with these T-cell determinants and the 3TD peptide. LNCs from P1-277-290-immunized mice proliferated against the 3TD peptide as well as against P1-277-290, but did not proliferate against P2-70-83 or P2-246-259 (Fig. 2a). LNCs from P2-70-83-immunized mice proliferated against the 3TD peptide and P2-70-83, but not against P1-277-290 or P2-246-259 (Fig. 2b). Finally, LNCs from P2-246-259-immunized mice proliferated against the 3TD peptide and P2-246-259, but not against P1-277-290 or P2-70-83 (Fig. 2c). Taken together, these results show that LNCs proliferated against specific T-cell determinants and the 3TD peptide. The results indicate that the three T-cell determinants in the 3TD peptide have the potential to induce T-cell proliferation against specific T cells *in vitro*.

Immunogenicity of T-cell determinants in the 3TD peptide *in vivo*

We next investigated the immunogenicity of each T-cell determinant in the 3TD peptide *in vivo*. Mice were immunized s.c. with the 3TD peptide. LNCs were removed from the mice and stimulated *in vitro* with P1-277-290, P2-70-83, P2-246-259 and the 3TD peptide. The LNCs from the 3TD peptide-immunized mice proliferated against all the three T-cell determinants, P1-277-290, P2-70-83 and P2-246-259, as well as against the 3TD peptide (Fig. 3). In addition, the degree of proliferation against P1-277-290 and P2-246-259 was strong, while that against P2-70-83 was relatively weak, which was consistent with the original immunogenicity of the three T-cell determinants in the native protein antigens, as shown in Fig. 1. This result indicates that the three T-cell determinants in the 3TD peptide have the potential to induce activation of specific T cells *in vivo*, and that they retain their original immunogenicity.

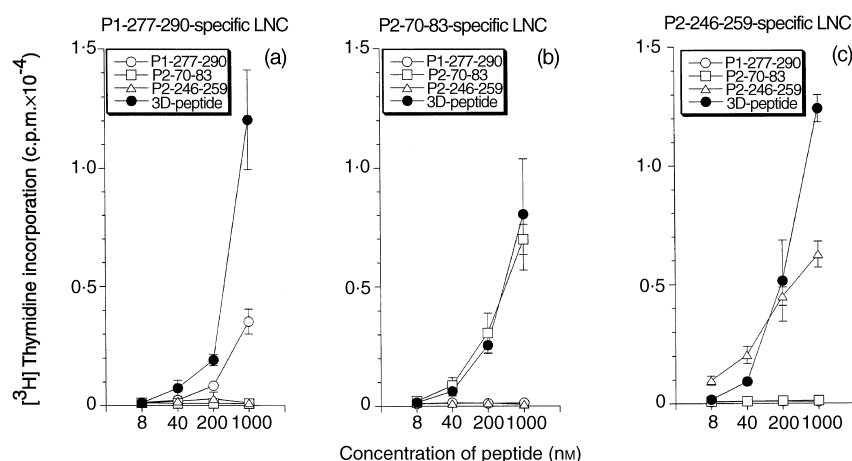


Figure 2. *In vitro* immunogenicity of three T-cell determinants in the 3TD peptide. Three groups of mice were immunized subcutaneously (s.c.) with 1 µg of P1-277-290 (a), P2-70-83 (b), or P2-246-259 (c) in complete Freund's adjuvant (CFA), and 10 days later lymph node cells were stimulated *in vitro* with different concentrations of the three T-cell determinants and 3TD peptide comprising them. Cell proliferation was measured as described in the Materials and methods. Results are expressed as mean counts per minute (c.p.m.) ± standard deviation (SD) and are representative of three identical experiments.

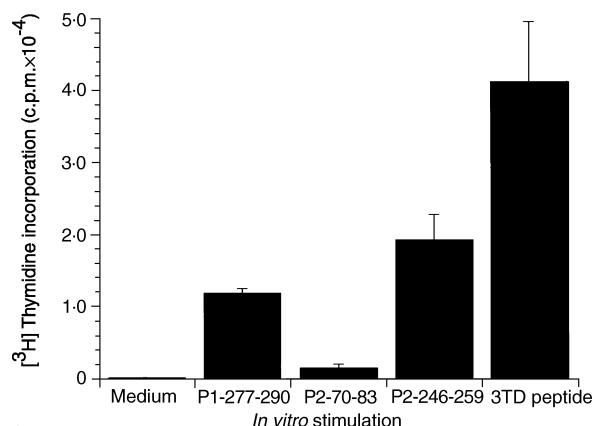


Figure 3. Immunogenicity of three T-cell determinants in the 3TD peptide. Mice were immunized subcutaneously (s.c.) with 1 µg of 3TD peptide in complete Freund's adjuvant (CFA) and 10 days later lymph node cells were stimulated *in vitro* with 10 µM of the three T-cell determinants (P1-277–290, P2-70–83 and P2-246–259) and with 10 µM of the 3TD peptide. Cell proliferation was measured as described in the Materials and methods. Results are expressed as mean counts per minute (c.p.m.) ± standard deviation (SD) and are representative of three identical experiments.

Tolerogenicity of T-cell determinants in the 3TD peptide *in vivo*

We finally investigated the tolerogenicity of each T-cell determinant in the 3TD peptide. We previously reported that oral administration of P2-246–259 to mice before they were immu-

nized i.n. with Cry j 2 inhibited proliferative responses of cervical LNCs against P2-246–259 and Cry j 2. In this report, we examined whether the 3TD peptide would also induce immunological tolerance in BALB/c mice. Groups of mice received orally 2.5, 25 or 250 µg of the 3TD peptide, or PBS alone, four times within 2 weeks and were subsequently immunized i.n. with Cry j 1 and Cry j 2, together with cholera toxin. After the mice were boosted with the same antigens, cervical LNCs from each of the four groups were removed and stimulated *in vitro* with P1-277–290, P2-70–83, P2-246–259, Cry j 1, Cry j 2 and concanavalin A (Con A), which was used as a positive control. LNCs from 3TD peptide-treated mice showed inhibited proliferation against the three T-cell determinants, P1-277–290, P2-70–83 and P2-246–259, compared to those from the PBS-treated control mice (Fig. 4). In addition, LNC proliferation against Cry j 1 and Cry j 2 was also inhibited in the 3TD peptide-treated groups. The inhibitory rate for each antigen was dependent on the dose of the 3TD peptide administered. As LNC proliferation against Con A was almost the same among the four groups, the inhibition was considered not to be caused by any non-specific effects of the 3TD peptide. In accordance with the inhibited cell proliferation, LNCs from the 3TD peptide-treated group showed inhibited interleukin-2 (IL-2) secretion against the three T-cell determinants, Cry j 1 and Cry j 2, compared to that in the control group (data not shown). Therefore, oral administration of the 3TD peptide induced specific T-cell tolerance for the three T-cell determinants and the native protein antigens (Cry j 1 and Cry j 2). The results indicate that the three T-cell determinants in the 3TD peptide have the potential to induce tolerance against specific T cells *in vivo*.

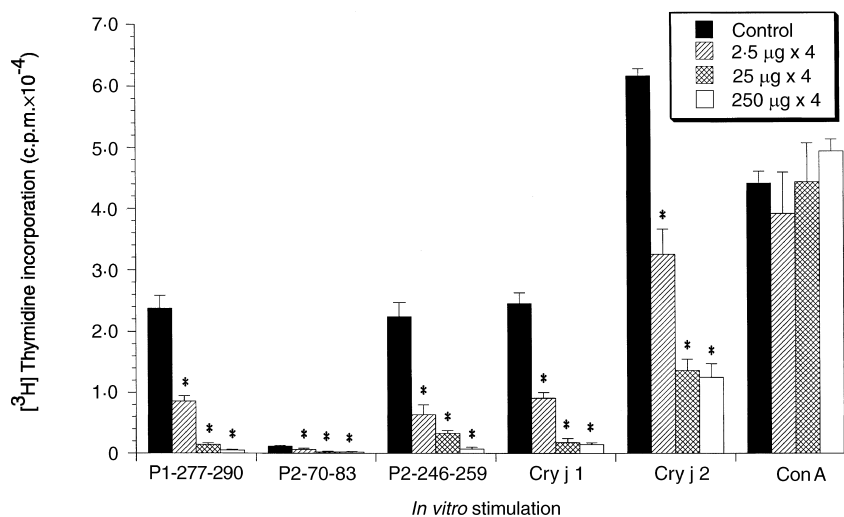


Figure 4. Tolerogenicity of three T-cell determinants in the 3TD peptide. Four groups of mice were administered orally with the 3TD peptide, four times at intervals of 3 or 4 days, within 2 weeks prior to being immunized intranasally with 1 µg of Cry j 1 and 1 µg of Cry j 2 in cholera toxin. Two weeks after the immunization, the mice were boosted with the same antigens, and lymph node cells were stimulated *in vitro* with 5 µM of the three T-cell determinants, 100 nM Cry j 1, 100 nM Cry j 2 and 5 µg/ml of concanavalin A (Con A) 1 week after the booster. The cell proliferation was measured as described in the Materials and methods. Results are expressed as mean counts per minute (c.p.m.) ± standard deviation (SD) and are representative of three identical experiments. The background responses in the control group and 3TD peptide-treated group (2.5, 25, 250 µg) are 86 ± 26, 39 ± 30, 39 ± 17 and 40 ± 14 c.p.m., respectively. The results for Con A are presented as quarter-sized bars. **P* < 0.01 versus control.

DISCUSSION

The purpose of this study was to investigate the potential of T-cell determinants in a sequentially linked peptide for inducing activation and tolerance of specific T cells. As a model for a linked peptide, we prepared a synthetic peptide comprising three murine T-cell determinants of Cry j 1 and Cry j 2, and we investigated the immunogenicity and tolerogenicity of the T-cell determinants in a 3TD peptide by means of LNC proliferation assays.

In terms of immunogenicity, it was first demonstrated that each T-cell determinant in the 3TD peptide has the potential to induce specific T-cell proliferation *in vitro*. Three types of peptide-specific LNCs were prepared from mice immunized with the three T-cell determinants. Specificity of the LNCs was confirmed because the cells proliferated only against the immunized T-cell determinant and not against the other two T-cell determinants. Under the same conditions, a similar dose-dependent T-cell proliferation was observed between the 3TD peptide and each of the three T-cell determinants. This result suggests that each of the T-cell determinants in the 3TD peptide was processed and presented by APCs included in the LNCs in a similar manner to the individual T-cell determinants *in vitro*.

In terms of immunogenicity, it was also demonstrated that each T-cell determinant in the 3TD peptide has the potential to induce specific T-cell activation *in vivo*. LNCs from mice immunized s.c. with the 3TD peptide proliferated against all the three T-cell determinants (P1-277–290, P2-70–83 and P2-246–259). This suggests that local APCs, such as dendritic cells, captured the 3TD peptide, migrated to draining lymph nodes and appropriately presented each of the T-cell determinants in the 3TD peptide to specific T cells. The activated specific T cells in the lymph nodes were detected by proliferation against the three T-cell determinants *in vitro*. Notably, the degree of LNC proliferation to each T-cell determinant appears to be correlated to its original T-cell immunogenicity in the native protein antigens; LNCs from the 3TD peptide-immunized mice proliferated significantly against the dominant T-cell determinants (P1-277–290 and P2-246–259) and showed a weak, but positive, proliferation against P2-70–83. These results from experiments assessing the potential of each T-cell determinant in the 3TD peptide for inducing activation of specific T cells both *in vitro* and *in vivo*, revealed that the T-cell determinants retain their own immunogenicity, even after they are integrated into a linked peptide.

Ria *et al.*⁶ reported that in a peptide comprising two T-cell determinants, the T-cell response was directed mainly towards the more immunogenic T-cell determinant, whereas the less immunogenic T-cell determinant elicited little or no T-cell response. Our data appear inconsistent with their results in terms of an emphasized hierarchy of T-cell immunogenicity in a sequentially linked peptide. They stated that the enhanced hierarchy of T-cell determinants might be caused by competitive binding to the MHC molecules between the two peptides. Their peptide comprised 13- (the less immunogenic) and 15- (the more immunogenic) amino acid peptides, whereas our 3TD peptide comprises three 14-amino acid peptides. Thus, length of peptide in addition to intermolecular competition

might influence accessibility to MHC molecules and consequently the T-cell immunogenicity of T-cell determinants in a linked peptide, as it has been reported that MHC class II molecules preferentially bind to longer peptides in the process of antigen presentation.^{14–16} Further investigation is necessary to clarify whether accessibility of MHC molecules to T-cell determinants in a linked peptide would influence their immunogenicity. It should be noted, however, that immunogenicity of the three T-cell determinants in the 3TD peptide was not so different from their original immunogenicity.

In terms of tolerogenicity, it was finally demonstrated that each T-cell determinant in the 3TD peptide has the potential to induce specific T-cell tolerance *in vivo*. Oral administration of the 3TD peptide to mice before they were immunized i.n. with Cry j 1 and Cry j 2, significantly inhibited LNC proliferation and IL-2 secretion against the three T-cell determinants (P1-277–290, P2-70–83 and P2-246–259), depending on the dose of the 3TD peptide administered. This result suggests that APCs in gut-associated lymphoid tissues, such as Peyer's patch, captured the 3TD peptide, appropriately presented the three T-cell determinants in the 3TD peptide to specific T cells, and rendered them tolerant. Although the mechanism of the oral tolerance is not clear, it appears evident that each T-cell determinant in the 3TD peptide has tolerogenicity because T cells from cervical lymph nodes showed an inhibited response against the three T-cell determinants.

Rogers *et al.*⁸ also reported tolerogenicity of a linked peptide comprising three human T-cell determinants of Fel d 1. They showed that s.c. injection of the linked peptide to mice before they were immunized with antigenic peptides from Fel d 1 significantly inhibited LNC proliferation against the linked peptide, a mixture of three T-cell determinants, and the immunogen. However, they did not determine the LNC proliferation against the three human T-cell determinants separately and thus tolerogenicity of each T-cell determinant in the linked peptide was not clearly demonstrated.

Sufficient tolerogenicity of T-cell determinants in a linked peptide has practical significance. As a result of inhibition to T cells specific for dominant T-cell determinants, LNC proliferation against Cry j 1 and Cry j 2, native antigens the T-cell determinants were derived from, was also inhibited. The contribution of T cells specific for the subdominant T-cell determinant to induce tolerance against Cry j 2 might be limited. Our study is the first, as far as we know, to demonstrate immunological tolerance against two protein antigens using a single peptide. There is a possibility that T-cell responses against multiple protein antigens can be inhibited by a single peptide comprising many dominant T-cell determinants of the antigens. Indeed, we have developed a peptide comprising seven human T-cell determinants of Cry j 1 and Cry j 2 as a candidate immunotherapeutic peptide.¹⁴

In summary, we have demonstrated that each of the three T-cell determinants in a 3TD peptide retains its immunogenicity. We have also demonstrated that each of the three T-cell determinants in the 3TD peptide has tolerogenicity. Our results have clarified potential T-cell immunogenicity and tolerogenicity of T-cell determinants in a linked peptide which comprises multiple T-cell determinants. These findings imply that a linked

peptide could be a novel immunotherapeutic tool for regulating several antigen-specific T cells.

ACKNOWLEDGMENTS

We thank Mihoko Watanabe for her contribution to this work. We also thank Kazuhiro Ito, PhD, DVM, for critical review of this article.

REFERENCES

- 1 Briner TJ, Kuo M, Keating KM, Rogers BL. Peripheral T-cell tolerance induced in naïve and primed mice by subcutaneous injection of peptides from the major cat allergen, Fel d 1. *Proc Natl Acad Sci USA* 1993; **90**:7608–12.
- 2 Hirahara K, Saito S, Serizawa N *et al.* Oral administration of a dominant T-cell determinant peptide inhibits allergen-specific T_{H1} and T_{H2} cell responses in Cry j 2-primed mice. *J Allergy Clin Immunol* 1998; **102**:961–7.
- 3 Hoyne GF, Jarnicki AG, Thomas WR, Lamb JR. Characterization of the specificity and duration of T cell tolerance to intranasally administered peptides in mice: a role for intramolecular epitope suppression. *Int Immunol* 1997; **9**:1165–73.
- 4 Liu JQ, Bai XF, Shi FD *et al.* Inhibition of experimental autoimmune encephalomyelitis in Lewis rats by nasal administration of encephalitogenic MBP peptides: synergistic effects of MBP 68–86 and 87–99. *Int Immunol* 1998; **10**:1139–48.
- 5 Porgador A, Staats HF, Faiola B, Gilboa E, Palker T. Intranasal immunization with CTL epitope peptides from HIV-1 or ovalbumin and the mucosal adjuvant cholera toxin induces peptide-specific CTLs and protection against tumor development *in vivo*. *J Immunol* 1997; **158**:834–41.
- 6 Ria F, Chan BMC, Scherer MT, Smith JA, Geftler ML. Immunological activity of covalently linked T-cell epitopes. *Nature* 1990; **343**:381–3.
- 7 Perkins DL, Berriz G, Kamradt T, Smith JA, Gaftier ML. Immuno-dominance: intermolecular competition between T cell epitopes. *J Immunol* 1991; **146**:2137–44.
- 8 Rogers BL, Bond JF, Craig SJ *et al.* Potential therapeutic recombinant proteins comprised of peptides containing recombined T cell epitopes. *Mol Immunol* 1994; **31**:955–66.
- 9 Sone T, Morikubo K, Miyahara M, Komiyama N, Shimizu K, Tsunoo H, Kino K. T cell epitopes in Japanese cedar (*Cryptomeria japonica*) pollen allergen: choice of major T-cell epitopes in Cry J 1 and Cry J 2 towards design of the peptide-based immunotherapeutics for the management of Japanese cedar pollinosis. *J Immunol* 1998; **161**:448–57.
- 10 Hirahara K, Tatsuta T, Takatori T *et al.* Preclinical evaluation of immunotherapeutic peptide comprising 7 T-cell determinants of Cry j 1 and Cry j 2, the major Japanese cedar pollen allergens. *J Allergy Clin Immunol* 2001; **108**:94–100.
- 11 Yasueda H, Yui Y, Shimizu T, Shida T. Isolation and partial characterization of the major allergen from Japanese cedar (*Cryptomeria japonica*) pollen. *J Allergy Clin Immunol* 1983; **71**:77–86.
- 12 Sakaguchi M, Inouye S, Tanai M, Ando S, Usui M, Matuhashi T. Identification of the second major allergen from Japanese cedar pollen. *Allergy* 1990; **45**:309–12.
- 13 Williams DA. The comparison of several dose levels with a zero dose control. *Biometrics* 1972; **28**:519–31.
- 14 Lo-Man R, Leclerc C. Parameters affecting the immunogenicity of recombinant T cell epitopes inserted into hybrid proteins. *Hum Immunol* 1997; **54**:180–8.
- 15 Rudensky AY, Preston-Hurlburt P, Hong SC, Barlow A, Janeway CA Jr. Sequence analysis of peptides bound to MHC class II molecules. *Nature* 1991; **353**:623–7.
- 16 Nelson CA, Vidavsky I, Viner NJ, Gross ML, Unanue ER. Amino-terminal trimming peptides for presentation on major histocompatibility complex class II molecules. *Proc Natl Acad Sci USA* 1997; **94**:628–33.