

Original article

Survival of memory T cells specific for Japanese cypress pollen allergen is maintained by cross-stimulation of putative pectate lyases from other plants

In view of recent studies on the mechanisms of the survival of peripheral memory T cells, we tested the biologic role of pectate lyase, a pectin-degrading enzyme, as the cross-reactive antigen required for the recurring survival signals for human T cells specific for Cha o 1, a pollen allergen molecule of the Japanese cypress. We determined a 16-mer epitope peptide for the T-cell clone, and prepared synthetic oligopeptides of homologous regions in putative pectate lyase of other plants. Of these homologous peptides, ZePel (*Zinnia elegans*), ban 17 (banana), and Amb a 1.1 (short ragweed) induced strong proliferative responses of the Cha o 1-specific T-cell clone *in vitro*. In addition, suboptimal doses of peptide homologs derived from banana and short ragweed enhanced the survival potency of this T-cell clone without detectable proliferative responses to the peptides. When there was no antigen stimulation, the T-cell clone decreased in viable cell number and lost antigen-specific proliferation activity on day 6 during *in vitro* incubation. On the other hand, T-cell clones incubated with these survival-inducing peptides maintained proliferative activity to Cha o 1 even on day 9. Serum derived from the donor patient did not contain detectable levels of IgE specific to banana or short ragweed by CAP-RAST. These results show that human T cells specific for pollen allergen seem to use cross-reactive pectate lyase peptides to deliver survival signals even in the absence of pollen allergen, and memory T cells maintained in such a manner might be functioning at the onset of allergic pollinosis, although pollen allergens are seasonal.

**Y. Nakamura¹, S. Takagi¹,
M. Suzuki¹, H. Ito¹, S. Murakami¹,
N. Ohta²**

Departments of ¹Otorhinolaryngology and ²Medical
Zoology, Nagoya City University Medical School,
Nagoya, Japan

Key words: CD4-positive memory T cell; cell survival;
Cha o 1; Japanese cypress pollen; pectate lyase; T-
cell epitope; pollinosis.

Yoshihisa Nakamura
Department of Otorhinolaryngology
Nagoya City University Medical School
1 Azakawasumi, Mizuhocho, Mizuhoku
Nagoya 467-8601
Japan

Accepted for publication 5 December 2000

During the primary immune response, T cells are sensitized to antigens, and many of them differentiate into effector T cells under strict regulatory mechanisms. However, a small fraction of antigen-specific T cells survive as memory T cells and remain in the peripheral blood. Although the mechanisms for long-term survival of memory T cells are not fully understood, recent studies suggest that they are originally short-lived, and are maintained by recurring survival signals from environmental and/or internal cross-reactive antigens even in the absence of the original sensitizing antigen (1). Adoptive transfer experiments suggest that memory T cells can survive only several months without antigens (2). The essential role of the self-peptide-class I major histocompatibility complex (MHC) in the long-term survival of CD8⁺ memory T cells was clearly shown with TAP1-deficient mice (3), although it is not clear whether CD4⁺ memory T cells require the peptide-class II MHC complex.

Allergic pollinosis is caused by airborne pollen allergens, such as Japanese cedar or cypress pollen,

and the incidence of this disorder is increasing in Japan. It is possible to demonstrate that patients with allergic pollinosis show allergen-driven proliferation of CD4⁺ T cells all year round, indicating the presence of allergen-specific memory T cells in their peripheral blood for long periods. Pollen is a seasonal allergen, and the interval between its releases into the air is usually 8–10 months (4). Therefore, we may ask how memory T cells to pollen allergens are able to survive in the peripheral blood of allergic patients all year round. It is likely that cross-reactive antigens send survival signals to memory T cells, because those T cells are short-lived in the absence of survival signals.

We cloned Cha o 1, the major allergen of Japanese cypress, and homology analysis of the primary sequence suggests that this molecule is a pectate lyase (5). Similarly, many other plant-origin molecules are supposed to be pectate lyases from their primary sequences (6–15). It was proved that Cry j 1, the major allergen of Japanese cedar (16), has pectate lyase enzymatic activity (17). Pectate lyase is a pectin-

degrading enzyme, and it is thought that pollen degrades pectin in the cell wall during the formation of pollen tubes in pollination (6), or that fruit is ripened (13, 14) by this enzyme. Therefore, pectate lyase seems to be widely distributed in the environment. If there is a cross-reaction at the T-cell level, it is possible that pectate lyase from other plants might act as the source of survival signals for Cha o 1-specific memory T cells.

In the present study, we isolated a Cha o 1-specific Th2-cell clone from a patient with allergic pollinosis caused by Japanese cypress. Using the T-cell clone, we tested the ability of the pectate lyase of various plants to release survival signals of the T cells *in vitro*. Some peptides of plant pectate lyase provided essential signals for T-cell survival. Details of the survival of memory T cells and a possible explanation for the onset of allergic pollinosis are discussed.

Material and methods

Cha o 1-specific human T-cell clone

We used peripheral blood from a Japanese patient with Japanese cypress pollinosis diagnosed by the clinical symptoms, and also by the presence of Japanese cypress-specific IgE detected by CAP-RAST (Pharmacia, Uppsala, Sweden). A Cha o 1-specific T-cell clone was established from the peripheral blood by the method described by Sone et al. (18) with minor modifications. Briefly, peripheral blood mononuclear cells (PBMC) were cultured in RPMI 1640 (Sigma, St Louis, MO, USA) supplemented with 10% heat-inactivated human male AB sera (Nabi, Boca Raton, FL, USA), 100 µg/ml of streptomycin, 100 U/ml of penicillin, and 50 mM of L-glutamine (GIBCO, Grand Island, NY, USA) with 50 µg/ml semipurified Cha o 1 for 5 days. Semipurified Cha o 1 was prepared by the method described by Ide et al. (19). On day 5, human rIL-2 (Behringer Mannheim, Mannheim, Germany) was added to the medium at a concentration of 20 U/ml. On day 10, cloning was done by the micromanipulation method, in which a single cell is microscopically sucked from the cell suspension with a microcapillary tube (20). We are convinced that the cell populations tested in the present study were clonal because of the method employed here. Single-cell culture was done in 96-well U-bottom plates (Corning Inc., Corning, NY, USA) in the presence of 5×10^4 of mitomycin C (Kyowa Hakko, Tokyo, Japan)-treated autologous PBMC as antigen-presenting cells (APC), 20 U/ml of rIL-2, and 1 µg/ml of semipurified Cha o 1 in the RPMI 1640-conditioned medium. When we microscopically observed clonal cell expansion, the cells were transferred to 24-well plates. APC and 0.04% phytohemagglutinin (Sigma, St Louis, MO, USA) were added every 2 weeks. The antigen specificity of the T-cell clone was confirmed by its proliferative response to recombinant Cha o 1 (r-Cha o 1), which was a kind gift from Dr Kino and colleagues (Meiji Institute of Health Science, Japan). Fifty days later, cloned cells were cryopreserved at -80°C . T-cell surface markers were analyzed with anti-CD3, CD4, CD8, TCR α/β (Becton Dickinson, San Diego, CA, USA), and anti-TCR γ/δ (T cell Sciences, MA, USA) using FACScan (Becton Dickinson, San Jose, CA, USA). As a negative control, we established T-cell lines reactive to PPD (purified protein derivative of tuberculin) (Nihon BCG Seizo, Tokyo, Japan) from the same donor by the same method.

Synthetic oligopeptides

We prepared synthetic oligopeptides to determine an epitope

recognized by a Cha o 1-specific T-cell clone. On the basis of screening data of bindings between oligopeptides and purified HLA-class II molecules on filter papers, as described by Fu et al. (21), we synthesized 16-mer peptides from candidate regions of Cha o 1 by the F-moc method. Since Cha o 1 is supposed to be a pectate lyase, its identified epitope sequence was applied to a homology search in a protein database, FASTA (Version 3.0t84). Each synthesized peptide was applied to a reverse-phase HPLC with a protein C4 column, and the main peak was collected (data not shown). The purity of each peptide was 85% or higher. After lyophilization, each peptide was dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mg/ml, and stored at -80°C before use.

Proliferative responses of cloned T cells

The proliferative responses of cloned T cells were tested by uptake of ^3H -TdR, as described elsewhere (20). The antigen concentrations used for proliferative responses were 0.1 µM for cypress or cedar peptides, and 10 and 100 µM for other homolog peptides. All cultures were set up in triplicate. A stimulation index (SI) was used for data analysis. The SI was calculated as follows: $\text{SI} = \text{cpm from (T cells plus APC plus antigen)}/\text{cpm from (T cells plus APC)}$. We tentatively evaluated $\text{SI} > 5$ as positive proliferation.

Antigen-induced survival of CD4^+ T cells *in vitro*

To test the survival of memory T cells in conditions without proliferation *in vitro*, we incubated T cells with low doses of oligopeptides. For cross-reactive peptides, we tested peptide-driven T-cell proliferation at doses of 10 or 100 µM and its 1.8 diluent series. We then found the maximum doses that gave no detectable proliferation ($\text{SI} < 1$) for each peptide. In the evaluation of T-cell proliferative responses, we previously assumed $\text{SI} > 5$ as positive proliferation. However, in this assay, we assumed $\text{SI} < 1$ as no proliferation to eliminate the influence of even slight proliferation on the results. In cases of non-cross-reactive peptides, testing was done at 10 µM. A Cha o 1-specific T-cell clone (1×10^4 cells/well) was cultured in U-bottom 96-well microtiter plates for 6 days with or without the tested peptides at the concentrations described above with 5×10^4 cells/well of autologous APC. Six days after starting the T-cell culture, we measured cytokines in the supernatants with ELISA kits for IL-2, IL-4, and interferon (IFN)- γ (Cytoscreen, Biosource International Inc., Camarillo, CA, USA) in line with the manufacturer's instructions. Then, for each well, 5×10^4 cells/well of autologous APC and 1 µM of original epitope peptide were added. Three days later, the original epitope peptide-driven proliferation was assessed as described above. To assess survival periods, we cultured T cells with peptides for 9 or 14 days before testing the original epitope peptide-driven proliferation of T cells.

Furthermore, T-cell survival *in vitro* was also assessed from two aspects; that is, a viable cell number and surviving cell activity. In 24-well plates, a Cha o 1-specific T-cell clone was adjusted to 3×10^5 cells/well, and incubated for 6 days with 6×10^5 cells/well of autologous APC and survival-inducing peptides at the low concentrations described above. Surviving cells were counted by trypan blue staining. Next, T cells were washed with RPMI 1640, and adjusted to 1×10^4 cells/well. Finally, Cha o 1-driven proliferation was tested.

Bcl- x_L and Bcl-2 protein expression

The Bcl- x_L and Bcl-2 protein expressions after 24-h incubation with peptides were measured with a sandwich ELISA. In 24-well plates, a Cha o 1-specific T-cell clone (1×10^6 cells/well) was incubated for 24 h with autologous APC (5×10^6 cells/well) and peptides at the low concentrations described above. These cells were lysed with an antigen extraction agent provided in the Bcl-2 ELISA kit (Oncogene, Boston, MA, USA). Lysates were centrifuged and the supernatants were collected. The Bcl-2 in these supernatants was measured with this kit in

Table 1. Proliferative response^a and cytokine production^b of ST12

Peptide	Concentration (μM)	Proliferation (cpm)	IL-4 (pg/ml)	IFN-γ (pg/ml)
None		449 ± 261	1.0 ± 0.4	0.3 ± 0.3
Semipurified Cha o 1	0.5	3945 ± 518	5.7 ± 0.1	1.5 ± 0.2
r-Cha o 1	0.1	30 565 ± 676	159.5 ± 0.9	1.2 ± 0.4
Cha o 1:202–217	0.1	23 037 ± 204	107.3 ± 0.1	1.4 ± 0.2
Cry j 1:202–217	0.1	26 452 ± 299	124.5 ± 0.6	1.5 ± 0.5

^a Results were expressed as mean ± SE. Assays were done in triplicate.^b Results were expressed as mean ± SE. Assays were done in duplicate.

line with the manufacturer's instructions. Next, Bcl-x_L in the same supernatants was measured. An affinity-purified goat polyclonal anti-Bcl-x_L antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was coated onto a 96-well plate (Nunc, Roskilde, Denmark) with a carbonate buffer (pH 9.6) overnight at 4°C. After blocking with PBS containing 1% casein, the plate was incubated with sample and a rabbit polyclonal anti-Bcl-x_L antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. After washing four times with PBS containing 0.05% Tween 20, the plate was subjected to detection with a biotinylated antirabbit antibody, a streptavidin-peroxidase conjugate (ImmunoCuruz Staining System, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and a substrate solution containing *O*-phenylenediamine (ICN Biomedicals Inc., Aurora, OH, USA), as described elsewhere (5).

Statistical analysis

Statistical analysis was done with Student's *t*-test.

Results

Cha o 1-specific T-cell clone, ST12

A T-cell clone, designated as ST12, was CD3⁺, CD4⁺, CD8[−], and TCR αβ⁺ (data not shown), and was shown to be Cha o 1-specific. Epitope analysis was done for ST12 cells, and we observed fine specificity to

Cha o 1:202–217 by testing synthetic peptide-driven proliferation *in vitro*. ST12 produced IL-4, but no detectable IFN-γ, in response to Cha o 1 *in vitro*, indicating that ST12 was a Th2 clone (Table 1).

Homolog peptides of the epitope sequence

Homologous sequences of the epitope peptide for the ST12 clone were sought in the FASTA database. Most sequences of high homology were found in putative pectate lyases, while a few were found in asparagine synthetase (22–24). We synthesized homologous peptides, as shown in Table 2. Although peptides of short ragweed origin (25, 26) were not fit by the FASTA search, we synthesized them because some studies suggested a 40–65% homology with other plants' putative pectate lyases (5, 16, 26). We tested specific IgE levels in the T-cell donor to the source plants of synthesized peptides including banana, short ragweed, tomato, and strawberry by CAP-RAST. However, no detectable level of specific IgE was found for any tested materials except Japanese cypress (4.94 U/ml) and Japanese cedar (41.3 U/ml).

Cross-reactive peptides recognized by a Cha o 1-specific T-cell clone

We analyzed the proliferative response of ST12 cells to Cha o 1:202–217 peptide as well as other homologous peptides *in vitro*. Cry j 1:202–217 made ST12 proliferate as strongly as Cha o 1:202–217 (Table 1). Three homologous peptides, ZePel:252–267, Ban 17:249–254, and Amb a 1.1:246–261 induced positive proliferation (SI > 5) of ST12 cells at a dose of 10 μM, suggesting a relatively strong cross-reaction, and three additional peptides also induced a positive proliferation at the dose of 100 μM. The other peptides tested did not

Table 2. Homologous peptides of epitope sequence (Cha o 1:202–217)

Peptide	Sequence	Source	Reference ^a
Putative pectate lyase			
Cha o 1:202–217	GHSDIYSDDKSMKVTV	Pollen of Japanese cypress	5
Ara-A10:187–202	GHSDTYSRDKNMQVTI	Thale cress	12
Ara-F1N20:245–260	GHNDNFVKDKMKVTV	Thale cress	AL022140-19 ^a
ZePel:252–267	GHSDSYTDKKNMQVTI	Leaf of <i>Zinnia elegans</i>	15
1912:253–268	GHSDSFTQDKGMQVTV	Pistil of tomato	7
PO149:300–315	GASDTYQDDKIMQITV	Alfalfa	11
njjs25:299–314	GHSDSYTRDKQMQVTI	Fruit of strawberry	13
ban17:249–254	GHSDELTSDKSMQVTI	Fruit of banana	14
Amb a 1.1:246–261	GAGDENIEDRGMLATV	Pollen of short ragweed	25
Amb a 1.2:248–263	GADDTHYQDKGMLATV	Pollen of short ragweed	25
Amb a 1.3:247–262	GADDTHVQDKGMLATV	Pollen of short ragweed	25
Amb a 2:247–263	GASDTHFQDLKMHVTL	Pollen of short ragweed	26
Pectate lyase			
Cry j 1:202–217	GHDDAYSDDKSMKVTV	Pollen of Japanese cedar	16
Putative asparagine synthetase			
AS1:59–74	GDQPLFNEDKSIIVTV	Pea	22
Asp:59–74	GDQPLYNEDKSIVVTV	Spear of asparagus	23
Ory:59–74	GDQPLYNEDKSVVTV	Rice	24

^a Accession number for FASTA.

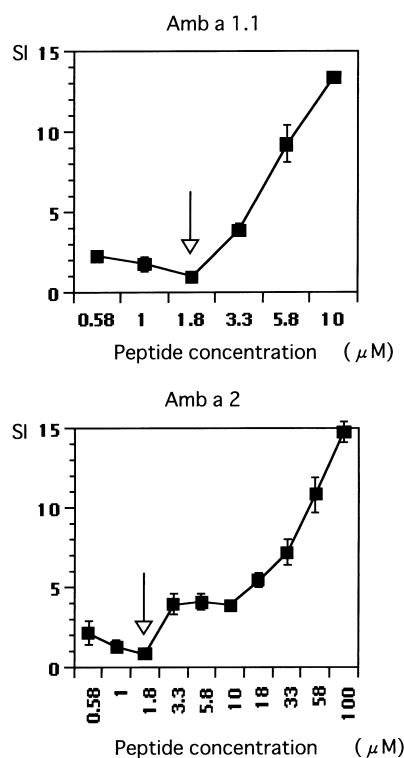


Figure 1. Proliferative response of ST12 to various concentrations of cross-reactive homolog peptides and doses used in survival assays. T-cell proliferation was assessed at dose of 10 or 100 μ M and its 1.8 diluent series for each peptide. Representative results are shown. Arrows in graphs indicate concentrations used in survival assay as maximum doses which gave no T-cell proliferation ($SI < 1$). These concentrations were determined for each cross-reactive homolog peptide (Table 4).

induce a positive response of ST12 cells at any doses tested here (Table 3). PPD-specific T cells did not show positive responses to these peptides at a dose of 10 μ M (data not shown).

Table 3. Cross-reaction of ST12 by homologous peptides

Peptide	SI at concentration of	
	10 μ M	100 μ M
Ara-A10:187-202	2.43	16.33
Ara-F1N20:245-260	4.54	14.24
ZePel:252-267	5.59	21.61
PO149:300-315	1.10	3.02
1912:253-268	0.94	1.28
njjs25:299-314	1.38	0.53
ban17:249-254	12.24	30.07
Amb a 1.1:246-261	13.33	36.06
Amb a 1.2:248-263	3.99	3.59
Amb a 1.3:247-262	3.38	1.46
Amb a 2:247-263	3.91	14.73
AS1:59-74	1.30	3.55
Asp:59-74	4.19	3.65
Ory:59-74	1.94	3.76

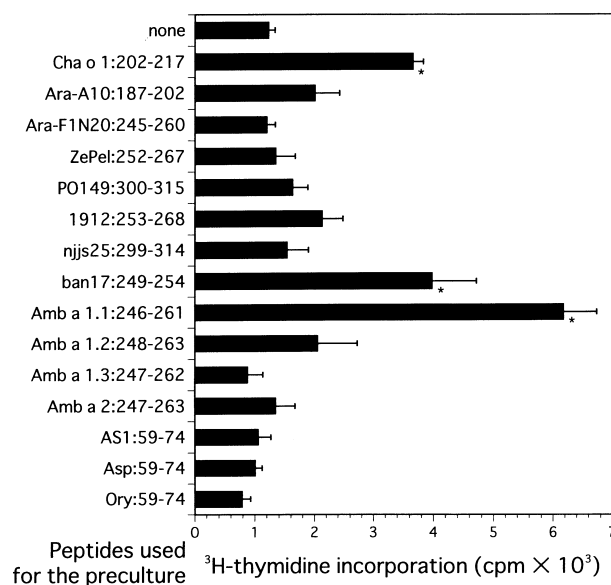


Figure 2. Survival of ST12 due to stimulation of homolog peptides. ST12 clone was cultured for 6 days with or without homolog peptides at maximum concentrations that gave no detectable proliferation (Table 4). Proliferative responses of surviving cells after restimulation with 1 μ M of Cha o 1:202-217 epitope peptide were then measured. Results are expressed as mean $\pm$ SE from triplicate experiments. Asterisks indicate significant difference between results with and without peptides ($P < 0.05$).

Survival assay on ST12 cells *in vitro*

First, positive or negative survival was assessed by proliferative responses after culture with low doses of peptides that deliver only survival signals, but no induction of proliferation. We found the maximum concentrations of cross-reactive peptides inducing no

Table 4. Proliferative response of ST12 at maximum concentrations of tested peptides that gave no detectable proliferation

Peptide	Concentration ^a (μ M)	Proliferation ^b (cpm)	SI
None		237 $\pm$ 50	
Cha o 1:202-217	3.3×10^{-3}	176 $\pm$ 70	0.74
Ara-A10:187-202	5.8	140 $\pm$ 25	0.59
Ara-F1N20:245-260	3.3	188 $\pm$ 27	0.79
ZePel:252-267	0.18	196 $\pm$ 26	0.83
PO149:300-315	10	261 $\pm$ 40	1.10
1912:253-268	10	222 $\pm$ 31	0.94
njjs25:299-314	10	326 $\pm$ 118	1.38
ban17:249-254	1.0	190 $\pm$ 29	0.80
Amb a 1.1:246-261	1.8	234 $\pm$ 24	0.99
Amb a 1.2:248-263	10	945 $\pm$ 370	3.99
Amb a 1.3:247-262	10	802 $\pm$ 182	3.38
Amb a 2:247-263	1.8	210 $\pm$ 35	0.89
AS1:59-74	10	307 $\pm$ 74	1.30
Asp:59-74	10	993 $\pm$ 259	4.19
Ory:59-74	10	460 $\pm$ 232	1.94

^a For non-cross-reactive peptides that did not show positive proliferation ($SI < 5$) even at 100 μ M (Table 3), 10 μ M was tested. For cross-reactive peptides, these concentrations were found as maximum doses which gave no detectable proliferation ($SI < 1$).

^b Results are expressed as mean $\pm$ SE. These assays were done in triplicate.

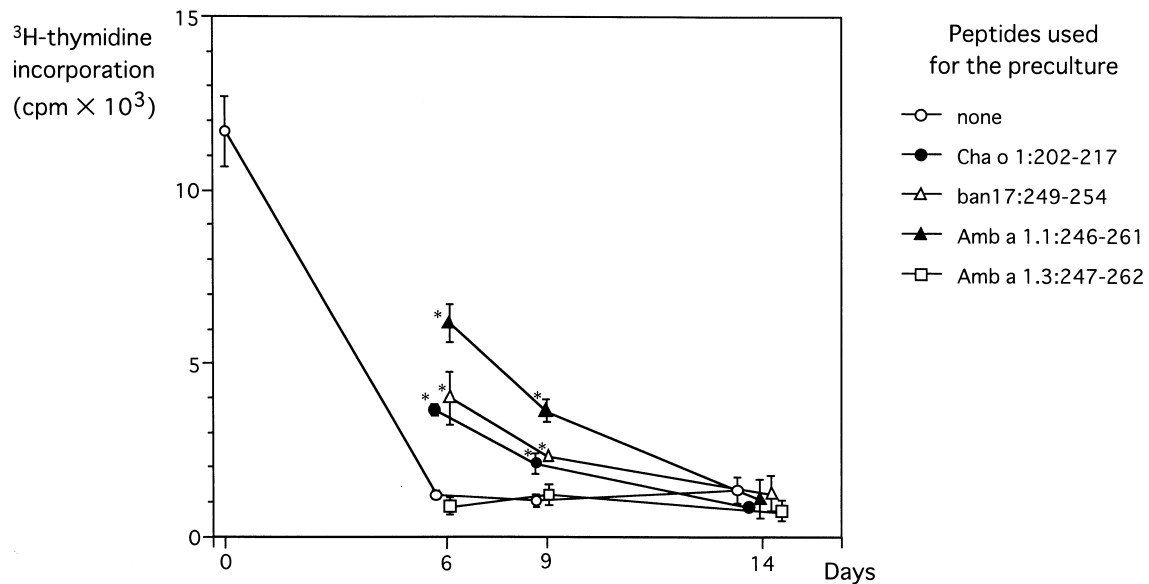


Figure 3. Time course of survival of ST12 after preculture with four different peptides. Graph shows results of survival assays after culturing with or without survival-inducing peptides for 6, 9, and 14 days. Results are expressed as mean $\pm$ SE from triplicate experiments. Asterisks indicate significant difference between results with and without peptides ($P < 0.05$).

detectable proliferation of ST12 cells ($SI < 1$), as shown by arrows in Fig. 1. After we found such maximum concentration for each peptide (Table 4), we cultured ST12 cells at such concentrations of peptides for 6 days. ST12 cells showed significant proliferation in response to 1 μ M of Cha o 1:202–217 when preincubated with Cha o 1:202–217, Ban 17:249–254, or Amb a 1.1:246–261 compared with the peptide-free culture (Fig. 2). Although ST12 cells survived for 9 days of preincubation, peptide-driven proliferation of ST12 cells was no longer observed after 14 days' preincuba-

tion (Fig. 3). During the incubation period, ST12 cells produced very faint or below detectable levels of IL-2, IL-4, and IFN- γ in their supernatants (Table 5).

When we assessed the surviving cell number and activity, respectively, significantly higher numbers and activities of viable cells were observed for preincubation with survival-inducing peptides compared with in peptide-free cultures or non-survival-inducing peptide cultures ($P < 0.05$) (Fig. 4). We confirmed that the remaining survival cells maintained the antigen-specific proliferative activity.

Table 5. Cytokine production^a in supernatants after 6-day preincubation with tested peptides

Peptide	Concentration (μ M)	IL-2 (pg/ml)	IL-4 (pg/ml)	IFN- γ (pg/ml)
None		0.0 $\pm$ 0.0	0.2 $\pm$ 0.2	1.4 $\pm$ 0.8
Cha o 1:202–217	3.3×10^{-3}	0.0 $\pm$ 0.0	0.9 $\pm$ 0.1	0.0 $\pm$ 0.0
Ara-A10:187–202	5.8	2.8 $\pm$ 0.6	0.2 $\pm$ 0.1	0.0 $\pm$ 0.0
Ara-F1N20:245–260	3.3	0.0 $\pm$ 0.0	0.1 $\pm$ 0.1	0.0 $\pm$ 0.0
ZePel:252–267	0.18	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
P0149:300–315	10	0.0 $\pm$ 0.0	0.3 $\pm$ 0.2	0.0 $\pm$ 0.0
1912:253–268	10	0.0 $\pm$ 0.0	0.9 $\pm$ 0.2	0.0 $\pm$ 0.0
njjs25:299–314	10	0.0 $\pm$ 0.0	0.3 $\pm$ 0.3	0.0 $\pm$ 0.0
ban17:249–254	1.0	0.0 $\pm$ 0.0	0.3 $\pm$ 0.3	0.0 $\pm$ 0.0
Amb a 1.1:246–261	1.8	0.0 $\pm$ 0.0	2.2 $\pm$ 0.2	0.0 $\pm$ 0.0
Amb a 1.2:248–263	10	0.0 $\pm$ 0.0	0.3 $\pm$ 0.1	0.0 $\pm$ 0.0
Amb a 1.3:247–262	10	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Amb a 2:247–263	1.8	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
AS1:59–74	10	0.0 $\pm$ 0.0	1.2 $\pm$ 0.1	0.0 $\pm$ 0.0
Asp:59–74	10	0.0 $\pm$ 0.0	1.0 $\pm$ 0.3	0.0 $\pm$ 0.0
Ory:59–74	10	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0

^a Results are expressed as mean $\pm$ SE. Assays were done in duplicate.

Bcl-x_L and Bcl-2 protein expression

The expression of Bcl-x_L was significantly higher in the culture with a relatively high dose of original epitope peptide (10 μ M of Cha o 1:202–217) compared with non-survival-inducing peptides (10 μ M of Amb a

Table 6. Bcl-x_L and Bcl-2 expression after 24-h incubation with peptides

Peptide	Concentration (μ M)	Absorbance at 450 nm ^a	
		Bcl-x _L	Bcl-2
No peptide		0.59 $\pm$ 0.00	0.13 $\pm$ 0.00
Cha o 1:202–217 ^b	10	0.97 $\pm$ 0.04*	0.13 $\pm$ 0.00
Cha o 1:202–217 ^c	3.3×10^{-3}	0.68 $\pm$ 0.01	0.12 $\pm$ 0.01
ban17:249–254 ^c	1.0	0.56 $\pm$ 0.03	0.11 $\pm$ 0.01
Amb a 1.1:246–261 ^c	1.8	0.63 $\pm$ 0.01	0.13 $\pm$ 0.00
Amb a 1.3:247–262 ^d	10	0.64 $\pm$ 0.02	0.12 $\pm$ 0.01
Blank		0.28 $\pm$ 0.04	0.08 $\pm$ 0.00

^a Results are expressed as mean $\pm$ SE. Assays were done in duplicate.

^b High dose of original epitope peptide.

^c Survival-inducing peptides.

^d Non-survival-inducing peptide.

Asterisk indicates significant difference from non-survival-inducing peptide (10 μ M of Amb a 1.3:247–262).

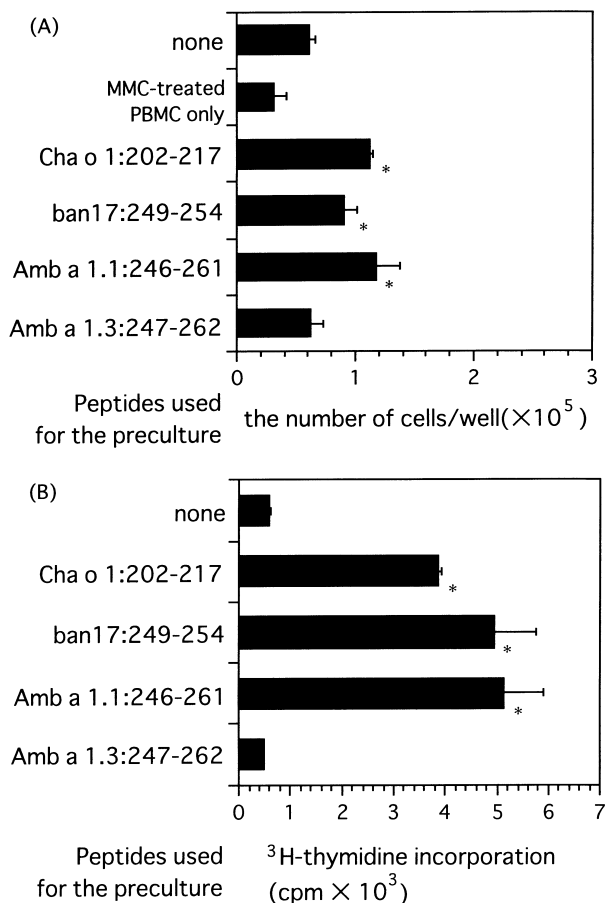


Figure 4. Number and proliferative activity of surviving ST12 cells after preculture with four different peptides. ST12 (3×10^5 cells/well) and MMC-treated PBMC (6×10^5 cells/well) were cultured for 6 days with or without homolog peptides at maximum concentrations that gave no T-cell proliferation (Table 4). (A) Number of surviving cells was counted with trypan blue on day 6. (B) After 6-day culture, cells were washed with fresh medium. Cells were then adjusted to 1×10^4 cells/well in all cell preparations. Proliferative responses of these cells after restimulation with $1 \mu\text{M}$ of Cha o 1:202-217 epitope peptide were measured. Results are expressed as mean $\pm$ SE from triplicate experiments. Asterisks indicate significant difference between results with and without peptides ($P < 0.05$).

1.3:247-262) ($P < 0.05$) (Table 6). However, at the low doses used in the survival assays, there was no significant difference between survival-inducing and non-survival-inducing peptides. The expression of Bcl-2 was not significantly different in any assays, including incubation with $10 \mu\text{M}$ of Cha o 1:202-217.

Discussion

In the present study, our observations strongly suggested that CD4^+ memory T cells could survive by continuous stimulation of cross-reactive epitope peptides, even in the absence of the original priming antigen. We used cloned T cells for our analysis of T-cell survival *in vitro*. Although memory cells have not

been fully characterized, it is likely that cloned T cells could be used for analysis of memory cell survival mechanisms because memory T cells share some characteristics with cloned T cells (27). It is thought that memory T cells are sensitized during the primary immune response, that cytokines secreted by memory T cells are important for induction of the secondary response, and that memory T cells require intermittent antigen stimulation for long-term survival.

Our data demonstrated that Cha o 1-specific memory T cells could be maintained even without obvious proliferation in response not only to Cha o 1, but also to cross-reactive pectate lyase peptide preparation from other plants. It is interesting to note that not all cross-reactive peptides induce positive survival. However, none of the peptides without cross-reactive activity provided survival signals to the cloned T cells. This suggests that survival signals arrive through TCR in the case of CD4^+ memory cells, although no direct evidence is available. Recent reports suggest that T-cell survival requires continuous expression of Bcl-2 family proteins, such as Bcl-2 and Bcl-x_L, to avoid apoptosis (1). It has been demonstrated that CD4^+ T cells incubated with anti-CD3 (TCR) plus anti-CD28 monoclonal antibodies expressed Bcl-x_L, and that Bcl-2 was upregulated by incubation with IL-2 (27). Using unique homolog peptides with lymphokine (IL-4) production-inducing activity, but not proliferation-inducing activity, Matsushita et al. (28) showed homolog peptide-induced CD4^+ T-cell clone survival by the same method as our study. In their study, using Western immunoblotting with chemiluminescence detection, they demonstrated that these unique peptides induced the upregulation of Bcl-x_L after 24-h incubation. In the present study, however, neither Bcl-x_L nor Bcl-2 expression was significantly upregulated even by survival-inducing peptides. It is possible that our assay was not sensitive enough to detect the Bcl-x_L expressions induced by the suboptimal doses of peptides. Alternatively, the survival mechanism might not be Bcl-2 family-mediated in our case, because we used the cross-reactive peptides at suboptimal doses and a different T-cell clone in comparison with the study by Matsushita et al. More detailed testing is still needed to reach a conclusion.

Another possibility was that cytokines produced by ST12 cells might have induced positive survival via a self-augmenting mechanism, because it has been reported that IL-2 can induce Bcl-2 independently (27), and that IL-2 and IL-4 can proliferate memory T cells even in the absence of antigens (29). However, we observed only very faint or no cytokine production in the culture medium. In addition, we also observed faint cytokine production by some peptides without survival-inducing activity. Therefore, cytokine-mediated cell survival was probably not the dominant mechanism in our case.

We have to consider whether cross-reacting epitope peptides are functioning in the survival of memory T cells required for the onset of Japanese cypress pollinosis in man. Our *in vitro* study showed that T cells survived for only up to 9 days, and this was far shorter than the survival period for pollen-specific T cells kept for a whole year *in vivo*. Reports from other laboratories also showed that T cells survived for almost 9 days (28), and our results are not inconsistent with other reports. This might be explained by the presence of other unknown cross-stimulating antigens in the environment, because we tested a relatively small number of peptides for survival induction. In the present testing *in vitro*, it was necessary to stimulate T cells with peptides at a dose of about 1–1.8 μ M, and this was rather high compared with common environmental antigens. Some of the peptides tested here were not from pollen, but from food material such as fruits or leaves. Therefore, they might be taken at much

higher concentrations into the human body than airborne peptides such as pollen. However, at present, not enough information is available to discuss *in vivo* conditions or natural environmental situations.

In conclusion, our data suggest that Cha o 1-specific memory T cells survive via the stimulation of cross-reactive pectate lyase antigens present in other plant materials, and this might explain the maintenance mechanism of pollen-allergen-specific memory T cells in man. More detailed analyses could help establish new prophylactic strategies against allergic pollinosis caused by seasonal allergens.

Acknowledgments

We thank Dr K. Kino, Meiji Institute of Health Science, for his kind gift of recombinant Cha o 1. We also thank Dr Y. Yabu, Dr J. Fu, and Ms M. Hato for their technical assistance.

References

1. JONATHAN S. Immunological memory. *Curr Opin Immunol* 1997;9:371–379.
2. GRAY D, MATZINGER P. T-cell memory is short-lived in the absence of antigen. *J Exp Med* 1991;174:969–974.
3. MARKIEWICZ MA, GIRAO C, OFFERMAN JT, et al. Long-term T cell memory requires the surface expression of self-peptide/major histocompatibility complex molecules. *Proc Natl Acad Sci U S A* 1998;95:3065–3070.
4. ITO H, NISHIMURA J, SUZUKI M, et al. Specific IgE to Japanese cypress (*Chamaecyparis obtusa*) in patients with nasal allergy. *Ann Allergy Asthma Immunol* 1995;74:299–303.
5. SUZUKI M, KOMIYAMA N, ITOH M, et al. Purification, characterization and molecular cloning of Cha o 1, a major allergen of *Chamaecyparis obtusa* (Japanese cypress) pollen. *Mol Immunol* 1996;33:451–460.
6. WING RA, YAMAGUCHI J, LARABELL SK, URSIN VM, MCCORMICK S. Molecular and genetic characterization of two pollen-expressed genes that have sequence similarity to pectate lyases of the plant pathogen *Erwinia*. *Plant Mol Biol* 1989;14:17–28.
7. BUDELIER KA, SMITH AG, GASSER CS. Regulation of a stylar-transmitting tissue-specific gene in wild-type and transgenic tomato and tobacco. *Mol Gen Genet* 1990;224:183–192.
8. ROGERS HJ, HARVEY A, LONSDALE DM. Isolation and characterization of a tobacco gene with homology to pectate lyase which is specifically expressed during microsporogenesis. *Plant Mol Biol* 1992;20:493–502.
9. TURCICH MP, HAMILTON DA, MASCARENHAS JP. Isolation and characterization of pollen-specific maize genes with sequence homology to ragweed allergens and pectate lyases. *Plant Mol Biol* 1993;23:1061–1065.
10. KIM SR, FINKEL D, CHUNG YY, AN G. Abundance patterns of lily pollen cDNA: characterization of three pollen-preferential cDNA clones. *Sex Plant Reprod* 1994;7:76–86.
11. WU Y, QIU X, DU S, ERICKSON L. PO149, a new member of pollen pectate lyase-like gene family from alfalfa. *Plant Mol Biol* 1996;32:1037–1042.
12. KULIKAUSKAS R, MCCORMICK S. Identification of the tobacco and *Arabidopsis* homologues of the pollen-expressed LAT59 gene of tomato. *Plant Mol Biol* 1997;34:809–814.
13. MEDINA-ESCOBAR N, CÁRDENAS J, MOYANO E, CABALLERO JL, MUÑOZ-BLANCO J. Cloning, molecular characterization and expression pattern of a strawberry ripening-specific cDNA with sequence homology to pectate lyase from higher plants. *Plant Mol Biol* 1997;34:867–877.
14. DOMÍNGUEZ-PUIGJANER E, LLOP I, VENDRELL M, PRAT S. A cDNA clone highly expressed in ripe banana fruit shows homology to pectate lyases. *Plant Physiol* 1997;144:1071–1076.
15. DOMINGO C, ROBERTS K, STACEY NJ, CONNERTON I, RUIZ-TERAN F, MCCANN MC. A pectate lyase from *Zinnia elegans* is auxin inducible. *Plant J* 1998;13:17–28.
16. SONE T, KOMIYAMA N, SHIMIZU K, KUSAKABE T, MORIKUBO K, KINO K. Cloning and sequencing of cDNA coding for Cry j 1, a major allergen of Japanese cedar pollen. *Biochem Biophys Res Commun* 1994;199:619–625.
17. TANIGUCHI Y, ONO A, SAWATANI M, et al. Cry j 1, a major allergen of Japanese cedar pollen, has pectate lyase activity. *Allergy* 1995;50:90–93.
18. SONE T, MORIKUBO K, MIYAHARA M, et al. T-cell epitopes in Japanese cedar (*Cryptomeria japonica*) pollen allergens: choice of major T-cell epitopes in Cry j 1 and Cry j 2 toward design of the peptide-based immunotherapeutics for the management of Japanese cedar pollinosis. *J Immunol* 1998;161:448–457.
19. IDE T, YAMAMOTO K, TABATA S, ASHIDA T. N-terminal amino acid sequence of a major allergen, Cha o 1, of Japanese cypress pollen [in Japanese]. *Jpn J Palynol* 1995;41:69–72.
20. OHTA N, REINSMOEN NL, BACH FH. Cellular basis of anti-SE response. *Hum Immunol* 1984;11:127–141.
21. FU J, HATO M, IGARASHI K, et al. A simple screening method for detecting bindings between oligopeptides and HLA-DR molecules on filter papers: possible application for mapping of putative helper T-cell epitopes on MSP1 of *Plasmodium falciparum*. *Microbiol Immunol* 2000;44:249–257.
22. TSAI FY, CORUZZI GM. Dark induced and organ-specific expression of two asparagine synthetase genes in *Pisum sativum*. *EMBO J* 1990;9:323–332.

23. DAVIES KM, KING GA. Isolation and characterization of a cDNA clone for a harvest-induced asparagine synthetase from *Asparagus officinalis* L. *Plant Physiol* 1993;**102**:1337–1340.
24. WATANABE J, HIGUCHI T, SAKAI T, YAMAYA T. Nucleotide sequence of cDNA encoding asparagine synthetase from rice callus. *Plant Physiol* 1996;**111**:347.
25. RAFNAR T, GRIFFITH IJ, KUO M, BOND JF, TOGERS BL, KLAPPER DG. Cloning of Amb a I (antigen E), the major allergen family of short ragweed pollen. *J Biol Chem* 1991;**266**:1229–1236.
26. GRIFFITH IJ, POLLOCK J, KLAPPER DG, POGERS BL, NAULT AK. Sequence polymorphism of Amb a I and Amb II, the major allergens in *Ambrosia artemisiifolia* (short ragweed). *Int Arch Allergy Immunol* 1991;**96**:296–304.
27. MUELLER DL, SEIFFERT S, FANG W, BEHRENS TW. Differential regulation of bcl-2 and bcl-x by CD3, CD28, and the IL-2 receptor in cloned CD4⁺ helper T cells. *J Immunol* 1996;**156**:1764–1771.
28. MATSUSHITA S, KOHSAKA H, NISHIMURA Y. Evidence for self and nonself peptide partial agonists that prolong clonal survival of mature T cells *in vitro*. *J Immunol* 1997;**158**:5685–5691.
29. LONDON AL, PEREZ VL, ABBAS AK. Functional characteristics and survival requirements of memory CD4⁺ T lymphocytes *in vivo*. *J Immunol* 1999;**162**:766–773.