

Analysis of antigenic determinants shared by two different allergens recognized by human T cells: house dust mite (*Dermatophagoides pteronyssinus*) and chironomid midge (*Chironomus yoshimatsui*)

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T. Nagano¹, N. Ohta², M. Okano¹, T. Ono², Y. Masuda¹

Departments of ¹ Otorhinolaryngology and ² Parasitology, Okayama University Medical School, Japan

To analyse the cross-reactivity of T-cell-mediated immunity between *Dermatophagoides pteronyssinus* (Dp) and *Chironomus yoshimatsui* (Cy), the most common allergens in Japan, we established antigen-specific human T-cell lines and clones. Some but not all of the Cy-induced T-cell lines showed a significant proliferative response not only to Cy, but also to Dp. No T-cell line responded to other unrelated antigens. When we stimulated the Dp-induced T-cell clones with Cy, 3 of the 40 clones (7.5%) showed a significant proliferation, and 2 of the 3 clones produced interleukin-4 and interferon- γ , indicating their helper function.

Cross-reactivity was diminished significantly after the absorption of Dp antigen in an anti-Cy affinity column. The cross-reactive epitopes were thought to be expressed on the Dp molecule of 45–53 kD. The presence of helper T cells reactive to both allergens suggests a possibility that this cross-reactivity might be involved in part in the high incidence of allergy to the 2 major allergens.

Key words: allergens, Japan; house dust mite; chironomid midge.

Toshiaki Nagano, MD
Department of Otorhinolaryngology
Okayama University Medical School
2-5-1, Shikatacho, Okayama-shi
Okayama 700
Japan

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The most important environmental allergens for inducing allergic diseases in Japan are the house dust mite and the chironomid midge (3, 5, 8). The high correlation of positive skin tests to the chironomid midge and house dust mite suggests a possible cross-reactivity of the antigens. However, serological studies have failed to document such cross-reactivity (6, 26). Hypersensitivity to these allergens can occur independently.

T cells are involved in allergic reactions with the helper or suppressor function on allergen-specific IgE production (4, 17). In specific immune responses, it has been generally accepted that T and B cells usually recognize the same antigenic molecules, but the recognition of epitopes differs (14, 21). During the regulatory T-cell response to allergens, the epitopes recognized by these cells are not necessarily identical to those of IgE-producing cells. Therefore,

it is possible that the production of antibodies to a given epitope is regulated by T cells that recognize a different epitope(s) (1). If the mites and midges share T-cell epitopes, even though serological cross-reactivity was not detectable, a helper T-cell clone with a single epitope specificity could respond to a cross-reactive antigenic determinant shared by Dp and Cy, resulting in the production of regulatory cytokines. The recent development of cloning methodology enabled us to isolate allergen-specific human T-cell clones (2, 16). By using T-cell clones, we investigated the possibility of cross-reactive epitopes of the 2 allergens being recognized by human T cells.

In this study, the specificity of the T-cell response to the mite and midge was analysed by *in vitro* testing of the proliferative response and the production of cytokines by atopic Japanese. We observed the presence of cross-reactive T cells in recognizing both

the mites and midges. The clinical and immunobiological roles of the T cells in the pathogenesis of allergic disease is discussed.

Material and methods

Four allergic Japanese were tested. Two had allergic rhinitis and 2 had bronchial asthma. All 4 had positive skin test to the house dust mite and their PRU (Pharmacia RAST Unit)/ml exceeded 3.5. None had been treated with immunosuppressive agents.

Mite antigen was obtained from the bodies of adult *Dermatophagoides pteronyssinus* (Dp) by the method of Sasa with slight modification (15). Two antigens from each of 2 chironomid midges, *Tokunagayusurika akamusi* (Ta) and *Chironomus yoshimatsui* (Cy), were prepared by the method described previously (12). As controls, we used purified protein derivative (PPD) (Nihon BCG Seizo Co., Tokyo, Japan) and the *Schistosoma japonicum* adult worm antigen (SjA) (19). A single lot of each antigen was used in these experiments. Protein content of the antigen was measured by the method of Lowry (10). All antigens were stored at -20°C until use.

We fractionated the crude Dp antigen by using a Sephacryl S-300 gel column (7). A total of 14 molecular weight fractions were tested: 2000 kD, 470 kD, 320 kD, 190 kD, 145 kD, 95 kD, 73 kD, 53 kD, 45 kD, 32 kD, 25 kD, 20 kD, 15 kD and 7.5 kD.

Peripheral blood mononuclear cells (PBMN) were separated from the heparinized peripheral blood of the patients by the Ficoll-Conray (Pharmacia, Uppsala, Sweden, and Daiichi Seiyaku Co, Tokyo, Japan) density gradient centrifugation method (specific gravity of 1.077) (23).

Antigen-specific T-cell lines and clones

Blastoid cell lines were generated from PBMN by the *in vitro* stimulation of mite and chironomid antigens. PBMN were stimulated by each antigen at a concentration of 10 $\mu\text{g}/\text{ml}$ for Dp and Ta, and 7.5 $\mu\text{g}/\text{ml}$ for Cy. The culture medium was RPMI-1640 (Gibco, Grand Island, USA) supplemented with 15% heat-inactivated pooled human sera (HS), 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 20 mmol l-glutamine (Gibco). After 7 days' incubation, the blastoid cells were further incubated in the presence of 5 $\mu\text{g}/\text{ml}$ of each antigen, 20 U/ml of recombinant human IL-2 (Behringer, Mannheim, Germany), and irradiated autologous PBMN (3000 rad) as antigen-presenting cells (APC). The medium was replaced every second day, and APC were added every 10 days. Surface phenotypes were determined

by a flow cytometer (EPICS, Hialeah, USA) using anti-CD3, CD4 and CD8 monoclonal antibodies (Becton Dickinson, Mountain View, USA). One of the blastoid cell lines was used for cloning by the micromanipulation method (18), and we obtained 40 clones from a bulk T-cell line. In brief, a single cell was picked up under the microscopic observation for incubation in a medium containing IL-2 and APC in 96-well, round-bottomed microtiter plates (Corning, USA). Cells of sufficient clonal expansion were then transferred to 24-well plates.

Antigen-induced proliferation of PBMN and T-cell lines

The specificity of cellular responsiveness was tested by antigen-driven, exogenous IL-2-independent proliferation *in vitro*. An amount of 100 000 of PBMN or 1×10^4 blastoid T cells suspended in 100 μl of RPMI-1640 with 15% HS was incubated with or without antigens in 96-well, flat-bottomed microtiter plates (Corning). The optimal antigen concentration had been determined in our previous study: 5 $\mu\text{g}/\text{ml}$ for Dp crude, 7.5 $\mu\text{g}/\text{ml}$ for Cy, 10 $\mu\text{g}/\text{ml}$ for Ta and SjA, and 2 $\mu\text{g}/\text{ml}$ for PPD. To test the Dp fraction-driven responsiveness, the concentration of all fractions was fixed at 5 $\mu\text{g}/\text{ml}$. PBMN were cultured for 7 days, and the T-cell lines for 3 days. T-cell lines, from which exogenous IL-2 was washed away by preincubation in an IL-2-free medium, were cultured with 5×10^4 APC. One μCi of ^3H -TdR was added 18 h before cell harvest. Uptake of ^3H -TdR was measured by liquid scintillation spectrometry (Aloka Co, Tokyo, Japan). The mean count per minute (cpm) of triplicate cultures or the stimulation index (SI) was used for data analysis.

Antigen-driven interleukin-4 and gamma interferon production by T-cell clones

In a 24-well plate, 1×10^6 T cells were incubated with 2×10^6 irradiated PBMN (3000 rad) for 36 h in the presence or absence of antigenic stimulation. After 36 h of incubation, the supernatant was obtained and tested for cytokine production. The production of interleukin-4 (IL-4) was determined by an EIA kit (R & D Corp., Minneapolis, USA). Gamma interferon (IFN- γ) was measured by an RIA kit (Centcor, Inc., Malvern, USA).

Preparation of rabbit anti-Cy IgG

We prepared rabbit anti-Cy antiserum as described elsewhere (12). Purification of anti-Cy IgG was done with a protein A affinity column (Kurabo Co, Osaka, Japan). Anti-Cy activity of the purified IgG was confirmed in Ouchterlony assay.

Absorption of Dp antigen with anti-Cy IgG affinity chromatography

We prepared an anti-Cy IgG affinity column, using Sepharose CL4B (Pharmacia, Uppsala, Sweden) according to the manufacturers written directions. Under the observation of a UV monitor, 2.5 ml of Dp crude antigen (1 mg/ml) was applied. The non-adherent fraction was eluted by PBS (pH 7.3), and Dp fraction or fractions, which were cross-reactive to Cy, were eluted by 6 mol guanidine HCl (pH 3.1). The PBS eluate was concentrated by using ultrafiltration (mol. wt. cutoff 10000), and was used for T-cell stimulation.

Statistical analysis

Data were analysed by t-test (24); $p < 0.05$ was considered statistically significant.

Results

PBMN proliferative response to chironomid and house dust mite antigens in vitro

PBMN obtained from allergic patients showed a strong proliferative response to Cy and/or Dp *in vitro*. However, the patterns differed: 3 responded to both Cy and Dp, whereas one responded only to Cy. Representative results are shown in Fig. 1.

T-cell lines induced by chironomid midge or house dust mite antigen

We established IL-2-dependent blastoid T-cell lines (CD3+, CD4+, CD8-) by the stimulation of Dp or Cy (data not shown). When we tested 2 Cy-induced T-cell lines, we observed vigorous proliferation in response to Cy and another chironomid antigen, Ta, in the absence of exogenous IL-2. One

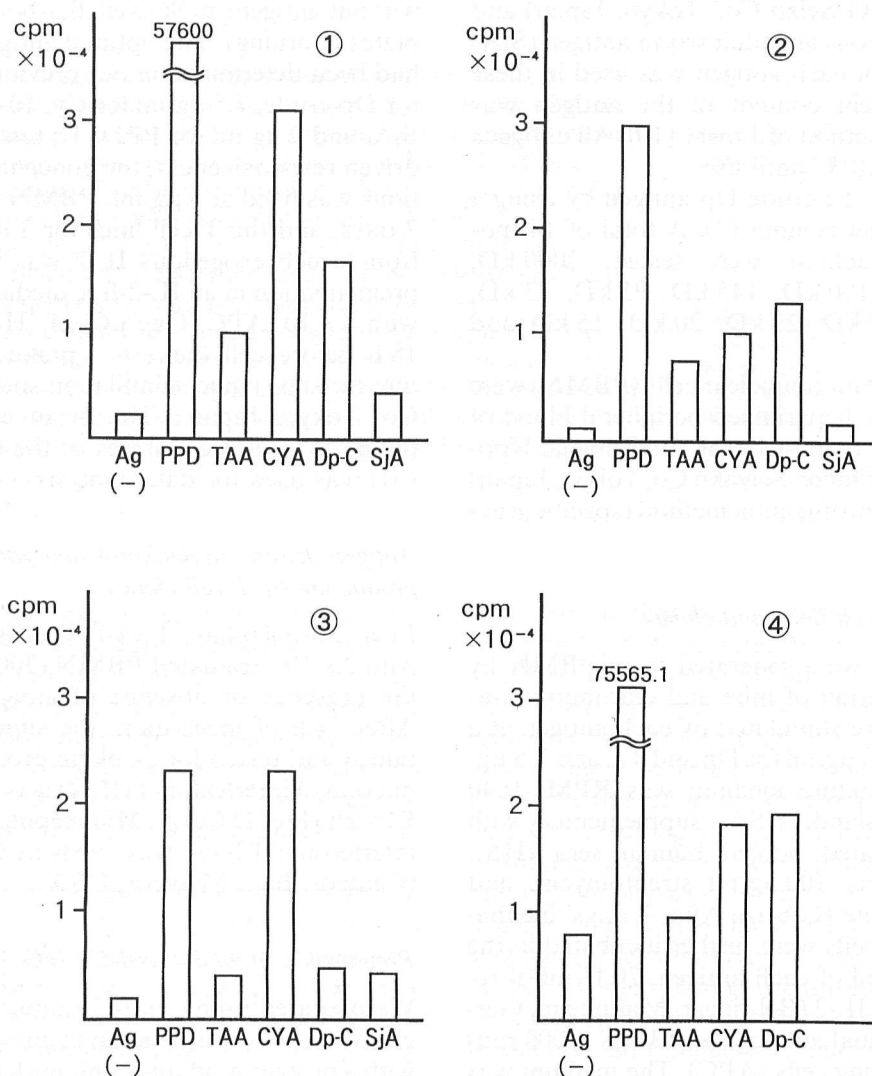


Fig. 1. Proliferation of PBMN from 4 patients to chironomid and house dust mite antigens *in vitro*. Subjects 1 and 4 have allergic rhinitis and subjects 2 and 3 have bronchial asthma.

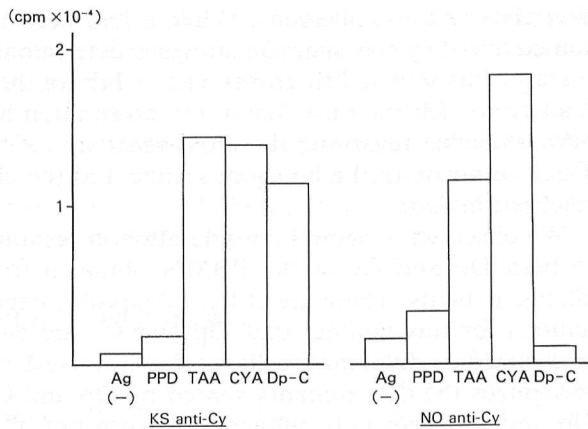


Fig. 2. Proliferative response of Cy-reactive T-cell lines stimulated with chironomid midge antigen and Dp-crude antigen.

of the 2 T-cell lines, KS T cells, showed a significant proliferative response not only to Cy, but also to Dp, whereas the other one (NO) did not respond to Dp (Fig. 2). A T-cell line induced by the stimulation of Dp crude antigen was also tested for its specificity. Although house dust mite antigen was used to establish the T-cell line, the T cells showed a proliferative response to both Cy and Dp. Those T-cell lines tested failed to respond to PPD or SjA (data not shown).

Antigen-induced proliferation of T-cell clones

We obtained 40 Dp-reactive clones (cloning efficiency 0.30), which were then stimulated with Dp-crude, PPD, SjA, Ta and Cy, and almost all clones showed a significant proliferative response only to Dp. The polyclonal parent T-cell line showed a proliferative response to almost all Dp fractions; however, the cloned T cells elicited responses to distinct

Dp fractions. The clones were subdivided into at least 5 groups based on their response to Dp fractions: type A exhibited a detectable response only to Dp-crude; type B to 45 kD and 53 kD; type C to 73 kD and 95 kD; type D to fractions of > 190 kD; and type E clones to low mol. wt. fractions of 20 kD and 25 kD, although this type also responded to high mol. wt. fractions.

T-cell clones cross-reactive to Cy and Dp

Three of the 40 clones tested showed a proliferative response not only to Dp, but also to chironomid antigens (Table 1). All 3 of these cross-reactive clones were categorized as type B.

Absorption of cross-reactive Dp antigen by an anti-Cy affinity column

The anti-Cy reactivity of the purified IgG was confirmed by Ouchterlony assay (data not shown). This IgG was used for preparing an affinity column. When the Dp-crude antigen was applied to the affinity column, we obtained an adherent and nonadherent Dp fraction. This suggests that some components of Dp-crude antigen were absorbed by the anti-Cy IgG affinity column, although the quantity was very low (data not shown). The protein concentration of the non-adherent fraction (Dp-absorbed) was adjusted, and we compared the proliferative response of the Dp-induced T-cell clones to the Dp-crude and Dp-absorbed fractions. By the stimulation of 5 µg/ml of the Dp-absorbed, the cross-reactive T-cell clones showed a significantly diminished response, as compared with that to Dp-crude, whereas T-cell clones without cross-reactivity exhibited a proliferative response to the Dp-absorbed fractions (Fig. 3).

Table 1. Proliferative response of T-cell clones to the antigens

Clone		Antigen (-)	PPD	SjA	Dp-C	7.5 kD	15 kD	20 kD	25 kD	32 kD	45 kD
NP 46	Mean cpm (× 10 ⁻³)	0.5	0.5	2.3	19.3	3.4	1.9	2.4	1.9	4.4	14.0
	S.D.	0.1	0.4	0.5	2.5	1.6	0.6	0.8	0.8	0.3	2.2
NP106	Mean cpm	3.2	6.2	2.4	22.0	2.9	2.2	4.4	3.1	4.1	10.9
	S.D.	2.9	2.7	0.8	2.5	1.5	1.2	0.8	2.3	0.7	2.6
NP133	Mean cpm	1.6	3.2	0.7	38.1	0.7	0.7	1.9	3.2	2.9	33.0
	S.D.	0.9	2.2	0.5	14.6	0.2	0.3	0.9	1.6	0.7	4.7
Clone		53 kD	73 kD	95 kD	145 kD	190 kD	320 kD	470 kD	2000 kD	Cy	
NP 46	Mean cpm	14.3	11.1	9.6	2.9	2.6	3.1	2.1	1.2	50.0	
	S.D.	0.5	3.1	1.1	0.2	0.6	0.9	1.2	0.5	1.4	
NP106	Mean cpm	13.3	9.3	7.7	4.3	1.6	1.1	2.0	1.6	15.0	
	S.D.	2.4	0.6	2.7	2.3	0.3	0.3	0.5	1.4	5.1	
NP133	Mean cpm	51.4	49.8	26.4	9.3	5.0	3.3	3.5	2.6	14.5	
	S.D.	4.7	2.0	0.2	3.7	0.8	1.6	0.7	2.0	19.7	

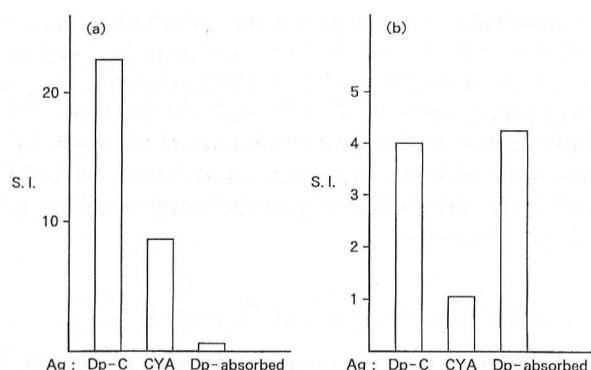


Fig. 3. Proliferative response of Dp-induced T-cell clones stimulated with the study antigens. Comparison of responsiveness to the Dp-absorbed antigen between cross-reactive (a) and non-cross-reactive (b) T-cell clones.

Cytokine production of antigen-stimulated T-cell clones

To analyse the function of the 3 anti-Dp T-cell clones showing cross-reactivity, we tested the production of IL-4 and IFN- γ . Stimulation with the Dp-crude antigen induced IL-4 production in 2 of 3 clones. Further, 2 clones also produced IL-4 and IFN- γ following stimulation with Cy (Table 2). No detectable cytokine activity was observed in the culture supernatants of APC stimulated by the antigens.

Discussion

Allergic disease is characterized by the production of allergen-specific IgE antibodies. Since the specific IgE response results from the interaction of specific T and B cells, information on the specificity of the T-cell response to allergens is essential, and could have some effect on the specificity of the IgE produced.

House dust mites and chironomid midges are the most important environmental allergens in Japan. A high correlation has been observed between positive

skin tests to these allergens. While it had been assumed that they may share an antigenic determinant, research has shown little cross-reactive IgE for these 2 allergens. On the other hand, no information has been available regarding the cross-reactivity of the T-cell response to the house dust mite and the chironomid midge.

We observed a vigorous proliferation in response to both Dp and Cy of the PBMN obtained from allergic patients. There are at least 2 possible explanations for this finding: that Dp and Cy are each recognized by different T cells, or that a single T cell recognizes the determinants shared by Dp and Cy. Dp and Cy were not mitogenic because not all T cells tested showed detectable response to those antigens. The presence of T-cell lines which showed a proliferative response to both Dp and Cy suggests that Dp and Cy share one or more T-cell-recognizing determinants. This possibility was tested by using cloned T cells. Only 3 of 40 clones tested (7.5%) showed a proliferative response to both Dp and Cy. This strongly suggests the presence of a cross-reactive T-cell determinant between Dp and Cy because cloned T cells express T-cell receptors of single specificity. All 3 cross-reactive clones recognized Dp molecules of 45–53 kD. This observation indicates that the Dp molecules of 45–53 kD express cross-reactive T-cell epitopes.

When we absorbed Dp-crude antigen onto an anti-Cy affinity column, the Dp-induced, cross-reactive T-cell clones showed a significantly diminished response to the absorbed fraction. This observation confirms that the T-cell recognition determinant was shared by Dp and Cy.

The next question was whether the cross-reactive T cells had any role in the onset of allergic disease. The published literature has shown that CD4+ T cells promote the production of IgE through such T-cell-derived cytokines as IL-4 (11, 22, 25). We then determined whether the cross-reactive T-cell clones would produce IL-4 after stimulation with Cy. By the stimulation of Dp-crude antigen, 2 clones produced detectable IL-4. Further, stimulation of Cy also induced the production of IL-4 in 2 of the 3 T-cell clones, although the amount was relatively low. We also observed Cy-driven IFN- γ production by the T cells.

The existence of a common T-cell recognition determinant on Dp and Cy suggests that the sensitization of Dp could promote a secondary IgE response to Cy, although there is no cross-reactive IgE between Dp and Cy. It is possible that Dp and Cy have similar epitopes recognized only by the T cells, but lack a cross-reacting B-cell epitope. There are several examples that show even non-cognate, or intermolecular T-B interaction, in antibody production (9, 20). Under such conditions, the Dp-sensitive

Table 2. Cytokine production of cross-reactive T-cell clones stimulated with Dp and Cy

Clone	Antigen	IL-4 (pg/ml)	γ -IFN (U/ml)
NP 46	Antigen(-)	0	0
	Dp-C	30.0	3.0
	Cy	N.D.	N.D.
NP 106	Antigen(-)	0	0
	Dp-C	0	20.7
	Cy	12.0	9.4
NP 133	Antigen(-)	0	0
	Dp-C	114.0	30.6
	Cy	38.0	N.D.

N.D.: Not Done.

T cells could help produce antibodies to Cy. The role of cross-reactive T cells in the immunopathogenesis of allergic disease remains to be clarified. It is not likely that cross-reactive T cells are the main cellular factors because of their low clonal frequency. We are presently conducting *in vitro* experiments to determine whether Dp-induced T cells could aid the production of Cy-specific IgE.

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