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Abstract

To analyze the possible major T cell recognition site(s) of chironomid antigens, we established human T cell lines and clones (CD3+ 4+ 8-) reactive to soluble extracts of the adult midge of Tokunagayusurika akamusi (TAA) and/or Chironomus yoshimatsui (CYA). All T cell lines and clones proliferated heavily in response to relatively large molecular weight fractions of TAA (MW greater than or equal to 15,000). Nine clones reactive to TAA were classified into 3 groups according to reactivity, indicating the existence of at least 3 distinct T cell recognition sites in TAA. Five T cell clones responded to both TAA and CYA, although the two chironomid antigens were serologically distinct. We conclude that T cell recognition sites of chironomid antigens are different from B cell recognition sites in humans.

KEYWORDS: allergen analysis, bronchial asthma, chironomid, lymphocyte

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Analysis of Chironomid Allergens Using Specific T Cell Lines and Clones in Humans

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To analyze the possible major T cell recognition site(s) of chironomid antigens, we established human T cell lines and clones ($CD3^+ 4^+ 8^-$) reactive to soluble extracts of the adult midge of *Tokunagayusurika akamusi* (TAA) and/or *Chironomus yoshimatsui* (CYA). All T cell lines and clones proliferated heavily in response to relatively large molecular weight fractions of TAA (MW≧15,000). Nine clones reactive to TAA were classified into 3 groups according to reactivity, indicating the existence of at least 3 distinct T cell recognition sites in TAA. Five T cell clones responded to both TAA and CYA, although the two chironomid antigens were serologically distinct. We conclude that T cell recognition sites of chironomid antigens are different from B cell recognition sites in humans.

Key words : **allergen analysis, bronchial asthma, chironomid, lymphocyte**

The chironomidae are one of the largest insect families including nearly 10,000 species. Since the larval stages are aquatic, they are distributed widely in all types of inland water. Although they had been thought to be merely nuisance insects, hypersensitivity to them has been reported since the 1920's (1-11). Dried extracts of the larvae induce immediate-type hypersensitivity reactions to fish-food factory workers and fishermen, in areas, mostly in Europe, where the larvae are commonly used as fish bait or food (6, 7). The adult chironomid antigens have also been demonstrated epidemiologically to be causative allergens in asthma. Anti-chironomid IgE antibodies have been detected in asthmatics by skin test or radioallergosorbent test (RAST) (8).

Analyses of several allergenic determinants in the chironomid have been reported. Low molecular weight hemoglobin of larvae is one candidate (5-7, 12). However, the details of chironomid allergens are still controversial. Moreover, our recent serological studies have shown that high molecular weight fractions, which are shared by several but not all chironomid species, are also important allergens.

It is likely that T cell immunity is involved in allergic reactions in humans. In cases of mite or ragweed pollen allergies, raised levels of antigen-driven T cell proliferation *in vitro* suggested cellular factors

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in allergies (10, 13-16). Recent developments in cloning methodology have enabled us to establish allergen-specific T cell clones. By using cloned T cells, it is possible to analyze T cell recognition sites of allergens as well as T cell mediated regulation of antibody production in atopic individuals (16). Although these methods can give us clues to understand the immunopathogenesis of allergy, we have little information about T cell responsiveness in chironomid hypersensitivity.

In this study we examined human T cell responses to two abundant chironomid species, *Tokunagayusurika akamusi* and *Chironomus yoshimatsui*. We used soluble extracts of adult midges of *T. akamusi* (TAA), *C. yoshimatsui* (CYA) and larvae of *T. akamusi* (TAL) for induction of peripheral blood lymphocyte (PBL) proliferation and to establish specific T cell lines *in vitro*. We obtained TAA- or CYA-induced, $CD3^+$ $CD4^+$ $CD8^-$ T cell lines. Then we established TAA-reactive T cell clones by micromanipulation. We analyzed possible major T cell epitope (s) of TAA, existence of interspecies shared T cell recognition sites, and the molecular weight of T cell recognition antigens.

Materials and Methods

Lymphocyte preparations. We investigated two healthy males; one (T. E.) with a positive skin prick test to chironomid midge antigens, and the other (N. O.) with a negative test. They had no history of bronchial asthma or other allergic diseases. PBLs were prepared from fresh heparinized blood by the Ficoll-Conray gradient solution method (specific gravity = 1.077) (17).

Antigens. Crude soluble antigens extracted from adult chironomid midges, TAA, CYA, and TAL were prepared by a method described elsewhere (9). As controls, purified protein derivative (PPD, Nihon BCG Seizo Co., Tokyo, Japan) and soluble schistosome egg antigen (SEA)(18) were used. Protein concentration was determined by Lowry's method (19), and all antigens were cryopreserved at −20°C before testing.

Lymphocyte proliferation assays in vitro. PBLs were cultured in 96-well flat-bottomed microtiter plates (Corning Co., Corning, NY, USA) in 0.2 ml of RPMI 1640 culture medium (CM), supplemented with 10% heat-inactivated pooled human male sera (HS), 100 μg/ml streptomycin, 100 U/ml penicillin, and 20 mM L-glutamine, in the presence or absence of antigens. The optimal conditions and antigen doses for antigen-driven PBL responses were determined in preliminary experiments (data not shown). In brief, 1×10^5 of PBL were cultured at 37°C in humidified 5% CO_2 and 95% air for 7 days. We used antigens at the concentration of 10 μg/ml for TAA and TAL, 7.5 μg/ml for CYA, 2 μg/ml for PPD, and 5 μg/ml for SEA. Wells were pulsed with 1 μCi of [^{3}H]-thymidine (specific activity = 10 Ci/mmol) (ICN Radiochemicals, Irvine, CA, USA) for the final 16 h, and [^{3}H]-thymidine incorporation was assessed by liquid scintillation spectrometry.

T lymphocyte and macrophage (Mϕ) preparation. PBLs (1×10^7 cells) suspended in RPMI 1640 CM were placed in a plastic dish (diameter; 100 mm)(Nunc, Rockilde, Denmark), and incubated for 60 min at 37°C in humidified 5% CO_2 and 95% air. Cells which did not adhere to the plastic dish were recovered by washing the dish with RPMI 1640 CM. Nonadherent cells were applied to a densely packed nylon-wool column (500 μg/2.5 ml) and incubated for 60 min at 37°C in humidified 5% CO_2 and 95% air. Cells were applied to a nylon wool column twice, and the cells which did not adhere to the nylon wool were used as T lymphocytes (20). Cells which adhered to the plastic dish were gently detached with a rubber policeman on ice and were used as Mϕ (20).

T lymphocytes-Mϕ coculture experiments. In T lymphocyte-Mϕ coculture experiments, 7×10^4 T lymphocytes and variable numbers of Mϕ were tested. The same number of T lymphocytes were also cocultured with 5×10^4 irradiated autologous PBLs (3000 rad). Coculture experiments were done under the conditions described for PBL proliferation assays.

Generation of chironomid midge antigen-induced T cell lines in vitro. PBLs (1×10^6) of T. E.

were suspended in 1.5 ml of RPMI 1640 CM in 24-well plates (Nunc), and were incubated with 10 μg/ml of TAA or 7.5 μg/ml of CYA for 7 days. Blast cells were harvested and were resuspended in RPMI 1640 CM with 3 μg/ml of appropriate antigens and 20 U/ml of purified human interleukin-2 (IL-2, Electro-Nucleonics, Silver Spring, MD, USA) in 24-well plates, and five times of autologous irradiated PBLs (3000 rad) were added as feeder cells. The culture media were exchanged every 2 days, and feeder cells were added to the culture every 10 days.

Cloning of T lympocytes. Ten days after the first addition of feeder cells, blast cells were cloned by a micromanipulation method described elsewhere (21). Single cells were sorted into individual wells of 96-well round-bottomed microtiter plates (Corning), and were cultured in RPMI 1640 CM with 3 μg/ml of TAA and 20 U/ml of IL-2. Irradiated autologous PBLs were also added at 1×10^5 per well as antigen presenting cells (APCs) every 10 days. Cells, which showed positive growth, were then transferred to 24-well plates.

Phenotyping of blastoid cell lines. The surface markers of cell lines were tested by membrane immunofluorescence using anti-human CD3, CD4 and CD8 monoclonal antibodies (Beckton Dickinson, Mountain View, CA, USA) as the first antibody and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Zymed Laboratory, Inc., San Francisco, CA, USA) as the second antibody. Viable blastoid cells (2×10^5/50 μl) were incubated with the first antibody at 4°C for 40 min, and then washed 3 times with phosphate-buffered saline. FITC-conjugated second antibody was then added to cells at 4°C for 40 min. Surface fluorescence of the cells was assessed by fluorescence microscopy.

Antigen-driven proliferation of T cell lines and clones. At least 10 days after the last addition of feeder cells, the cells from T cell lines and T cell clones (TLCs) were washed 3 times to wash away the remaining exogenous IL-2. Then 1×10^4 cells from T cell lines or TLCs were cocultured in 0.2 ml of RPMI 1640 CM, with optimal dose antigens and with 5×10^4 irradiated autologous PBLs (3000 rad) as APC, in 96-well flat-bottomed microtiter plates (Nunc) for 72 h. One μCi of [^{3}H]-thymidine was added for the final 8 h.

Fractionation of TAA. TAA was fractionated by gel filtration. We applied 16 mg of TAA on Sephacryl S-200 superfine (Pharmacia, Sweden). Gel filtration was performed at 4°C using a 26 mm×95 cm column equilibrated with Tris buffer (pH 7.4). Elution was performed with Tris buffer at a flow rate of 36 ml/h, and 5.0 ml fractions were collected. Absorbance of the fractions at 280 nm and 415 nm was measured with a spectrophotometer. Each fraction was used at the concentration of 5 μg/ml for proliferative response of PBLs, T cell lines and clones.

Results

PBL response to chironomid midge antigens in vitro. Two healthy volunteers were tested for *in vitro* PBL proliferation in the presence of various antigens. PBLs of both volunteers showed vigorous proliferative response to PPD, while we observed no detectable proliferative response to SEA. PBLs of both volunteers showed heavy proliferation in the presence of TAA, CYA, and TAL (Table 1).

T lymphocyte-Mϕ cooperation in vitro. To test whether T cells were involved in the proliferative response to TAA or CYA, we performed T-Mϕ coculture experiments using cells of one of the two subjects. Although cultures of T cells alone or Mϕ alone showed no proliferative response, significant proliferation was observed when Mϕ or irradiated PBLs were added to T cells (Fig. 1). We observed clear dose-dependency in the T-Mϕ interaction, and optimal responsiveness was observed at the T-Mϕ ratio of 10: 1. These results suggested that T lymphocytes were involved in the proliferative response to chironomid midge antigens *in vitro.*

Generation of chironomid midge-induced T cell lines. We obtained IL-2-dependent blastoid cell lines. The surface phenotypes of these cells were $CD3^+$ $CD4^+$ $CD8^-$, indi-

Table 1 *In vitro* PBL proliferative response of peripheral blood lymphocytes (PBL) to chironomid antigens[a, b]

PBL donor	Antigen added					
	None	PPD 2μg/ml	SEA 5μg/ml	TAA 10μg/ml	CYA 7.5μg/ml	TAL 10μg/ml
T.E.	2,623 ±915	28,767 ±7,566	3,021 ±498	20,002 ±6,729	11,329 ±1,040	14,683 ±3,796
N.O.	1,208 ±667	110,729 ±4,715	5,553 ±2,800	27,350 ±16,839	31,077 ±9,932	65,022 ±9,236

a : Peripheral blood lymphocytes from two donors, T.E. and N.O. were cultured at the concentration of 1×10^5/well for 7 days, at 37°C in humidified 5% CO_2 and 95% air.

b : [³H]-thymidine incorporation was assessed by liquid scintillation spectrometry. Results are expressed as the mean ± SD of three dishes.

Abbreviations : PPD, purified protein derivative (Nihon BCG Seizo Co.) used as control; SEA, soluble schistosome egg antigen; TAA, CYA and TAL, crude soluble antigens extracted from adult midges of *Tokunagayusurika akamusi, Chironomus yoshimatsui* and larvae of *Tokunagayusurika akamusi*, respectively.

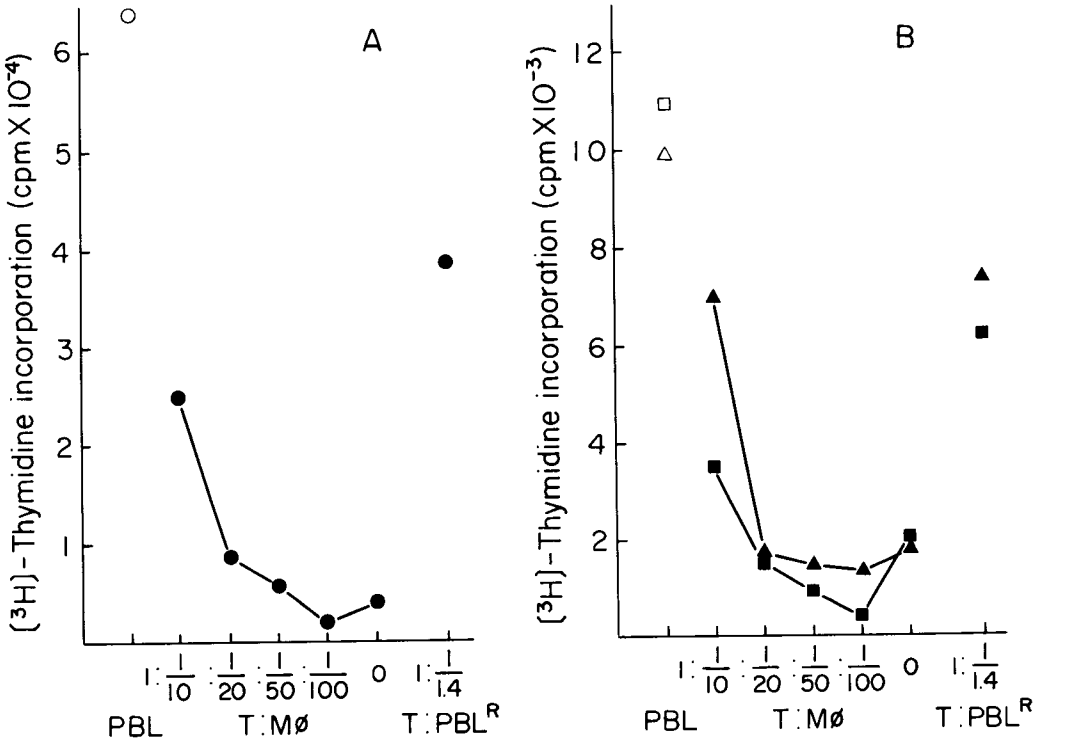

Fig. 1-A The T lymphocyte-macrophage (Mφ) interaction in T cell proliferation in the presence of purified protein derivative (PPD). The open circle (○) shows peripheral blood lymphocyte (PBL) proliferation in the presence of PPD, and closed circles (●) shows T cell proliferation in the presence of Mφ or irradiated autologous PBL. T cell proliferation is dose-dependent on Mφ.

Fig. 1-B The T lymphocyte-macrophage (Mφ) interactions in T cell proliferation in the presence of crude soluble antigens extracted from *Tokunagayusurika akamusi* (TAA) and *Chironomus yoshimatsui* (CYA). Proliferative response of peripheral blood lymphocytes (PBL) to TAA (△) and CYA (□), and that of T cell to TAA (▲) and CYA (■) in the presence of Mφ or irradiated autologous PBL are shown. Responsiveness of T cells depends on the number of Mφ. A T: Mφ ratio of 10 : 1 gave the most vigorous response.

Table 2 Specificity analysis of antigen-driven proliferation of T cell lines and clones[a,b]

Responder	Antigens added				
	None	PPD	TAA	CYA	TAL
Anti-TAA TCL	6,700	8,700	44,000[c]	26,000[c]	32,500[c]
TLC D12	1,100	800	27,000[c]	19,300[c]	11,900[c]
C4	500	400	8,400[c]	5,900[c]	5,100[c]
F5	700	1,700	7,600[c]	5,600[c]	6,000[c]
A10	900	1,000	12,400[c]	8,800[c]	10,400[c]
E11	1,100	1,500	13,300[c]	9,200[c]	11,700[c]
A1	600	600	3,200[c]	800	4,200[c]
G7	1,100	600	3,300[c]	1,700	3,000[c]
D8	1,000	400	3,000[c]	600	7,600[c]
B2	2,300	1,900	4,900[c]	700	800
Anti-GYA TCL	1,100	2,900	46,000[c]	45,000[c]	34,000[c]

a: T cell lines (TCLs) and clones (TLCs) were cultured at the concentration of 1×10^4/well for 7 days, at 37°C in humidified 5% CO_2 and 95% air.

b: See footnotes to Table 1.

c: Positive proliferative response.

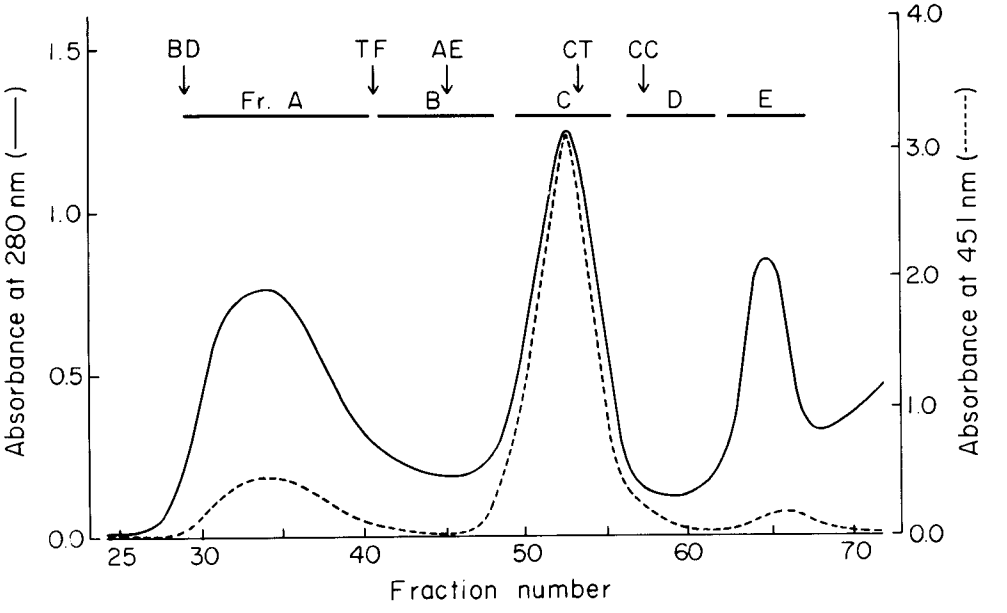

Fig. 2 Sephacryl S-200 gel filtration of crude soluble antigens from *Tokunagayusurika akamusi*. Blue dextran (BD), transferrin (TF), alubumin egg (AE), chymotrypsinogen (CT) and cytochrome C (CC) were used as markers. Fractions (Fr.) A (MW=2,000,000-80,000), B (MW=80,000-33,000), C (MW=33,000-15,000), D (MW=15,000-7,000) and E (MW=7,000 or less) were pooled.

cating that these were helper/inducer T cells. Regardless of the antigens used in establishing T cell lines, each one showed a proliferative response to TAA, TAL and CYA in the absence of exogenous IL-2, but failed to respond to PPD (Table 2).

These results suggested that these three chironomid midge antigens, TAA, CYA and TAL, shared cross-reactive determinants with each other.

TAA-reactive human TLCs. A TAA-induced T cell line was cloned by micromanipulation. Fifteen clones were obtained from 200 single cell-dispensed wells. All nine clones tested showed TAA-driven proliferation, but they were not reactive to PPD. Although these TLCs showed strong proliferation to crude TAA, we observed three distinct subgroups among them: five of nine TLCs responded to both CYA and TAL (category 1), three TLCs to TAL but not to CYA (category 2), and one did not respond to CYA or TAL (category 3)(Table 2).

Fractions of TAA. The elution pattern of TAA through a Sephacryl S-200 column is shown in Fig. 2. There were three main absorbance peaks at 280 nm, and one at 415 nm. We tentatively divided the eluate into five fractions. The molecular weight (MW) of each fraction was estimated to be: Fraction (Fr.) A, 2,000,000-80,000; B, 80,000-33,000; C, 33,000-15,000; D, 15,000-7,000; E, 7,000 or less. PBLs from the two individuals showed different patterns of proliferative response to TAA fractions *in vitro*. PBLs from T.E. showed heavy proliferation against three fractions (Frs. A, B and C, Fig. 3-A), whereas PBLs from N.O. were reactive against two fractions (Frs. A and B, Fig. 3-B).

Response of chironomid midge-induced T cell lines to TAA fractions. A TAA-induced T cell line and a CYA-induced one were stimulated with TAA and TAA fractions. Both T cell lines tested showed heavy proliferative response to Frs. A and B, but only the TAA-reactive one responded to Fr.C. Neither showed detectable proliferative response to Frs. D and E (Table 3).

Response of TAA-reactive human TLCs to TAA-fractions. Although all six TLCs tested showed strong proliferative response to crude TAA, three of the six TLCs responded to Frs.A and B (group 1), two responded only to Fr.C (group 2) and one responded only to Fr.B (group 3). All TLCs of group 1 responded to TAA, CYA and TAL, TLCs of group 2 responded to TAA and TAL but not to CYA, and a TLC of group 3 respond-

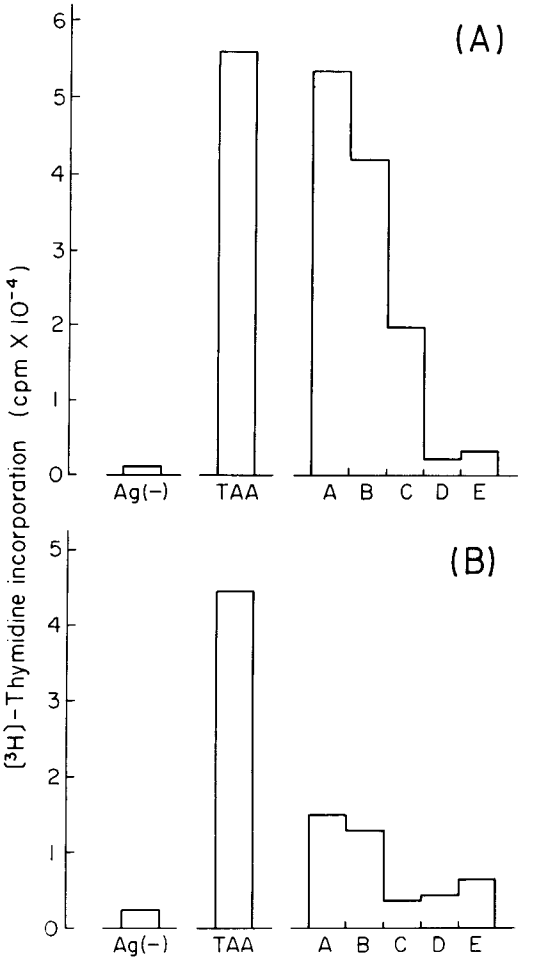

Fig. 3 *In vitro* proliferative response of peripheral blood lymphocytes (PBLs) from two donors T. E. (A) and N.O. (B) to fractions of crude soluble antigens from *Tokunagayusurika akamusi* (TAA)(Fig. 2). Proliferation was determined as described in the legend to Table 1. Results are expressed as cpm. Three fractions (Frs. A, B and C) induced heavy proliferation of PBLs from T. E (A) and two fractions (Frs. A and B) were stimulative to PBLs from N. O. (B).

Table 3 *In vitro* proliferative response of T cell lines and clones to TAA fractions(Fr)[a]

Responder	Response to			TAA fractions added (5 μg/ml)						
	TAA	CYA	TAL		PPD	Fr.A	Fr.B	Fr.C	Fr.D	Fr.E
Anti-TAA TCL	+	+	+	6,700	8,000	92,000[b]	42,000[b]	20,100[b]	2,500	3,600
TLC D12	+	+	+	1,100	800	16,000[b]	14,600[b]	600	200	600
E11	+	+	+	1,100	1,500	18,000[b]	12,000[b]	1,100	1,000	2,000
C4	+	+	+	500	400	5,100[b]	6,000[b]	900	200	1,400
G7	+	−	+	1,100	700	600	400	2,600[b]	200	400
D8	+	−	+	1,000	400	600	1,000	3,400[b]	400	400
B2	+	−	−	2,300	1,900	800	4,400[b]	1,100	900	1,000
Anti-CYA TCL	+	+	+	1,100	3,000	21,000[b]	20,000[b]	3,000	600	700

a : See footnotes to Tables 1 and 2.
b : Positive proliferative response.

ed only to TAA (Table 3).

Discussion

Hypersensitivity to the chironomid has become a health problem in the world(1-4, 11). In Europe, there is evidence that larval hemoglobins of *Chironomus riparius* cause occupational allergic diseases (5-7). In Japan, larvae of the chironomid are not so important allergens as in Europe. However, increased numbers of the chironomid result in high incidence of hypersensitivity to the insect. It has been demonstrated that many patients with bronchial asthma have a high titer of chironomid midge-specific IgE antibodies (8). Although serological analysis by Tee *et al.* indicated that larvae and pupae have stronger antigenicity than adults in European cases of chironomid allergy (5), we observed that a major allergen(s) was located in a high molecular weight portion of adult midges rather than in larval extracts and was apparently different from hemoglobin (22). Those differences might have come from differences in the chironomid species studied or in the sensitization manner. The allergenic fraction of chironomid is thus still controversial.

T cell dependency in reaginic responses are well documented in man (10, 13-16, 23, 24). Antigenic determinants recognized by T cells are not necessarily analogous to those recognized by B cells, so the characterization of T cell recognition sites is also essential for uncovering the immunopathogenesis of allergic reactions. O'Hehir *et al.* showed that T cells recognize a species-specific epitope expressed on 9,000-13,000 MW fractions of house dust mites, *Dermatophagoides farinae* (15), whereas B cells seem to recognize other epitopes (4-10, 13, 15, 25-27). HLA-DR-restricted helper T cells, which recognize such unique T cell recognition sites, control immunoglobulin secretion (13, 14, 16). Serological studies of allergenicity in cases of chironomid allergy have been done(5, 9, 28), but analysis of T cell recognition sites or T cell regulation has not yet been done.

Regardless of response to the skin prick test of the two healthy volunteers in this study, the PBLs of both individuals were reactive to TAA, CYA and TAL in a PBL proliferation assay. TAA and CYA are not mitogenic because only 11 of 35 healthy control PBLs showed vigorous proliferation to

TAA and CYA (manuscript in preparation). We established TAA-induced and CYA-induced two T cell lines from one of the individuals. Inasmuch as both lines were reactive to three antigens, these crude antigens seemed to share a cross-reactive determinant. Together with our previous findings that TAA and CYA are serologically distinctive from each other (9), the present results indicate that T cell recognition sites in chironomid antigen were clearly different from B cell recognition sites. TAA-reactive TLCs gave us more detailed information. Nine TLCs tested seemed to be classifiable into three categories. TLCs of category 1 responded to a determinant expressed on all three crude antigens. This T cell recognition site seems to be shared by *T. akamusi* and *C. yoshimatsui* regardless of the stage of development. Clones of category 2 recognized a determinant expressed on both TAA and TAL but not on CYA, suggesting that this T cell recognition site is *T. akamusi*-specific, but stage-nonspecific. The TLC of category 3 was reactive only to TAA. This site seems to be stage- and species-specific. Crude TAA has at least three distinct T cell recognition sites. Although only nine clones were tested, category 1 and category 2 were relatively common in TAA-reactive TLCs.

In the PBL proliferation assay, we observed heterogeneity in response to molecular weight fractions of TAA. Frs. A, B and C had antigenicity for PBLs of T.E., but only Frs. A and B had antigenicity for N.O. The PBLs from both persons were not reactive to either Fr. D or E. This suggests that relatively large molecular weight fractions of TAA activate T cells. Both TAA- or CYA-induced T cell lines showed heavy proliferation to Frs. A and B. However, Fr. C was stimulatory only to the TAA-induced line. No T cell line showed detectable proliferative response to Fr. D or E. This result again suggests that a major T cell stimulating determinant(s) is expressed on relatively large molecular weight fractions, and that the T cell epitope(s) expressed on Fr. C might be species-specific. At 415 nm, at which wave length hemoglobin is detected (29), we observed a major absorbance peak at Fr. C. This result suggests that hemoglobin is not likely to be the common T cell recognition site shared by *T. akamusi* and *C. yoshimatsui* in the T cell response.

To analyze further details, we tested TLCs for fraction-induced proliferation. Of six TLCs tested, three were of category 1, two of category 2 and one of category 3. We observed three distinct response patterns in six clones, and it was interesting to see that each group corresponded to a particular category. All TLCs of category 1 responded to Frs. A and B. Two TLCs, which responded to only Fr. C, were included in category 2. Although Fr. C is hemoglobin-rich, it is not clear whether or not the hemoglobin molecule is responsible for the category 2 response. The TLC of category 3 responded to the TAA-specific antigenic site expressed on Fr. B. Although only a small number of T cell clones were tested, it is possible that Frs. A and B have a common determinant(s) for TAA and CYA. On the other hand, Fr. C contains a *T. akamusi*-specific determinant, and a TAA-specific site might be on Fr. B. The physicochemical nature of the interspecies shared determinant is not clear. We do not know which MW fraction of CYA expresses the sharing determinant, nor do we know whether the same molecule expressing the common determinant is shared by TAA and CYA or is expressed on different molecules. This seems to be the first report of T cell recognition sites during the human immune response to chironomid antigens, and of cross-reactive or species-specific T cell recognition sites in this insect allergen.

The existence of a common T cell recognition site(s) on *T. akamusi* and *C. yoshimatsui* suggests the possibility that sensitization to *T. akamusi* also produces a strong secondary response to *C. yoshimatsui* even in individuals who have no sensitization to the latter. Baldo *et al.* also supported this possibility by showing the presence of common B cell recognition sites in different species of insects (30). This might be important in interpreting epidemiological data of chironomid allergy.

Further investigations are needed to analyze the physicochemical nature of allergenicity of the chironomids. Presently, functional analysis of chironomid-specific human T cell lines and clones is under way.

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