

Koichiro Kawai^a
Hiromi Tagoh^a
Kazuyuki Yoshizaki^b
Gyokei Murakami^c
Atsushi Muraguchi^a

^a Department of Immunology,
Toyama Medical and Pharmaceutical
University, Toyama,

^b Department of Medicine III,
Osaka University Medical School,
Osaka and

^c Department of Pediatrics,
Toyama Red Cross Hospital, Toyama, Japan

Purification and Characterization of an Allergenic Monomeric Hemoglobin from a Chironomid Distributed Worldwide, *Polypedium nubifer*

Abstract

The *Pol n* component MV, a potent experimental allergen for mice, was purified to homogeneity from extracts of a chironomid distributed worldwide, *Polypedium nubifer* (PN). The *Pol n* I component MV was shown to have cross-reactivity to hemoglobins (Hb) derived from all species of chironomids tested. Determination of the amino acid sequence of the first 37 N-terminal residues revealed that it had 30–59% homology to Hb of an European chironomid, *Chironomus thummi thummi*, which had been known as an important allergen for humans. By Western blot analysis, we showed that sera from asthmatic patients, which had positively reacted to the extract of the adult PN midge, bound to the purified *Pol n* I component MV. Furthermore, using rabbit polyclonal antibodies raised against synthetic polypeptides corresponding to the N-terminal residues, it was demonstrated that the N-terminal amino acid sequence between position 15 and 35 contained antigenic epitope(s) for human IgE. The results indicate that the *Pol n* I component MV is an allergen for human beings as well as for mice, and useful as a diagnostic tool for chironomid allergy.

Key Words

Chironomid
Allergen
Hemoglobin
IgE
Asthma

Introduction

Recent studies have shown that chironomid midges are important occupational and environmental allergens. In Germany, people exposed to larvae of chironomids through their work have been reported to suffer from type I allergic reactions [1]. In Sudan, it has been reported that a small midge, *Cladotanytarsus lewisi*, emerged in enormous numbers from the Nile basin and caused severe respiratory diseases in people living close to the Nile [2]. In Japan, several cases of severe bronchial asthma induced by direct inhalation of chironomid midges have been reported [3]. Japanese researchers claim that about 30% of asthmatics showed positive reactions to extracts from a midge, *Chironomus plumosus*, at prick tests, indicating that the chirono-

mid midges are an important cause of bronchial asthma in Japan [4].

We have been involved in characterizing the allergens derived from many species of chironomids distributed in the urban areas in Japan. We have found that some species of chironomids cultured in our own laboratory have a potent experimental allergenic activity in mice [5, 6]. Furthermore, we have demonstrated that the major allergens of the chironomid larvae for mice are the monomeric and dimeric hemoglobins (Hb) in their body fluid [5]. In the present study, we purified to homogeneity the allergen for mice of a chironomid, *Polypedium nubifer* (PN) distributed worldwide, and determined the sequence of 37 N-terminal amino acid residues. We have shown that the sequence has a homology to the Hb of *Chironomus thummi thummi* (CTT),

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Correspondence to: Dr. A. Muraguchi
Department of Immunology
Toyama Medical and Pharmaceutical University
Sugitani 2630
Toyama City 930-01 (Japan)

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which have been found to be major allergens for the allergic patients exposed to either adults or larvae of CTT in Germany [1, 7]. We have also shown that human serum IgE of the patients with bronchial asthma bound to the purified allergen and that rabbit antisera against the N-terminal residues inhibited the binding of human IgE to the purified allergen.

Materials and Methods

Chironomid Midges and Larvae

Larvae and adult midges of PN were cultured in our own laboratory. Larvae were usually collected at the last instar stage. Adults were collected after natural death in a container.

Animals

Male BALB/c mice (5 weeks old) and male Wistar rats (7–9 weeks old) were purchased from Sankyo Labo Service (Tokyo, Japan) and used in this study.

Preparation of Crude Extracts from Larvae and Midges

Crude extracts from adult midges and larvae were prepared as follows. For midge extracts, frozen dried midges were triturated. After adding a 20-fold volume of PBS as powder weight, ether extraction was performed for 48 h at 4°C. The aqueous phase was centrifuged and the supernatant was dialyzed against PBS. After centrifugation, the supernatant was used as midge extracts. For larval extracts, frozen larvae were thawed at 4°C, homogenized, centrifuged, and the supernatant was filtered through a 0.45- μ m membrane filter (Millipore, Bedford, Mass., USA). The filtrate was dialyzed against PBS, centrifuged, and used as larval extracts.

Gel Filtration

Gel filtration through Sephadex G-75 (Pharmacia Biotech, Uppsala, Sweden) was carried out in a column (3.1 $\times$ 64 cm) preequilibrated with 0.1 M sodium acetate, 0.035 M magnesium chloride, pH 7.0. Sample solution was applied on the column and eluted at a flow rate of 7 ml/h. Four-milliliter fractions were collected and absorbance at 280 and 415 nm was measured. Standard markers used were bovine serum albumin (M_r 67,000), ovalbumin (M_r 43,000), chymotrypsinogen A (M_r 25,000), myoglobin (M_r 17,000) and cytochrome c (M_r 12,000). Each of the fractions or pooled fractions were assayed for the allergenic activity and/or IgE-inducing potency.

Anion Exchange Chromatography

Ion exchange chromatography on DE-32 cellulose (Whatman Biochemicals, Maidstone, UK) was performed in a column (2.8 $\times$ 40 cm) equilibrated with 0.005 M potassium phosphate, pH 8.6. After samples were applied on the column, elution was performed with a linear gradient of 0–0.19 M KCl and 7.5-ml fractions were collected.

Assay for IgE-Inducing Potency in Mice

A group of 5 individual male BALB/c mice were immunized 3 times intraperitoneally with 1 μ g protein of an antigen with 4 mg of aluminium hydroxide gel per animal at 3-week intervals. The sera were collected a week after the last injection and the titration of antigen-specific mouse IgE was determined by the standard passive cutaneous assay (PCA) in rat skin.

Assay for Allergenic Activity

Allergenic activity of fraction(s) was examined by the Prausnitz-Küstner reaction (PK)-type skin test in rat skin as described by Kawai and Konishi [6]. Briefly, rats were intradermally (i.d.) sensitized with mouse antisera to PN midge extract or *Pol n* I components (1:160 or 1:320 dilution). Four hours later, 1% Evans blue solution (1 ml) was intravenously (i.v.) injected into the rats, and 5 min later serially diluted antigen solution was i.d. injected at the same point as the antisera. Thirty minutes later, dorsal skin was evaluated, and the diameter of bluing was measured. A spot larger than 5 mm in diameter was regarded as a positive reaction. The allergenic activity was represented as a reciprocal of a maximum dilution of 320 μ g/ml showing a positive reaction.

Test for Cross-Reactivity

Allergenic cross-reactivity was examined by PK-type skin tests as described above. Briefly, rats were i.d. sensitized with mouse antiserum to a monomeric component of *Pol n* I, and 4 h later, serial dilutions of several monomeric Hb components of a species were i.d. challenged. The reactivity of a species was represented as a reciprocal of the highest dilution of 320 μ g/ml showing a positive reaction among all the components examined for the species.

SDS-PAGE

For SDS-PAGE, samples (20 μ g) were mixed with 3-fold the volume of sample buffer containing 5% 2-mercaptoethanol, boiled for 3 min, put onto a gel plate composed of a stacking gel (4% acrylamide in 0.125 M Tris-HCl, pH 6.5) and a separation gel (17.5% acrylamide in 0.375 M Tris-HCl, pH 8.8), and electrophoresed at 30 mA for 4 h. Then gels were stained with Coomassie brilliant blue R-250.

N-Terminal Amino Acid Determination and Analysis

Approximately 1.5 nmol of purified *Pol n* I component MV in 50 μ l of distilled water were used for analysis of amino acid sequence. Automatic Edman degradation was performed with a Model 477A protein sequencer (Applied Biosystems, Foster City, Calif., USA). The 37 N-terminal amino acid sequence was determined.

Patients' Sera

Sera from peripheral blood of 43 asthmatic children (from 6 to 19 years old, 27 males and 16 females) and sera from cord blood of 15 individuals were obtained in the pediatric and obstetrics departments of the Toyama Medical and Pharmaceutical University and Toyama Red Cross Hospital. All patients had symptoms of recurrent acute attacks of wheezing, despnæ, cough, and expectoration of mucoid sputum. All patients examined in the present study were atopics and showed positive reactions to a mite, *Dermatophagoides pteronyssinus*, with RAST EIA. Induction of asthmatic symptoms by the mite allergen or chironomid allergen was not tested. None of the selected serum donors had received hyposensitization therapy. Sera were stored at –20°C in aliquots of 0.3 ml until used.

ELISA

ELISA inhibition was performed in a 96-well microtiter plate (Nunc, Roskilde, Denmark). Briefly, wells were coated with the purified *Pol n* I component MV at a concentration of 10 μ g/ml. After washing, anti-peptide antibodies were added at various concentrations and incubated overnight at room temperature. Patients' sera (1/5 dilution) were then poured an incubated overnight at room temperature. Wells were washed and reacted with HRP-conjugated antihuman ϵ -chain antibodies. O-Phenylenediamine was used as a substrate. A reverse in-

hibition assay was performed as follows. After coating with the purified *Pol n* I component MV, wells were filled with various dilutions of patients' sera or cord blood sera and incubated overnight. Wells were then reacted with antibodies against N-terminal synthetic peptides (N2, N3; 200 ng/ml), followed by incubation with biotin-conjugated antirabbit immunoglobulins and HRP-conjugated avidin. Percent inhibition was calculated according to the following formula: % inhibition = (A492 under absence of inhibitors - A492 under presence of an inhibitor) / A492 under absence of inhibitors $\times$ 100.

Western Blot

Western blot was performed by the ECL Western blotting system (Amersham, Buckinghamshire, UK). Briefly, protein samples were electrophoresed in 15–17.5% polyacrylamide gel under denaturing or nondenaturing conditions and transferred to Clear blot-P membrane strip (Atto, Tokyo, Japan). Blotted membrane strips were blocked with PBS containing 0.25% Tween 20 (PBS-T) in the presence of 5% skim milk for 2 h at room temperature, washed 3 times with PBS-T and incubated with allergic patient's sera diluted with PBS-T overnight at room temperature. After washing, the strips were then washed and incubated with HRP-labeled antihuman ϵ -chain antibodies for 2 h at room temperature. The strips were reacted with ECL-Western blotting reagents for 1 min and exposed to X-ray films (Fuji Photo Film, Tokyo, Japan) and the films were developed.

Preparation of Antisera against the Synthesized Peptides

Synthesized peptides (N1, N2 and N3 in fig. 7A) were prepared by the Peptide Institute (Osaka, Japan). The purity was over 80% when examined by mass spectroscopy. They were coupled with rabbit serum albumin (RSA) by the carbodiimide method [8]. Three different peptide-coupled RSA preparations (1 mg/ml) were each emulsified with 1 ml of Freund's complete adjuvant and injected subcutaneously into several dorsal points of a rabbit. Rabbits were boosted for several times by subcutaneous injections of peptide-RSA in saline (0.1–4 mg/2 ml) at 2-week intervals and bled weekly. Titration of antipeptide antibodies in each serum was performed by ELISA using alkaline phosphatase-conjugated goat antirabbit immunoglobulins as a second antibody in 96-well microtiter plates. Sera with high titer of antipeptide antibodies were pooled, and applied to a column of Affi-Gel 10 (Bio Rad Laboratories, Hercules, Calif., USA) that had been coupled with the peptides. Peptide-coupled Affi-Gel 10 columns were prepared according to the manufacturer's instruction, by mixing Affi-Gel 10 (2.5 ml) with 0.1 M MOPS-Na, pH 7.0 (2 ml) containing 2.5 mg of peptide, shaking at 4°C overnight, blocking with 0.25 ml of 1 M ethanolamine-HCl, pH 8.0, packing into a column and washing with 3 M NaSCN. Antibodies absorbed onto columns were eluted with 3 M NaSCN, immediately dialyzed against PBS and lyophilized.

Results

Partial Purification of the Allergen from the Extract of Adult PN

In a previous study [6], we have shown that the extract of adult PN midges cultured in our own laboratory has a high allergenic activity in mice. In order to purify the allergenic moiety in the extract of the adult PN, the crude extract obtained from adult PN was applied to a Sephadex G-75 co-

lumn and allergenic activities in each of the fractions were analyzed. As shown in figure 1A, high allergenicity was eluted from the G-75 gel filtration column in the fraction B (10.5–37.6 kD). Very low or only negligible allergenicity was observed in fraction A with a higher molecular weight, or fraction C with a lower molecular weight. The fraction B was then dialyzed against the buffer, applied to a DE-32 column and eluted with 0–0.25 M KCl. As shown in figure 1B, significant allergenic activity was found in fraction B-II (tube 78–124). This fraction showed a much higher absorbance at 415 nm, which corresponded to the γ -peak of oxygenated Hb, than the absorbance at 280 nm, suggesting that major allergen(s) of the adult PN may be the remnant Hb(s) of the adult midge. Further purification and characterization of the allergen from PN was hampered by the scarcity of remnant Hb(s) in the extract of adult midges. We thus tried to purify the allergenic Hb(s) from larvae PN which contain a more than a 100-fold higher amount of Hb(s) in the body fluid compared to that of the adult midge [5].

Purification and Isolation of Allergenic Hb(s) from the Larvae PN

Purification and isolation of allergenic Hb(s) from larvae PN were performed by sequential chromatography of Sephadex G-75 and DE-32 ion exchange column. Sephadex G-75 gel filtration of the extract of larva PN revealed that larval Hbs consisted of dimeric and monomeric forms, whose molecular weight corresponded to 33.7 and 16.8 kD, respectively (fig. 2A). Allergenicity was identified only in the fractions corresponding to those dimeric and monomeric Hb(s) [data not shown]. The fractions containing the dimeric Hbs (tube 61–70) and those containing the monomeric Hbs (tube 80–91) were then separately pooled, applied to a DE-32 column and eluted with 0–0.19 M KCl in the buffer. Dimeric Hbs were separated into two components and monomeric Hbs were separated into seven components (fig. 2B). The components were designated as DI–DII for dimeric Hbs and MI–MVII for monomeric Hbs according to the eluted order and assayed for the allergenicity as well as IgE-inducing potency. Both the highest allergenic activity and IgE-inducing potency were found in the MV and MVI fraction (fig. 2B). The results indicate that MV and MVI are major allergenic Hb(s) of the larval chironomid, PN.

Since both the MV and MVI fraction showed a similarly high allergenicity, we compared the cross-reactivity of MV and MVI to the different Hb(s) obtained from the various chironomid species. As shown in figure 3, MV had a cross-reactivity with Hbs of virtually all species of the chironomid tested, while MVI did not. The data suggest that MV may

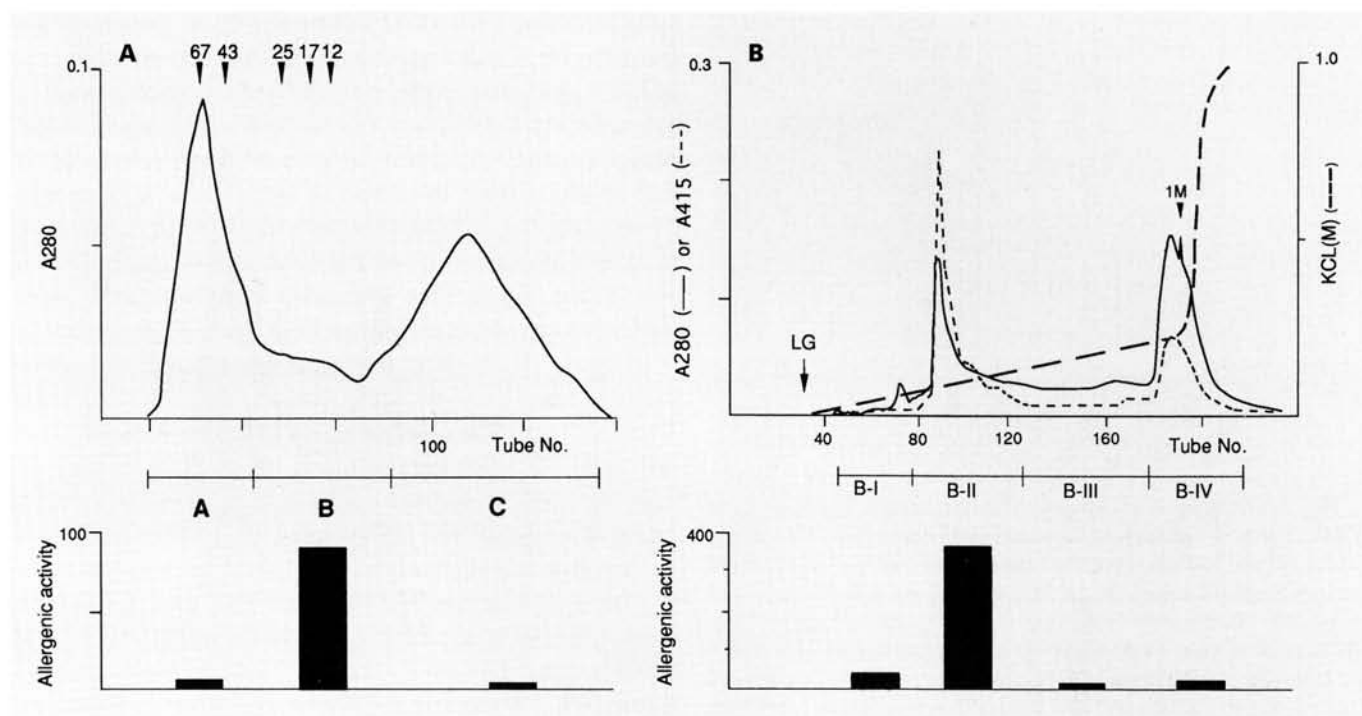


Fig. 1. Partial purification of allergen from extract of adult PN. **A** Crude extracts obtained from adult PN were applied to a Sephadex G-75 column and allergenic activity in pooled fractions (A–C) were determined. Standard markers used were bovine serum albumin (M_r 67,000; 67), ovalbumin (M_r 43,000; 43), chymotrypsinogen A (M_r 25,000; 25), myoglobin (M_r 17,000; 17), cytochrome c (M_r 12,000; 12). **B** Fraction B in **A** was applied to a DE-32 column, eluted with 0–0.25 M KCl and allergenic activity in each of the pooled fraction (B-I to B-IV) was determined. Allergenic activity was represented as reciprocal of a maximum dilution of 320 μ g protein/ml eliciting a positive PK-type skin reaction.

have common allergenic epitope(s) to taxonomically distantly related species. The data also show that MV, but not MVI, cross-reacted with Hb(s) of *Tokunaga surika akamusi*, which was found to be an important allergen of bronchial asthma in Japan [9]. Thus, we chose the MV fraction for further purification.

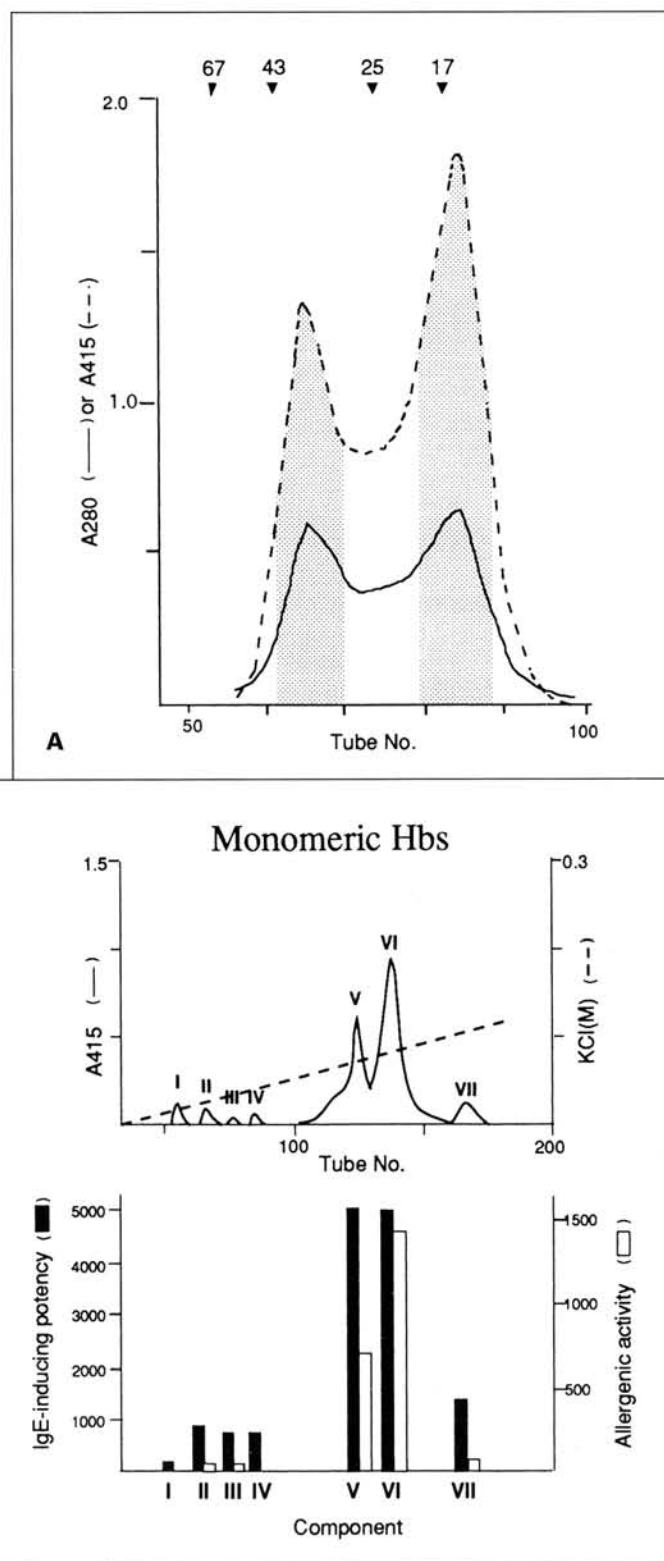
The purification of allergenic Hb in the MV fraction was performed by sequential chromatography on a DE-32 ion exchange column and a G-75 gel filtration column. After application of the MV fraction to a DE-32 ion exchange column, a main 415-nm absorption peak was eluted in fractions 138–145 (fig. 4A). This fraction was again gel-filtered through Sephadex G-75. The main peak was recovered in fractions 74–85 (fig. 4B). A pool of the fractions 74–85, designated as the *Pol n I* component MV, was then analyzed for homogeneity with SDS-PAGE. Only one band with a molecular weight of approximately 13,000 was detected by SDS-PAGE (fig. 4C), showing the homogeneity of the *Pol n I* component MV. The difference of the molecular sizes obtained by G-75 gel filtration and SDS-PAGE may be due

to the conformational change of globin(s) by the SDS treatment. In fact, the molecular size of myoglobin (M_r 17 kD) used as a standard marker in this experiment was approximately 13 kD in SDS-PAGE analysis [data not shown].

Determination and Analysis of NH_2 Terminal Amino Acid Sequence of *Pol n I* Component MV

The *Pol n I* component MV was applied to the amino acid sequencer and the sequence of 37 N-terminal amino acid residues was determined. The amino acid homology search indicated that the sequence of the *Pol n I* component MV was 30–59% identical with those of monomeric and dimeric Hbs of a European species of the chironomid, CTT. The *Pol n I* component MV showed 30–54% homology with the monomer CTT Hbs and 43–59% homology with the dimer CTT Hbs. Figure 5 shows the aligned N-terminal amino acid sequence of 5 different CTT monomer Hbs and 7 different CTT dimeric Hbs. Four conserved positions among N-terminal sequences of the *Pol n I* component MV and 12 CTT Hbs were observed in a 43-alignment. Two of these,

Fig. 2. Purification of allergenic Hb(s) from larva PN. **A** Extracts of larva PN were applied to a Sephadex G-75 column and the absorbance at 415 and 280 nm in each of the tubes was determined. Standard markers used are shown in the legend of figure 1. **B** Dimeric and monomeric Hb fractions in **A** were separately pooled, applied to a DE-32 column, eluted with 0–0.19 M KCl. The allergenic activity (□) and IgE-inducing (■) in each of the eluted fraction (designated as D-I and D-II in dimeric Hbs and M-I to M-VII in monomeric Hbs) were determined.



valine at position 29 and proline at 40, were estimated to be situated with interhelical contact, corner or bend positions of the globin molecule. Two other hydrophobic amino acids, isoleucine at 31 and leucine at 32, were both considered to be at interior sites. An almost completely conserved position at 42 was estimated to be one of haem contacts. Replacement only by hydrophobic amino acids at positions 17, 21, 24, 35 and 36 suggests that these positions are at interior sites. Three positions conserved only among monomers, glutamine at 13, lysine at 25 and valine at 34, may contribute to the stability of monomeric Hbs.

Clinical Study of Pol n I Component MV

Baur et al. [1, 7, 10] demonstrated that CTT Hb(s) are potent allergen(s) in humans. To examine whether the purified Hb from PN, *Pol n I* component MV, has any allergenicity to humans, we have first examined the reactivity of IgE antibody in sera from 43 asthmatic patients to the extract of adult PN by ELISA. The sera of 47% (20 of 43) of the asthmatic children in our clinic had IgE antibody reacted to the extract of the adult PN, a result indicating that PN extract contains moieties allergenic to humans [data not shown].

The sera of 20 asthmatic patients, which had positively reacted to the extract of the adult midge of PN, were then examined for the reactivity to the purified protein, *Pol n I* component MV. Five out of 20 sera of the asthma patients reacted positively to the *Pol n I* component MV [data not shown], the data suggesting that some, but not all patients, had IgE antibodies reactive to this purified Hb. To further investigate whether the IgE antibodies in the seropositive patients react to the *Pol n I* component MV molecule, a Western blot analysis was performed. The sera from all *Pol n I* component MV-seropositive patient reacted to the molecule with the same electrophoretic mobility as the *Pol n I* component MV (fig. 6), confirming that the patient's IgE antibodies recognize the *Pol n I* component MV.

Antibodies Recognizing the N-Terminal Amino Acid Residues Inhibit the Binding of Patients' IgE to Pol n I Component MV

In order to examine whether the N-terminal sequence contains the antigenic epitope(s) recognizable by the patients' IgE antibody, polyclonal antibodies against three different synthesized polypeptides corresponding to the N-terminal sequence (N1, N2, N3 in fig. 7A) were generated in rabbits. These antibodies (anti-N1, N2, N3) were then examined for their ability to inhibit the binding of the patients' IgE to the *Pol n I* component MV using the ELISA inhibition assay. As shown in figure 7B, anti-N2 antibody inhibited the binding of IgE to the *Pol n I* component MV. Anti-

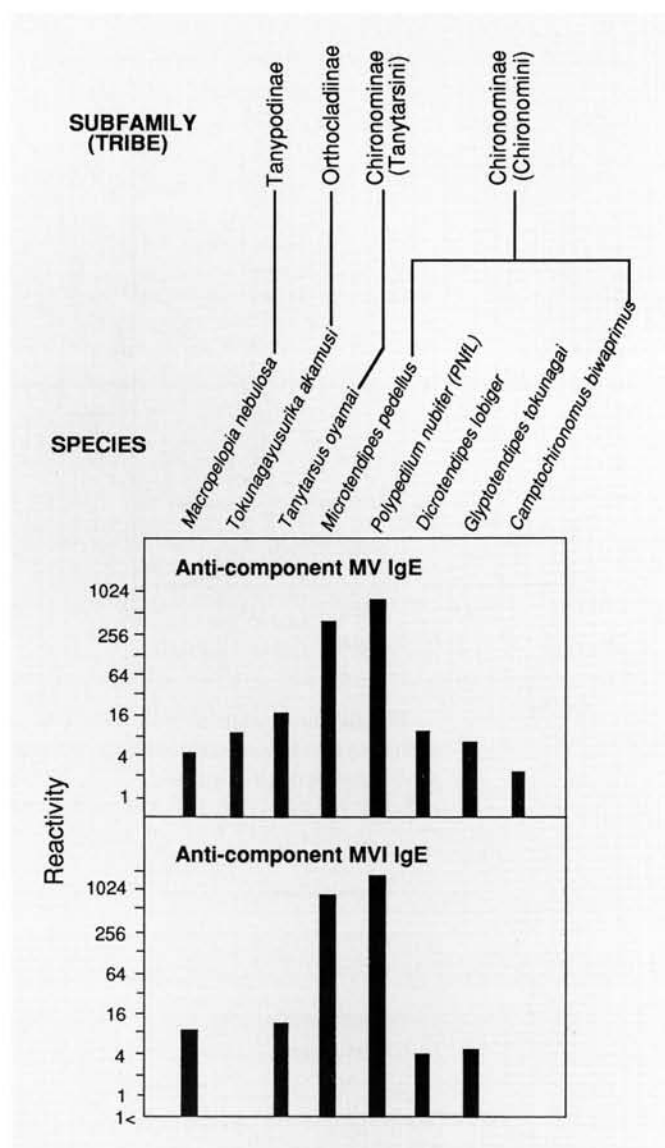


Fig. 3. Comparison of cross-reactivity of fraction MV and MVI to different Hbs from various species of chironomids. Murine IgE antibodies were raised against the *Pol n I* component MV and the *Pol n I* component MVI and were examined for reactivity with monomeric Hbs from different chironomid species. The reactivity of a species was represented as a reciprocal of the highest dilution of 320 µg/ml showing positive P-K-type skin reaction on rat skin among all Hb components examined for species.

N3 antibody also weakly inhibited the binding, suggesting the presence of IgE epitope(s) on N-terminal sequence between positions 15 and 35. In order to confirm these results, we performed reverse inhibition assays in which patients' sera were used as inhibitor. As shown in figure 8, the serum of patient 1 inhibited almost 100% of binding of anti-N2 and

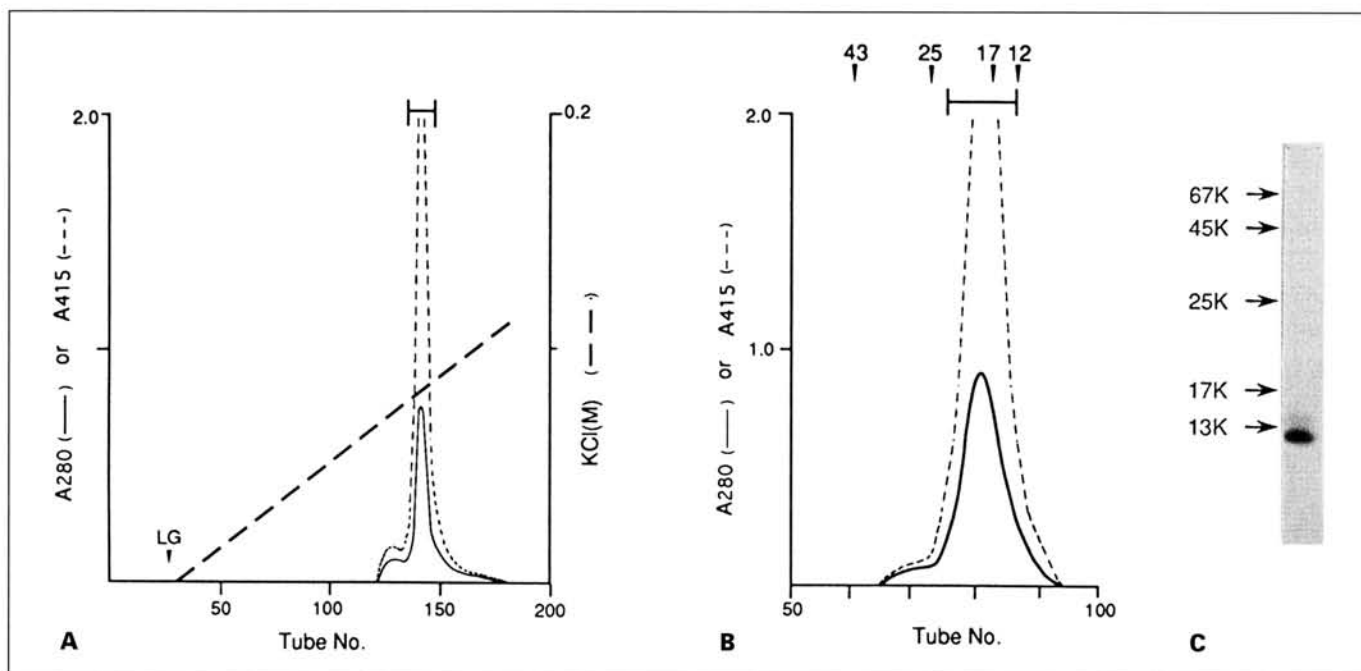


Fig. 4. Purification and isolation of the *Pol n I* component MV from larva PN. **A** MV fraction in figure 2B was applied to DE-32 ion exchange column and eluted with 0–0.19 M KCl. **B** Fractions (tube 138–145) in **A** were pooled and gel-filtered through a Sephadex G-75 column. **C** Fractions containing the peak of A415 (tube 74–85) in **B** were pooled and analyzed by SDS-PAGE. The molecular weight of the standard markers is shown.

<i>Pol n I</i> component MV	DFLTADQAGVLQSAWSNIKNSEVDILYAVFKAYPDIQ	Homology to <i>Pol n I</i> component MV (%)
CTT monomers		
IA	GP SGDQIAAAKASWNTVKNNQVDILYAVFKANPDIQ	51
I	GP SGDQIAAAKASWNTVKNNQVDILYAVFKANPDIQ	51
IIIA	VATPAMPSTDAQVAAVKGDWEKIKSGVEILYFFLNKFPGNF	30
III	LSADQISTVQASFDKVGDPVGIILYAVFKADPSIM	46
IV	LTADQISTVQSSFAGVKGDVAVGIILYAVFKADPSIQ	54
CTT dimers		
X	DPEWHTLDAHEVEVQATWKAVSHDEVEILYTVFKAHPDIM	43
VIII	AVTPMSADQLALFKSSWNTVKHNEVDILYAVFKANPDIQ	57
VI	AVLTTEQADLVKKTWSTVKFNEVDILYAVFKAYPDIM	59
VIIIB	SPLTAEASLVQSSWKAVSHNEVDILAAVFAAYPDIM	57
IIB	APLSADEASLVRGSWAQVKHSEVDILYYIFKANPDIM	51
IX	DPVSSDEANAIIRASWAGVKHNEVDILAAVFSDFPDIM	46
VIIA	APLSADQASLVKSTWAQVRNSEVEILAAVFTAYPDIM	59

Fig. 5. N-terminal amino acid sequence of the *Pol n I* component MV and its comparison to those of CTT Hbs. Open square shows completely conserved amino acids among all 13 Hbs in a 43-alignment.

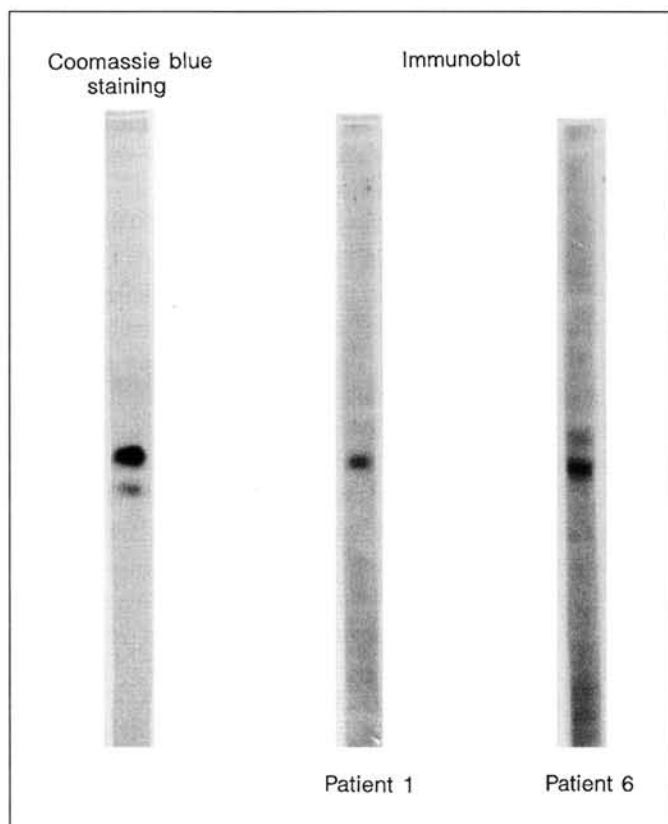
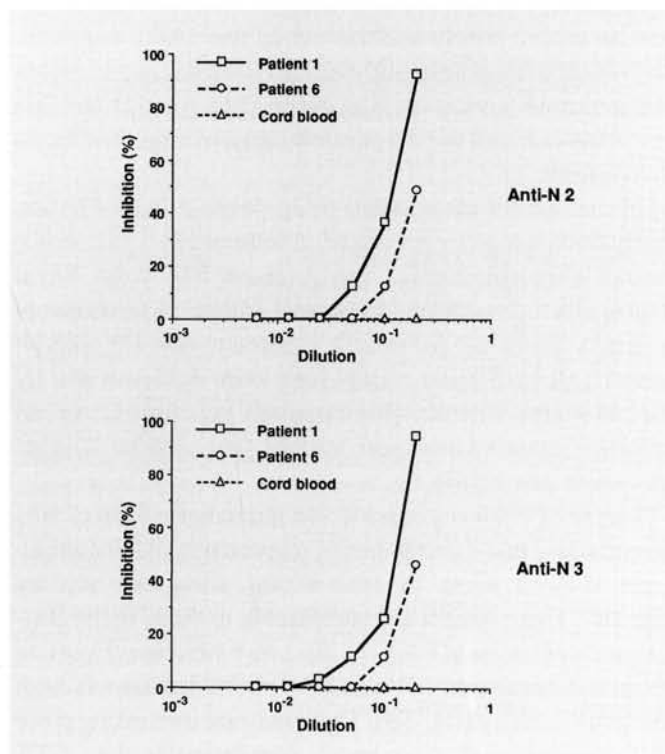
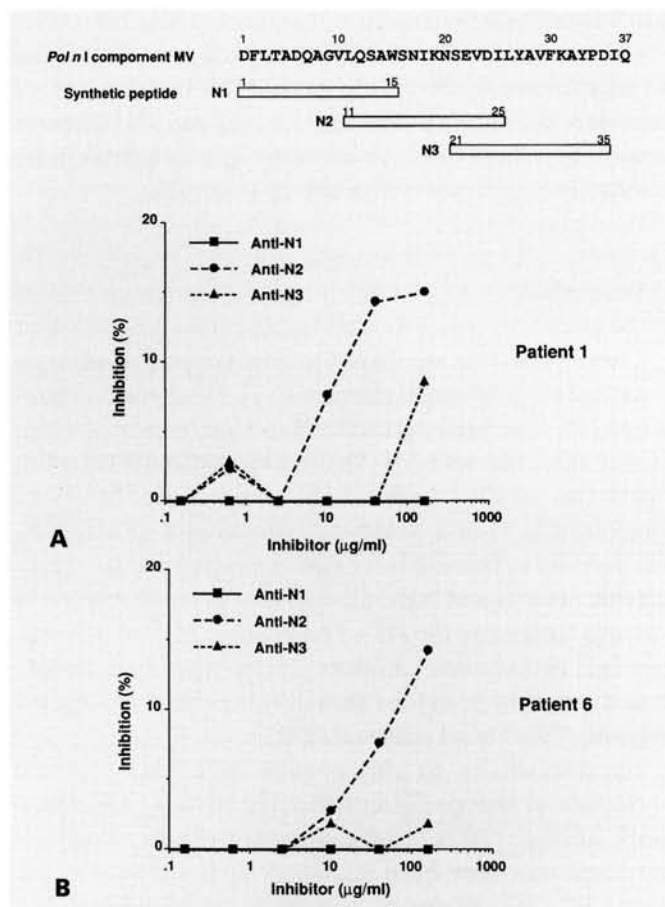


Fig. 6. PAGE and ECL Western blot. The purified *Pol n I* component MV was subjected to PAGE under nondenaturing conditions using 17.5% acrylamide gel and transferred onto a PVDF membrane. The membrane was incubated in 1/2 dilution of *Pol n I* component MV-positive patient sera.

Fig. 7. Inhibition of the binding of the patient IgE to *Pol n I* component MV by purified antibodies generated against synthesized polypeptides corresponding to the N-terminal amino acid residues. **A** Individual rabbits were immunized with the three different synthesized polypeptides (N1, N2, N3 consisting of 15 amino acids) corresponding to the N-terminal sequence. **B** Various concentrations of purified antibody (anti-N1, N2, N3) were added to plates on which the *Pol n I* component MV had been coated. After overnight incubation, plates were washed and the patient's serum (1/5 dilution) was added. After washing, human IgE bound to the *Pol n I* component MV was measured by ELISA. Inhibition was calculated as described in the Materials and Methods.

Fig. 8. Inhibition of the binding of antipeptide antibodies to *Pol n I* component MV by patient's serum. Various concentrations of patient's serum or cord blood serum were added to plates on which the *Pol n I* component MV had been coated. After overnight incubation plates were washed and antibodies against N-terminal peptides (N2 or N3; 200 ng/ml) were added. After washing, rabbit immunoglobulins bound to the *Pol n I* component MV were measured by ELISA. Inhibition was calculated as described in the Materials and Methods.



anti-N3 antibodies to the *Pol n* I component MV. The serum of patient 6 inhibited about 50% of binding of anti-N2 and N3 antibodies to the *Pol n* I component MV. In contrast, cord blood sera did not show any inhibitory effect. These results confirm that there exists at least one IgE epitope(s) in N-terminal sequence between positions 15 and 35.

Discussion

Chironomid Hbs are known to be an important occupational and environmental allergen [1, 2]. However, no chironomid Hbs have been purified and its significance as a sensitizing agent has not been clarified, except for Hbs of a European species, CTT. In this study, a monomeric Hb (*Pol n* I component MV) of a worldwide chironomid species, PN, was purified to homogeneity and shown to have strong allergenic activity and high IgE-inducing potency in mice. It was also shown that the *Pol n* I component MV binds to human IgE of asthmatic children. Furthermore, we demonstrated that epitopes(s) for human IgE exist at N-terminal residues of the *Pol n* I component MV.

Hypersensitivity to chironomids have been reported worldwide: in Europe [7], in Africa [2], in Asia [3, 4] and in North America [11]. Chironomid species of several distantly related taxa have been shown to be potent sensitizing agents [12, 13]. However, ecological morphological and biochemical characteristics of allergenically important species have been poorly understood. In this study, a species, PN, which is ubiquitous and emerges in large numbers from eutrophicated lentic waters in Japan [14], was for the first time demonstrated to be a possible causative agent of bronchial asthma.

In the current study, adult midge extract from PN was firstly fractionated by gel filtration followed by an anion exchange chromatography. The remnant Hbs from larval stages, although obtained in a small amount, was shown to be highly allergenic for mice compared to non-Hb components (fig. 1A). Similar results have been demonstrated by clinical studies with sensitized patients' sera for a European species, *Camptochironomus tentans* [15], and an African species, *C. lewisi* [16].

Larvae of PN had a dimeric and monomeric form of Hbs comprising 2 and 7 components, respectively. All 9 components showed more or less strong allergenic activity (fig. 2B). These results are comparable to those in the clinical study of larvae of CTT [1]. Among 9 Hb components on PN larvae, monomers MV and MVI showed extremely high allergenic activity (fig. 2B). These data are interesting since CTT monomers showed lower allergenicities than CTT

dimers [1]. Regarding the non-Hb components, non-Hb fractions of larva PN had negligible allergenicities to mice in this study [data not shown]. The data are consistent with our previous data demonstrating that non-Hb larval components from *Chironomus* as well as *Glyptotendipes* species have an almost negligible allergenicity [5].

Baur et al. [17] demonstrated that IgE antibodies from patients clinically sensitized by Hbs of *Chironomus plumosus* and CTT did not show cross-reactivity to Hbs of distantly related species to these two species. In the present study, however, murine IgE antibodies raised against the *Pol n* I component MV showed distinct cross-reactivity even to distantly related taxa of PN, i.e., species of *Tanytarsini*, *Orthocladiinae* and *Tanypodinae* (fig. 3). From these data, we speculate that the *Pol n* I component MV might be a promising diagnostic agent of hypersensitivity to a wide variety of chironomid species, although we have not tested this possibility in humans.

The sequence of N-terminal 37 amino acids was determined for the *Pol n* I component MV and compared with 12 CTT Hb components. The *Pol n* I component MV showed 30–54 and 43–59% homology to CTT monomers and to CTT dimers, respectively (fig. 5). The homology of at most 59% was lower than those observed between each 2 of 12 CTT Hbs (at most 99%) according to Goodman et al. [18]. This reflects a rather distant evolutionary relationship between *Polypedium* and *Chironomus*. Why did the *Pol n* I component MV, a monomeric Hb, show a higher homology to CTT dimers rather than to CTT monomers? One explanation is that an ancestor monomeric Hb common to CTT dimers and *Pol n* I component MV monomers had branched from an earlier ancestor before another ancestor of CTT monomers branched. Four conserved positions at 29, 31, 32 and 40, and an almost completely conserved position at 42 in 43 alignment of these 13 Hb species were considered to be directly or indirectly involved in Hb functionality. Positions 17, 21, 24, 35 and 36, where replacement only by hydrophobic amino acids were observed, were estimated to participate in the functionality as interior positions. Positions at 13, 25 and 34, conserved only among monomers, may contribute to the stability of monomers.

We have shown in this study that about 12% of asthmatic children living in the Toyama Prefecture had serum IgE antibodies reactive to the *Pol n* I component MV. The *Pol n* I component MV was considered to have allergenic epitopes common to or cross-reactive among a variety of Hb-bearing species belonging to distantly related taxa. Thus, the rates of individuals with IgE reactive to the *Pol n* I component MV may indicate the frequency of hypersensitivity to Hb-bearing chironomids. Considering that chironomid midges

emerge in a greater number and during a much longer period in south-western Japan, where eutrophication or hypertrophication of various water systems is rapidly developing due to the intensive human activities, compared to north-eastern Japan such as the Toyama Prefecture, Hb-bearing chironomids, who are dominant components of the benthic community in eutrophicated waters [19], might become a major allergen throughout the Japanese archipelagoes.

Baur et al. [1, 10] demonstrated that IgE epitopes are situated within the sequences comprising the surface-exposed or bend portion and with high flexibility in molecules of CTT Hbs, CTT III, IV and VIII. More recently, Kampen et al. [20] demonstrated that the N-terminal 5-amino acid sequence [13–17] of CTT III is essential for antibody binding, using monoclonal antibody and synthetic peptides. In this study, we have shown that there is at least one IgE epitope(s) between position 15 and 35 in the N-terminus of the *Pol n* I component MV. This sequence corresponds to A–C, com-

prising a bend portion A–B as estimated on the basis of a report by Goodman et al. [18].

Determination of antigenic epitopes of the allergen for either IgE or T cell receptors is prerequisite for further understanding and manipulation of IgE production in mice as well as in human beings. Molecular cloning of cDNA encoding the *Pol n* I component MV may give direct access to determine such antigenic epitopes. Bearing this in mind, we are now trying to clone the cDNA from the cDNA library of larva PN using oligonucleotide probes corresponding to the sequence of N-terminal residues.

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