

Hypersensitivity to larvae of chironomids (non-biting midges).

Cross-sensitization with crustaceans

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In 2,368 consecutive adult patients with asthma and/or rhinitis the incidence of positive skin prick test (SPT) with a chironomid extract (CHIR) (produced from "red feather mosquito larvae" used as fish food) was 14% (26% in atopics and 4% in non-atopics). RAST with chironomid was positive in 4% of 110 consecutive sera (8% in atopic sera). Significant correlations were found between RAST and SPT results with chironomid and between SPT results with CHIR and with various crustaceans. Correlations were also found reciprocally among SPT results with different crustaceans and between some crustaceans and molluscs (clam and oyster) as well as among RAST results with chironomid, shrimp and crab. Inhibition experiments showed that chironomid extracts inhibited RAST with shrimp, and vice versa. It is concluded that *Chironomidae* might be allergens of clinical importance in asthma and rhinitis in Sweden, that cross-allergy exists between chironomids and shrimp and that cross-allergy also might occur among chironomids, crustaceans and molluscs.

Key words: allergens; chironomids; cross- reactions; crustaceans; insects; skin test; RAST.

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CLINICAL ASPECTS

The high incidence of positive SPT with a chironomid extract indicates that it may include allergens of clinical importance in Sweden. The clinical relevance of the test results, however, has to be documented by provocation tests in a larger number of patients. Further, the positive test results with CHIR might only indicate sensitization via an immunologically related allergen which could be more important.

Inhalant allergy against non-biting midges belonging to the family *Chironomidae* has earlier been reported as a common cause of bronchial asthma in Sudan (8, 12). Recently, widespread allergy against chironomid has also been described in Japan (9, 10, 13) and USA (11). In Europe allergy against chironomids until now has been reported only in persons handling chironomid larvae as fish food.

One of us (7) observed allergy in four patients using chironomids as fish food. One of

these patients was found to have hypersensitivity to crustaceans. In subsequent clinical practice concomitant allergy with chironomids and crustaceans was observed in several patients. As a result of these observations, the present study was conducted in order to find: 1) the incidence of chironomid sensitization in the west coast of Sweden, and 2) the interrelationships in the cross-sensitization pattern between chironomids, crustaceans, molluscs and common inhalant allergens.

Table 1
Results of skin prick tests in consecutive patients

Allergen	Incidence (%) of positive test results	
	1-2 +	≥ 3 +
<i>Common inhalant allergens</i>		
<i>(n = 2,368)</i>		
<i>D. pteronyssinus</i>	6	11
<i>Cladosporium</i>	3	1
<i>Alternaria</i>	2	1
Cat	5	17
Dog	8	13
Horse	4	6
Birch	5	15
Timothy	6	18
Mugwort	6	15
<i>Insect allergens</i>		
Chironomid (<i>n</i> = 2,368)	7	7
Chironomid Hb (<i>n</i> = 410)	2	< 1
<i>Crustaceans used as fish food (n = 300)</i>		
<i>Daphnia</i>	5	2
<i>Artemia</i>	5	1
<i>Mysis</i>	4	1
Krill	3	1
<i>Crustaceans used as human food (n = 115)</i>		
Shrimp	3	3
Crab	2	3
Cray fish	1	3
Lobster	2	3
<i>Molluscs (n = 115)</i>		
Clam	1	< 1
Oyster	< 1	< 1

PATIENTS AND METHODS

Patients

Adult patients with asthma and/or rhinitis, referred for allergy testing at the Department of Medicine, Halmstad, were studied. During 1983-1986, 2,368 consecutive patients were submitted to skin tests with a chironomid extract (CHIR) in addition to common inhalant allergens. A number of consecutive patients underwent SPT with other allergens as well, according to Table 1. Further, SPT were performed on selected patients who had a positive

history of allergy to shell fish or a positive SPT to CHIR.

Skin tests

Skin prick tests were performed on the volar side of the forearm. A drop of allergen was placed on the skin, and a prick test lancet with a 1 mm point was jabbed through the drop. The wheal reactions were read after 15 min and recorded in accordance with the recommendations of the Standardization Committee of the Northern Society of Allergology (1). Thus a wheal reaction of the same size as that of a histamine reference (histamine HCl 1 g/l in 1983-1985 and 3 g/l in 1986) was recorded as three plus (3+). The mean (± 1 SD) diameters of the wheals elicited by the histamine reference were 3.9 ± 0.9 mm with histamine 1 g/l and 5.0 ± 1.2 mm with histamine 3 g/l.

Provocation tests

Nasal provocation tests were performed on selected patients. With a 1 ml disposable syringe, 0.1 ml of extraction fluid was instilled into the one nostril. In the absence of reactions CHIR allergen extract 1/100 w/v was instilled into the other nostril after 10 min. Reactions were scored as doubtful or positive depending on the intensity of sneezing, rhinorrhea, itching, or blocking of the nose.

A few patients underwent conjunctival tests. They were performed by administering one or two drops of CHIR extract 1/100 w/v in the conjunctival sac of one eye and control solution in the other. Congestion of the conjunctival mucosa and itching in the eye were regarded as signs of positivity.

RAST

Specific IgE antibodies in the patient sera were measured using the Phadebas RAST (Pharmacia Diagnostics AB, Uppsala, Sweden) standard procedure in duplicate with chironomid and

Table 2
Relation between SPT and RAST with chironomid in 420 patients

SPT	RAST class					Positive RAST (%)
	0	1	2	3	4	
≥ 3 +	17	11	7	4	2	59
2 +	14	1	6	0	0	33
1 +	5	2	0	0	0	29
Neg.	344	4	2	1	0	2
Positive SPT (%)	12	78	87	80	100	

shrimp allergens on sera from 110 consecutive patients as well as on sera selected because of positive test results with chironomid, shrimp or crab. The results were expressed as PRU/ml and Phadebas RAST classes 0-4.

RAST inhibition

RAST inhibition experiments were performed essentially as previously described by Yman et al. (20) using 30 µl of allergen extract and buffer. The allergen extracts were incubated with 20 µl of patient serum for 3 h in duplicate at room temperature.

Nitrocellulose immunoblotting experiments

The chironomid and shrimp allergens were separated by SDS-g PAGE and transferred to a nitrocellulose membrane by electrophoretic

blotting. Specific IgE antibodies to the chironomid and shrimp were utilized as probes for detecting the allergens. The bands were localized in the nitrocellulose membrane by means of enzyme-labelled anti-IgE.

The SDS-g PAGE gel was moulded in BIO-RAD, Protein Dual Slab Cell equipment according to operating instructions. Electrophoresis was performed essentially as described by Neville (16), run at 6mA/gel for 18-20 h. Electrophoretic blotting, performed in a Trans-blot cell (BIORAD) and the immunological detection, using enzyme-labelled anti-IgE (Phadezym RAST) and a chromogenic substrate were performed as earlier described by Bengtsson et al. (4) and Yman et al. (21).

The nitrocellulose immunoblotting inhibition experiments were conducted as follows: the allergens studied were transferred to nitrocellulose strips as described above. Before serum incubation of the strips, the serum (0.5 ml) was inhibited with 0.5 ml 1/10 or 1/3 (w/v) of the allergen extract for 3 h before continuing as described above.

Table 3
Relationship between SPT results with shrimp and chironomid in 310 patients

SPT result shrimp	SPT result chironomid			Positive chironomid (%)
	neg.	1-2 +	≥ 3 +	
≥ 3 +	3	2	17	74
1-2 +	8	4	1	8
Neg.	259	6	10	4
Positive shrimp (%)	1	17	56	

Allergens

SPT were performed with the allergens shown in Table 1.

Mite, animal dander and pollen allergens were biologically standardized glycerinated Soluprick extracts from Allergologisk Laboratorium, Copenhagen, Denmark. During 1983-1985 extracts with an activity of 5 HEP were

Skin test	RAST class				
	0	1	2	3	4
↗ 3+	○ ● ● ● ●	○ ○ ■ ■ ■ ■ ●	○ ■ ● ● ●	● ●	● ●
2 +	■ ●	○	○ ■ ●		
1 +	○ ●	○ ■			
Neg.		○	○	○	

- negative provocation test
- doubtful provocation test
- positive provocation test

Fig. 1. Results of SPT, RAST and provocation tests with chironomids in 34 patients.

used and in 1986 extracts with 3 HEP activity. (HEP = Histamine Equivalent Prick). The shrimp, lobster, crab, crayfish, clam and oyster allergens used were 1/20 w/v extracts from the same laboratory. Test results are presented without any correlation for different strength of allergen extracts or histamine controls.

The chironomid, krill, *Mysis*, *Daphnia* and *Artemia* extracts were produced at the Allergy Laboratory, Sahlgren's Hospital, Göteborg, Sweden) (Ass. Prof. Lars Belin) in the following way: Deep frozen suspensions of "larvae from red feather mosquitos" (*Chironomus* species), krill, *Mysis*, *Daphnia* and *Artemia* were purchased as food for aquarium fish at a pet shop. Nothing was known about the media used for cultivation of the larvae, etc. After thawing, the products were disintegrated by freeze-pressing according to Edebo (5). As the chironomid fish food contained an undetermined amount of fluid, no buffer solution was added. Jensen's buffer solution (1.45 g KH₂PO₄, 7.6 g Na₂HPO₄, 4.8 g NaCl in 1 l aqua destilata) in the proportion 1/5 w/v was added to the other fish foods which contained less fluid. The particulate residues were removed by centrifugation and filtration using 0.65 µm and finally 0.22 µm Millipore filters. The fluid was diluted 10 times with 50 % glycerol and 0.5 % phenol in Jensen's buffer before being used as a prick test reagent. These poorly characterized extracts were labeled 1/100 w/v. Extracts used for the provocation test were diluted with Jensen's buffer without glycerol.

The chironomid hemoglobin allergen extract was kindly supplied by Dr. X. Baur, München, GFR (3) as freeze dried extract and used in the SPT in a concentration of 0.1 g/l.

Table 4

Correlations between different allergens according to SPT tests. The table is symmetrical. The upper right shows significance level of correlations according to chi square tests, the lower left shows the values of the corresponding correlation coefficients.

	Chironomid	<i>Daphnia</i>	<i>Mysis</i>	Krill	<i>Artemia</i>	Shrimp	Lobster	Crab	Crayfish	Clam	Oyster
Chironomid		NS	NS	**	***	***	*	NS	*	NS	NS
<i>Daphnia</i>	0.45		**	***	***	***	NS	*	**	NS	*
<i>Mysis</i>	0.51	0.55		***	***	*	NS	NS	NS	NS	*
Krill	0.61	0.33	0.53		***	**	***	***	***	***	***
<i>Artemia</i>	0.70	0.54	0.58	0.66		***	***	***	***	NS	NS
Shrimp	0.63	0.65	0.52	0.63	0.80		***	**	***	NS	NS
Lobster	0.56	0.35	0.40	0.78	0.71	0.75		***	***	NS	*
Crab	0.52	0.33	0.38	0.69	0.68	0.60	0.67		***	NS	NS
Crayfish	0.49	0.44	0.41	0.68	0.72	0.79	0.76	0.62		*	*
Clam	0.32	0.18	0.32	0.60	0.38	0.35	0.37	0.37	0.44		***
Oyster	0.26	0.27	0.50	0.60	0.37	0.36	0.42	0.34	0.47	0.59	

* = P < 0.05; ** = P < 0.01; *** = P < 0.001.

Table 5
Relationship between RAST with shrimp and with chironomid in 212 patients

RAST class shrimp	RAST class chironomid				Positive chironomid (%)
	0	1	2	3-4	
3-4	0	0	4	4	100
2	5	3	19	7	85
1	6	5	7	3	71
0	118	12	17	1	20
Positive					
Shrimp (%)	8	40	64	93	

Preparation of allergen extracts for RAST inhibition and nitrocellulose immunoblotting

For the immunoblotting experiments, extraction was performed at a concentration of 1/10 (w/v) for chironomid and 1/5 (w/v) for shrimp. The material was homogenized in 0.1 M phosphate buffer, pH 7.4, and then agitated at + 4°C for 2 h. After centrifugation at 20,000 rpm for 20 min and filtration, the extracts were desalted and then lyophilised. The extracts used in the inhibition experiments were produced in the same way but the shrimp extract was used at a concentration of 1/3 (w/v). These solutions were neither desalted nor lyophilised but used immediately.

Statistics

The significance of correlations between different allergens was tested with chi-square test on 3 × 3 tables (negative, doubtful and positive tests (Table 3). In these calculations patients with only negative tests to all allergens used were excluded. Correlation coefficients were calculated as a rough estimation of the degree of correlation between different allergens. In these calculations figures from test results plotted in 3 × 3 tables were also used.

RESULTS

Of the 2,368 patients skin tested, 1,118 (50 %) were regarded as atopic, i.e. they had positive

test results with at least one common inhalant allergen. The SPT results with the various allergens are shown in Table 1.

Incidence of positive test results with chironomid extracts

The CHIR extract gave a slight positive (1 + or 2 +) SPT reaction in 164 and a clearly positive (≥ 3 +) reaction in 171 patients, corresponding to positive SPT in 14 % of all patients tested and 26 % of the atopics. Forty-seven patients (4 % of the non-atopics) had an isolated positive reaction with CHIR.

Table 6
RAST-inhibition of sera from 14 patients, positive to both shrimp and chironomid

Patient No.	% inhibition of chironomid RAST with shrimp extract	% inhibition of of shrimp-RAST with chironomid extract
1	73	58
2	94	70
3	92	1
4	73	48
5	99	78
6	69	56
7	93	76
8	32	88
9	29	16
10	96	45
11	80	14
12	89	92
13	29	38
14	33	87
Mean value	71	55

The CHIR extract was used for SPT on 20 healthy persons without any known allergic disease. All of them showed negative test results.

The chironomid hemoglobin extract gave a 1+ or 2+ skin reaction in 10 and a $\geq 3+$ reaction in only three of 410 consecutive patients. All patients showing positive SPT with the hemoglobin extract also had positive reaction with the CHIR extract.

RAST with chironomid and shrimp were performed on the sera of 110 consecutive patients. Positive results ($\geq$ Phadebas class 1) were obtained in four patients (8% of the atopics) with chironomid and in six patients (12% of the atopics) with shrimp.

Relationship between SPT and RAST results with chironomids

When SPT results with chironomid were compared with those of RAST in selected sera (most of which were negative) a positive correlation was found (Table 2). Of the disagreeing cases, a negative RAST in combination with a positive SPT was more common than in the opposite.

Relationship between provocation tests and other test results

Nasal or conjunctival provocation tests were performed in 35 selected patients. As shown in Fig. 1 a rough correlation was found between the results of provocation tests and SPT and RAST, but several patients with positive skin test to chironomid had a negative provocation test.

Relationship between test results with different allergens

A significant positive correlation was found when 320 SPT results with chironomid were compared with those of shrimp (Table 3).

In a number of patients, skin tests were performed with other crustaceans and with clam and oyster. Significant correlations were found

also between chironomid and some of these, as well as with most of these allergens reciprocally (Table 4). Of common inhalant allergens, only mite showed significant correlation with CHIR ($P < 0.05$, $r = 0.34$).

RAST was performed with chironomid, shrimp and crab allergens. Statistically significant correlations were found between RAST results with chironomid and shrimp ($P < 0.001$, $r = 0.49$, chironomid and crab ($P < 0.001$, $r = 0.46$) and between shrimp and crab ($P < 0.001$, $r = 0.67$). The data in Table 5 show the correlation between chironomid and shrimp. All patients with shrimp-RAST $\geq$ class 3 had positive RAST with chironomid.

Inhibition studies

Inhibition studies were performed with shrimp and chironomid on 14 sera which were RAST positive to these two allergens. It was found that the shrimp extract inhibited the anti-CHIR IgE antibodies and vice versa (Table 6). The shrimp extract inhibited the chironomid uptake much more than the chironomid extract inhibited the shrimp uptake. One explanation could be that the chironomid extract was too diluted. Some of the sera were very weakly inhibited by the chironomid extract (patients 3, 9 and 11, Table 6).

Fig. 2 shows immunoblotting inhibition on two sera (patients 11 and 13). These results correlate well with the RAST inhibition results (Table 8). They indicate that there is some cross-reactivity between chironomid and shrimp.

DISCUSSION

The study showed that 26% of our atopics and 4% of non-atopics with asthma and/or rhinitis were sensitized against CHIR. We have no explanation for this high prevalence. As far as we know, they have not been exposed earlier to chironomid used as fish food. However, they have certainly been exposed to other chironomid species, since these insects are widespread in our country. Another study from the Dept.

of Medicine, Halmstad, indicates that the prevalence of positive SPT to CHIR is higher among farmers than among urban patients (5). This might mean that people in rural areas are more exposed to chironomid or related allergens than people in urban areas.

It should be kept in mind that our CHIR extract, produced from "larvae from red feather mosquitoes" used as fish food, is not a standardized allergen extract and might contain various impurities, e.g. other animals, bacteria, fungi or plant debris. The possibility that the extract contains histamine cannot be ruled out. Thus, some of the positive test results could be non-specific, without relation to sensitization to chironomid allergens.

In Europe, allergy to chironomid has so far been reported only in patients using larval chi-

ronomids as fish food (7). In other parts of the world, however, chironomid (midges or larvae) has been reported to be a common cause of atopic disease: in Sudan (8, 12), Japan (9, 10, 13) and USA (11).

Baur et al. (2, 3), who reported hypersensitivity to *Chironomus riparius* in patients who were in contact with chironomid larvae used as fish food, identified sensitization to chironomid hemoglobin. Antigenic determinants could be localized to certain polypeptide sequences of the hemoglobins.

Tee et al. (19) have shown a cross-reactivity between various species of chironomid midges. However, in another study (15) it was found that the allergenicities of chironomid midges change during the metamorphosis from larva to adult and are not common among all species. A



Fig. 2. Immunoblotting inhibition of patient sera Nos. 11 and 13. Before serum incubation of the strips, the serum was inhibited with the allergen extract for 3 h.

study, to date presented only in parts, shows that concomitant sensitization to chironomid and to a common biting mosquito, *Aedes communis*, is common (6). This result indicates that cross-reactivity also might exist between chironomid and mosquitoes belonging to a different family.

We found a high degree of cross-sensitization among CHIR, various crustaceans, clam, oyster and house dust mite. The existence of significant correlations between test results with two allergen extracts can mean that partial immunological identity exists between the extracts, but obviously does not prove such an identity. The correlation can depend on concomitant sensitization to two allergens, both occurring in the patients' environments. For two of the allergens, chironomid and shrimp, however, our study clearly indicates true cross-reactivity, since the two extracts inhibited each other in inhibition experiments.

Cross-allergy among crustaceans has previously been described by Lehrer (14). However, cross-reactions have not earlier been reported between chironomid, crustaceans, molluscs and mites, but have been reported between cockroach antigens/allergens and several species of crustacea (17).

To conclude, the high incidence of positive SPT with CHIR indicates that chironomids might contain allergens of clinical importance in Sweden. The clinical relevance of the test results, however, has to be documented by provocation tests in a larger number of patients. The cross-sensitization which was found between chironomid and crustaceans indicates that a relationship between inhalant allergy and ingestion allergy may occur.

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