

Extracts of larval and adult midges of *Tokunagayusurika akamusi* Tokunaga (Diptera: Chironomidae) in asthmatic patients of the metropolitan area of Tokyo

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Prick test and radioallergosorbent test to extracts of larval and adult midges, *Tokunagayusurika akamusi* (Tokunaga) were done in randomly selected asthmatic patients in the metropolitan area of Tokyo, Japan. Thirty-eight percent of 303 asthmatic patients were prick-test positive to either larval or adult midges, and 32.4% of 105 asthmatic patients were positive to either extracts by RAST, while only one out of 20 normal individuals was positive to RAST. These positive rates in the asthmatic patients were the second to the positive rates of prick test and RAST using mite antigen. Antigens from midges seem to be the second important allergens in the metropolitan area of Tokyo.

ABBREVIATIONS

AM	= adult midges
¹²⁵ I-anti IgE	= ¹²⁵ I-anti-human IgE
PBS	= phosphate buffered saline
PRU	= pharmacial RAST unit

INTRODUCTION

In Tokyo, the house dust mites are the main allergens in perennial asthma and rhinitis. In seasonal allergy, Japanese cedar in early spring and ragweed in late summer and autumn are other important aller-

gens. Allergic patients sensitive to various fungi are also found.

Various species of chironomid midges (non-biting midges) have been recorded in Tokyo. These midges swarm and die. The dead bodies accumulate on the ground, break up and might be inhaled; however, there has been no report concerning hypersensitivity to these insects in this area. Hypersensitivity to chironomid midges was extensively studied in the northern Sudan, and radioallergosorbent test (RAST) was carried out by Gad El Rab in patients allergic to "green nimitti" midge, *Cladotanytarsus lewisi*.² These studies were done in the district along the White, Blue, and Main Nile rivers in Africa.

The purpose of our study is to examine IgE antibodies to chironomid midges in asthmatic patients of the metropolitan areas of Tokyo.

MATERIALS AND METHODS

Extract of Adult Midge

Adult midges of *Tokunagayusurika akamusi* were collected in early November on the shore of the Lake Kasumigaura. They were lyophilized and stored at -20 °C. The ly-

ophilized midges were homogenized in an ice cold bath. Defatting was done in the following manner. Ether was added to the homogenized material at a ratio of 30 mL of ether to 1 g of the lyophilized midges, and the homogenizing was continued. The whole material was centrifuged at 15,000 rpm for 30 minutes in 4 °C. The supernatant was discarded and the sediment was lyophilized again. Such procedures of defatting was repeated, and the defatted material was added with PBS of pH 7.4 at a ratio of 20 mL of PBS to 1 g of the original lyophilized midges, stirred with a magnetic stirrer for 48 hours at 4 °C and centrifuged at 15,000 rpm for 30 minutes. The supernatant was dialyzed against PBS for 24 hours at 4 °C with several changes of the buffer, and centrifuged at 15,000 rpm for 30 minutes. The supernatant was lyophilized and stored at -20 °C until use. The lyophilized material contained 52.1% protein when examined by Lowry's method.³

Extract of Larvae

Larvae of *T. akamusi* were collected from mud in the Lake Kasumigaura. The procedure for preparing

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extract was the same as that for the AM-extract. The material contained 80.0% protein.

Extract for Prick Test

The lyophilized larval or AM extract was dissolved in glycerinated Coca's solution (NaCl 0.5 g, NaHCO₃ 0.25 g, glycerin 100 mL, adjusted to a total volume of 200 mL with distilled water) at a ratio of 20 mL solution to 1 g of the lyophilized midges and filtrated through a millipore filter (Millex-HA, 0.45 µm, Filter Unit, Millipore Corporation, Bedford, MA, USA). The filtered material was used as a 1:20 extract. A 1:100 extract was prepared similarly as above using 100 mL glycerinated Coca's solution in place of 20 mL. The protein concentration of 1:20 AM extract was 8.8 mg/mL and that of the larval extract was 16.3 mg/mL.

Skin test

Prick tests using 1:20 and 1:100 larval or AM extracts were done in 303 adult (165 female, 138 male) asthmatic patients in the Allergy Clinic, Tokyo University Hospital and in the Allergy Clinic, Teikyo University Hospital, both in Tokyo. The range of age was 17 to 76 years, and 92% of the patients were 20 to 55 years old. Four percent were over 55, and 4% were less than 20 years old. In the same patients, other 30 different allergens including house-dust mite (*Dermatophagoides farinae*), ragweed, Japanese cedar, other pollens, food allergens, and fungi were examined for prick test. Evaluation of the skin reaction was done according to the criteria described by Vanselow.⁴ Prick tests with larval and AM extracts were also done in 20 normal adult individuals who had never suffered from asthma, allergic rhinitis, atopic dermatitis, or drug allergy.

Patient Sera

One hundred and five sera were randomly collected from asthmatic patients of the two allergy clinics whose skin reactions to the midge were known. Blood samples were obtained by venous puncture and

maintained at room temperature for two to three hours. Sera were separated and stored at -20 °C until use. Another 15 sera were collected from asthmatic patients who were prick-test positive to larval and/or AM extracts. Twenty normal sera were collected from the same normal individuals as described above.

Radioallergosorbent Test (RAST)

RAST was done according to the modified method described by Ceska et al⁵ with some modifications. Paper discs, 5-mm diameter, were activated with CNBr. Five hundred pieces of activated paper disc were suspended in 10 mL of 10 mg protein/mL solution of the larval or AM extract in a 50-mL test tube. The tube was rotated end-over-end overnight at 4 °C. Then the paper discs were washed and treated as described by Ceska. A paper disc was suspended in 0.05 mL of patient serum and incubated for 24 hours at 4 °C. The paper disc was washed with RAST incubation buffer and 0.05 mL of ¹²⁵I-anti IgE was added and incubated for 24 hours at 4 °C. The ¹²⁵I-anti IgE was purchased from Pharmacia Chemicals, Co. Ltd, Uppsala, Sweden and used as instructed by the company. After the paper disc was washed, the radioactivity was counted by an automatic gamma counter.

Rast for house-dust mite (*Dermatophagoides farinae*) was done by the use of RAST kit purchased from Pharmacia Chemicals, Uppsala, Sweden. A reference serum containing IgE antibodies to birch pollen was also purchased from the company. All counts of radioactiv-

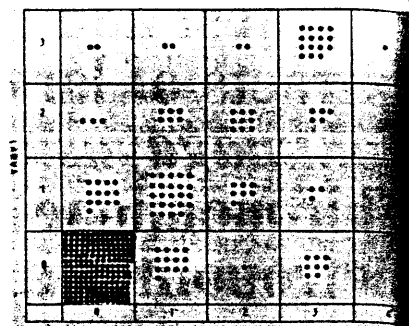


Figure 1. Correlation between skin reaction to extracts of larvae and adult midges. Grades of reactions to prick test are those described by Vanselow.⁴ The correlation is low ($y = 0.329x + 1.418$, $r = 0.302$, $F = 11.3$, $P < .01$). Patients negative (0) to both antigens were not included in the calculation.

ity in RAST for the midge or the mite were converted to PRU from a standard curve obtained by the use of the reference serum and the reference discs. Rast score was determined as 0 to 4 from PRU as follows: PRU < 0.35: RAST score = 0; 0.35 to 0.70: 1; 0.70 to 3.5: 2; 3.5 to 17.5: 3; 17.5 < : 4. RAST scores of 2 and over were defined as positive.

RESULTS

Prick Test with Midge Allergens and Other 30 Allergens

In 303 patients with asthma, the rate of positive reaction to chironomid midge, mite, ragweed, and Japanese cedar are shown in Table 1. One hundred and fifteen patients (38.0%) were positive to either of the 1:20 larval or AM extract. Out of the 115 patients, 75 were positive to both larval and AM extracts, 22 were positive to only AM, and 18 were positive to larval extract only.

Table 1. Skin Reaction to the Larval and Adult Midges of *T. akamusi*, Mite (*D. farinae*), Ragweed, and Japanese Cedar in 303 Asthmatic Patients*

		<i>T. akamusi</i>	Mite	Ragweed	Japanese Cedar
Positive to both larval and adult midge	75	115 (100%)	91 (79.1%)	8 (6.9%)	21 (18.3%)
Positive to only adult midge	22				
Positive to only larva	18				
Negative to both larval and adult midge	188 (100%)		93 (49.5%)	16 (8.5%)	41 (21.8%)
Total	303 (100%)	115 (38.0%)	184 (60.7%)	24 (7.9%)	62 (20.5%)

* Rate of mite-positive patients is higher than in midge-positive patients and midge-negative patients. The rate of ragweed-positive or Japanese cedar-positive patients is not different in both groups.

As shown in Figure 1, the correlation between skin reactions to larval and AM extracts was low ($r = .30$, $P < .001$) when calculated without completely negative patients. Two out of the 115 patients (1.7%) were positive only to the midge (AM and larval) and negative to other 30 allergens. When 1:100 extracts were used in prick test, positive patients decreased from 97 to 53 with AM, and from 93 to 48 with the larval extract.

On the other hand, 184 out of 303 patients (60.7%) showed positive reaction to the mite extract, 24 (7.9%) to ragweed, and 62 (20.5%) to Japanese cedar. The positive rate to the other 27 common allergens was lower than that to Japanese cedar. Fifty-nine out of 303 patients (19.5%) were negative to all 30 allergens and midges. Of 115 midge-positive patients, 79.1% were also positive to mite, whereas 49.5% of 188 midge-negative patients were positive to mite. The difference was significant in χ^2 test ($P < .001$). The positive rate of skin reaction to ragweed or Japanese cedar was not different between midge-positive and negative groups.

The correlation between the strength of skin reaction to AM extract and that to mite extract was low ($r = 0.315$, $P < 0.01$). A similar correlation was observed between skin reaction to larval extract and that to mite extract.

In the Kasumigaura Hospital near the Lake Kasumigaura, skin test to AM extract was positive in two out of four asthmatic patients, and two out of ten normal individuals. The results of prick test to larval extract was completely consistent with the results of the prick test to AM extract.

RAST

RAST was done in 105 randomly selected asthmatic patients and 20 normal volunteers. Relation between skin reactions to 1:20 extract and RAST is shown in Table 2. Out of 105 patients, 41 patients were prick test-positive to AM. RAST to

AM was positive in 26 patients out of the 41 prick test-positive patients, while RAST was positive in only five patients out of 64 prick test-negative patients. A total of 31 out of 105 asthmatic patients (29.5%) were positive to AM RAST.

In tests to larvae, prick test was positive in 33 out of 105 patients and RAST was positive in 12 out of the 33 prick test-positive patients. Only seven out of 72 prick-test negative patients were positive in RAST. A total of 19 out of 105 asthmatic patients (18.1%) were positive to larval RAST. A total of 34 out of 105 patients (32.4%) were positive to either of AM or larval RAST.

The relation between skin reaction to 1:100 extract and RAST is shown in Table 3. The rate of positive reactions to the prick test were close to that of RAST, although patients who were skin test-negative but RAST-positive somewhat increased.

Details of the results in RAST are shown in Table 4. When 1:20 extract was used, skin test was more sensitive than RAST; however, exceptions were observed as follows. Two patients who were skin test-positive to only AM extract were positive to larval RAST. In the group of 62 patients who were negative to both allergens, four were positive to both RAST, one was

Table 2. Relation Between Results of Prick Test with 1:20 Extracts and RAST

SKIN REACTION TO ADULT MIDGE

RAST TO ADULT MIDGE

	+	-	TOTAL
+	26 [*]	5	31 (29.5%)
-	15	59 ^{**}	74
TOTAL	41 (39.0%)	64	105 (100%)

RATE OF COINCIDENCE

$$= (* + **) / 105$$

$$= (26 + 59) / 105 = 0.81$$

SKIN REACTION TO LARVA

RAST TO LARVA

	+	-	TOTAL
+	12 [*]	7	19 (18.1%)
-	21	65 ^{**}	86
TOTAL	33 (31.4%)	72	105 (100%)

RATE OF COINCIDENCE

$$= (* + **) / 105$$

$$= (12 + 65) / 105 = 0.73$$

Table 3. Relation Between Results of Prick Test with 1:100 Extract and RAST

SKIN REACTION TO ADULT MIDGE

		+	-	TOTAL
RAST TO ADULT MIDGE	+	25 [*]	7	32 (30.5%)
	-	1	72 ^{**}	73
	TOTAL	26 (24.8%)	79	105 (100%)

RATE OF COINCIDENCE

$$= (* + **) / 105$$

$$= (25 + 72) / 105 = 0.92$$

SKIN REACTION TO LARVA

		+	-	TOTAL
RAST TO LARVA	+	12 [*]	7	19 (18.1%)
	-	8	78 ^{**}	86
	TOTAL	20 (19.0%)	85	105 (100%)

RATE OF COINCIDENCE

$$= (* + **) / 105$$

$$= (12 + 78) / 105 = 0.86$$

Table 4. Prick Test and RAST to Larval and Adult Midges in 105 Asthmatic Patients Using 1:20 and 1:100 Extracts*

Skin Reaction to Prick Test	To Adult Midge						To Larva					
	Prick Test		RAST				Prick Test		RAST			
	1:20	1:100	≥2	1	0		1:20	1:100	≥2	1	0	
Positive to both larval and adult midge (31)	31	22	21	7	3		31	20	12	9	10	
Positive to only adult midge (10)	10	4	6	3	1				2†	2	6	
Positive to only larval (2)						2 ←	2					2
Negative to both larva and adult midge (62)			5†	9	48				5†	2	55	
			62						62			
Total (105)	41	26	32	19	54		33	20	19	15	71	
			105						105			

* Numbers are cases. L† indicates results of RAST in the individuals who were prick-test positive to 1:20 extracts. ← and → means the same patient. Prick test is more sensitive than RAST when 1:20 extract was used.

† Some discrepancies between skin test and RAST.

positive only to AM RAST and one was positive only to larval RAST. A total of 17 out of 19 patients positive to larval RAST were included in 32 patients positive to AM RAST. Only two patients were positive to only larval RAST. Twenty-one patients who were skin test-positive to both 1:100 AM and larval extract and four patients who were skin test-positive only to 1:100 extract AM were also positive to AM RAST.

Other than 105 patients described above, 15 were selected from asthmatic patients who showed positive reaction to 1:20 midge extract. This group contained patients who showed relatively strong reactions (+++) to prick test with 1:20 AM or/and larval extract, whereas the group of 105 patients described above contained only ten who showed strong reactions (+++) to the same extracts. Eleven patients were prick test-positive to both larval and AM extract, three patients were positive to only AM extract, and one patient was positive to only larval extract.

Out of 15 patients, nine (60%) were positive to AM RAST and seven (46.7%) were positive to larval RAST. A discrepancy between prick test and RAST was found in two patients. One patient was skin-test

positive to only larval, but was positive to only AM RAST. One was skin-test positive to only AM, but was positive to both RAST. Nine of all 15 patients were positive to either of AM or larval RAST. The positive rate of RAST in this group was higher than in the group of 105 patients already described ($P < .05$, χ^2 test).

In 20 normal individuals, three (15%) were prick test-positive to both 1:20 larval and AM extracts in grade 1 but negative to 1:100 extracts. Two out of the three individuals were positive to both larval and AM RAST in score two, and one was positive to both larval and AM

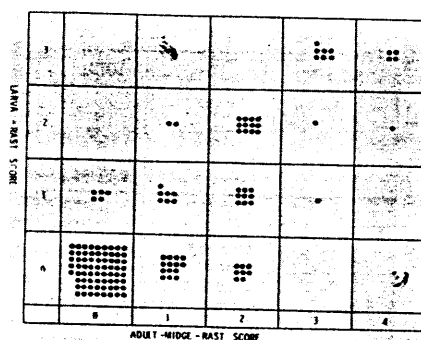


Figure 2. Correlation between adult midge RAST scores and larva-RAST scores. The correlation is highly significant ($y = 0.822x - 0.201$, $r = 0.697$, $F = 65.2$, $P < 0.001$). Patients negative (0) to both antigens were not included in the calculation.

RAST in score 1. Prick test-negative individuals in the normal group did not show positive reaction to RAST.

Correlation Between Strength of Skin Reaction and RAST

Correlation between reactions to prick test and RAST was examined. The correlation was low between the strength of prick-test reactions and that of RAST scores when the AM extract was used as antigen ($r = 0.39$, $P < .001$), and the correlation was very low when the larval extract was used as antigen ($r = 0.08$, N.S.).

Correlation Between AM and Larval RAST

Correlation between the result of RAST using AM extract and that of RAST using larval extract is shown in Figure 2. A high correlation was observed between RAST scores for the both antigens ($r = 0.70$, $P < .001$). RAST scores of larval RAST, however, were lower than those of AM RAST.

Correlation of RAST Using AM and Mite Extracts from *D. farinae*

In a randomly selected group of 105 asthmatic patients, 52 sera were available for RAST using *D. farinae* as the antigen. Thirty-four sera were positive to RAST; therefore, at least 34 out of 105 patients were positive to mite-RAST. A total of 67 sera

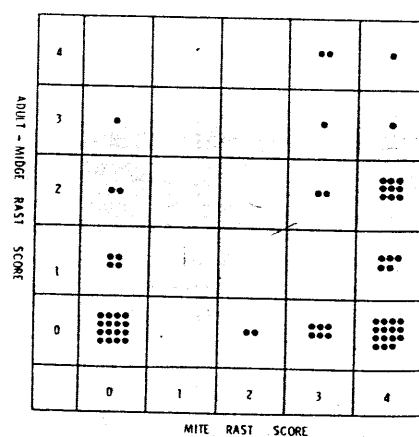


Figure 3. Correlation between adult midge RAST scores and mite RAST scores. The correlation coefficient is very low ($y = -0.100x + 3.226$, $r = 0.090$, $F = 0.4$ NS). Patients negative (0) to both antigens were not included in the calculation.

were available for both RAST to the mite extract and to the AM or larval extract. These 67 sera consisted of 52 sera described above and 15 sera from asthmatic patients who were prick test-positive to AM and/or larval extract, as described in patient sera in "Materials and Methods."

The correlation between AM RAST scores and mite-RAST scores was very low as shown in Figure 3 ($r = .09$, N.S.). A similar correlation was found between the results of mite-RAST and larval RAST.

DISCUSSION

In Japan, 169 species of chironomid midges had been recorded up to 1977.⁶ In Lake Kasumigaura, 100 km north of Tokyo, the emergence of adult midges were observed four times in a year: March, May to June, September, and October to November. A different species can be seen each time. AM of *T. akamushi* emerge in late October to early December.

The Tama river, which flows in the suburbs of Tokyo, contains several species of chironomid midges.⁷ The distribution of species depends on pollution of the water. *Chironomus yoshimatsui* Martin et Sublette have been observed in the Kanda River, which is located in Central Tokyo. This species emerges in all seasons except winter. All these midges swarm and die. The dead bodies break up and might be inhaled.

There has been no report about hypersensitivity to chironomid midges in a big city such as Tokyo. In this study, it was unexpectedly shown that approximately 40% of asthmatic patients were prick test-positive to either of the extracts of larval and AM of *T. akamushi*. In RAST, 32.4% of asthmatic patients were positive to either of the larval RAST or the AM RAST. The rate of positive RAST is higher than that of positive skin-reaction to other allergens such as Japanese cedar (20.5%); therefore, the midges seem to be important allergens next to

mites in asthmatic patients in Tokyo. At this time only the extract of *T. akamushi* was available for the experiment. But the high rate of positive skin tests and RAST in asthmatic patients in Tokyo indicates that there is some allergenic similarity between *T. akamushi* and midges in Tokyo such as *C. yoshimatsui*.

Approximately 60% of asthmatic patients were prick test-positive to mite (*D. farinae*) and approximately 80% of midge-positive patients were also positive to mite. The correlation coefficient between the results of mite-RAST and that using AM or larval extract was low. This means that the main allergenicity of the midges is different from that of mites; however, some common antigenicity between mites and midges cannot be completely ruled out.

Coincidence between the results of skin test and those of RAST using midges was high; however, strength of skin reaction did not correlate with RAST score. The reason is not clear. It was reported by Miyamoto et al¹¹ that IgE antibody levels correlate well with thresholds of skin reaction to mite. Evaluation of skin reaction by Vanselow's criteria may be different from evaluation by threshold determination.

A highly significant correlation was observed between larval RAST score and AM RAST score. From these results, there seems to exist common antigenic components between larvae and AM. But in 115 prick test-positive individuals, there were 22 individuals who were positive to only AM and 18 individuals who were positive only to larvae. These results suggest that there might also exist different antigens between larvae and AM. Strength of skin reaction to AM extract did not correlate with that to larval extract. It might also reflect existence of different antigens between them, or might reflect some problem in classification of skin reaction as mentioned above. Details of the antigenic relationship between larvae

and AM, and also between the two antigens and the mites will be published in another paper. It should be also investigated how IgE antibodies to the midges are related to asthmatic attacks. In a preliminary experiment, a patient who was strongly positive to RAST, had an asthma attack following provocation with AM extract. Many trials, however, are needed for confirmation. It should also be investigated whether there is a difference in the rate of positive skin-tests between Tokyo and the area around Lake Kasumigaura, where tremendous numbers of the midges emerge every year.

It was reported by Baur⁸ that some of the individuals handling dried larvae of chironomid midges as fish food, and workers engaged in the production of fish food became allergic to the larvae. The author fractionated an extract from larvae of *Chironomus thummi thummi* and demonstrated that hemoglobins of the larvae were responsible for the allergy, and demonstrated also that there existed immunologic cross reactivity between larvae of different species of Chironomidae.⁹ The author also succeeded in locating the antigenic determinant of one of these hemoglobins, with a defined amino acid sequence. It was reported recently by Tee et al¹⁰ that the major allergen of green nimiti midges *Cladotanytarsus lewisi* was associated with molecules of approximately 17,000 daltons. Another allergen of weaker allergenicity of molecular weight of 66,000 daltons was also found. Their preliminary observation indicated that there was variable inhibition of RAST to the *C. lewisi* by hemoglobin from *Chironomus thummi thummi*.

A similar analysis of allergens in larvae and adult midge of *T. akamushi* and investigation on their allergenic relationship to midges of other species should be carried out. If there are common allergenicities between many species of chiron-

omid midges that emerge in different seasons, allergy to these insects may be a very important problem.

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