

Evidence for haemoglobins as common allergenic determinants in IgE-mediated hypersensitivity to chironomids (non-biting midges)

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Summary

Chironomids (non-biting midges) are known to cause IgE-mediated hypersensitivity in man. This study compares the cross-reactivity between the chironomid midge *Cladotanytarsus lewisi* ('green nimitti'), a widespread cause of allergy in the Sudan and *Chironomus riparius* (= *thummi*, CTT) where larvae are used as pet fish food and where haemoglobins were previously shown to be major allergens. As with *C. riparius*, immature forms of *C. lewisi* also contain allergenic material since skin test responses to larval, pupal and adult extracts were obtained in Sudanese individuals. Crossed radioimmuno-electrophoresis of the *C. lewisi* larval and pupal extracts indicate that they contain a higher proportion of the allergenic fractions than adults. Further evidence of common allergen determinants between *C. lewisi* and *C. riparius* were obtained by the demonstration of positive skin-prick tests, in Sudanese patients, to extracts of larval, adult and isolated haemoglobin extracts of *C. riparius*. Cross-reactivity between *C. lewisi* and *C. riparius* was also demonstrable by RAST inhibition studies. A dose-dependent inhibition was observed using both the *C. lewisi* adult midge RAST and the *C. riparius* haemoglobin RAST, the two respective antigens, and sera from individuals hypersensitive to either *C. lewisi* or *C. riparius*. Due to the immunological cross-reactivity found between these distantly related species, we conclude that chironomids should be seen as significant environmental and occupational allergens.

Introduction

Hypersensitivity to the 'green nimitti' midge, *Cladotanytarsus lewisi* (Diptera: Chironomidae), is an important and widespread cause of bronchial asthma and allergic rhinitis in the Sudan [1-4]. The problem of midge nuisance has been recognized since the 1920s and is probably partially the result of the production of increased breeding areas by the building of dams that have produced lake-like conditions on parts of the White, Blue and main Nile rivers. Symptoms are considerably higher in the winter months when the midges emerge in large numbers. A positive relationship has been found between the severity of patients' symptoms and big emergences of midges as measured in light-trap catches [5].

There is now substantial evidence that haemoglobins of certain larval chironomids (*Chironomus riparius* = *Chironomus thummi*) are important occupational allergens [6, 7]. Freeze-dried larvae of this species, widely used in Europe as pet fish food, produce IgE-associated allergic symptoms in exposed individuals. By RAST inhibition experiments Baur [6] and Baur *et al.* [8] provided evidence that *C. lewisi* and *C. riparius* shared common allergens since Sudanese patients had specific IgE antibodies to extracts of larvae, adults, and isolated haemoglobin of *C. riparius* (as CTT).

The present paper extends the evidence of cross-reactivity between allergens of *C. lewisi* and *C. riparius* by RAST inhibition; *in vivo* confirmation was obtained by positive skin-prick tests in *C. lewisi* hypersensitive patients using extracts of larvae, adults and isolated haemoglobin of *C. riparius*.

Since immunological cross-reactivities exist between different chironomid species in different developmental stages, the clinician should be aware of the potential worldwide nature of this environmental allergen, especially in water-rich regions where problems of nuisance chironomids are steadily increasing. Examples of such hypersensitivity has been found in lakes in Central Florida (Cranston, unpublished observations) and Wisconsin [9].

Materials and Methods

Collection of Chironomidae

Adult *C. lewisi* midges were collected by light-trap in the Sudan, identified as comprising over 99% *C. lewisi* and dried at 26°C.

Larvae and pupae of Nilotic Chironomidae were collected by aquatic drift nets partially immersed in the White Nile at Khartoum. The samples comprising up to 75% *C. lewisi* were dried on filter paper at 26°C.

Larvae and adults of *C. riparius* were collected from cultures maintained by Dr P. Credland and dried as above. A commercially available tropical fish food containing predominantly *C. riparius* larvae, killed by ultraviolet radiation and packed by 'Gamma Foods' was purchased from an aquarist's shop and dried as above.

Preparation of extracts

The chironomids were defatted in ether and 48 hr aqueous extracts made as described previously [10]. The *C. riparius* (CTT) total haemoglobin fraction was prepared by ammonium sulphate precipitation of a homogenised larval extract [8].

Skin-prick testing

Sudanese patients hypersensitive to *C. lewisi* were chosen for two series of tests.

(i) To find if patients were also hypersensitive to immature stages of *C. lewisi*, sixteen patients hypersensitive to *C. lewisi* were tested with unfractionated extracts of *C. lewisi* adults, pupae and larvae.

(ii) To estimate the evidence for common allergen determinants between *C. lewisi* and *C. riparius*, the response in twenty-six patients with unfractionated adult *C. lewisi* extract was compared with the response to three unfractionated *C. riparius* adult and larval extracts and with *C. riparius* total haemoglobin fraction. The *C. riparius* laboratory larval and adult extracts were chosen in order to determine whether *C. lewisi* hypersensitive patients were also hypersensitive to *C. riparius* antigens. The commercial larval antigen was included to compare it with the laboratory extract and the partly purified haemoglobin, since it was the proven major allergen in *C. riparius* hypersensitivity.

All skin test solutions were prepared in 50% Coca's solution and 50% glycerol at 1 mg/ml concentration, except the *C. riparius* total haemoglobin, which was prepared at 0.1 mg/ml, and passed through a 0.45 μ m filter before use. Histamine (dihydrochloride) at 1 mg/ml was used as a positive control solution and the diluent as a negative control. To compensate for the varying degrees of sensitivity of the arm, the tests were performed in duplicate and positioned in reverse order on the second arm. The weal area (maximum $\times$ minimum diameter) of resultant weals was measured after 15 min and any reaction provoked by the negative control deducted from that caused by the test solution.

Human sera

Sudanese patients with bronchial asthma and/or allergic rhinitis and a positive skin-prick test to *C. lewisi* were bled for antibody studies. Similarly, serum was obtained from German patients with allergic symptoms and a positive skin-prick test to *C. riparius* following their occupational exposure to larvae of chironomids.

RAST/RAST inhibition

C. lewisi-specific IgE antibodies in human sera were measured using the radioallergo-sorbent test (RAST) described previously [10], where lyophilized antigen was coupled to CNBr activated Sepharose 4B (10 mg/gm Sepharose). RAST inhibition studies were performed by 16 hr pre-incubation of positive sera from hypersensitized patients with various concentrations of unfractionated *C. lewisi* extract and CTT total haemoglobin extract and comparison made in the RAST assay with the same sera unabsorbed.

C. riparius-specific IgE antibodies were measured in a RAST where *C. riparius* haemoglobin was coupled to activated paper discs [8]. RAST inhibition tests were performed after positive sera had been pre-incubated for 12 hr with Chironomid extracts as for *C. lewisi* above.

Crossed radioimmuno-electrophoresis (CRIE)

These tests were performed following the method of Lowenstein [11] and are detailed in Tee *et al.* [10]. An autoradiograph of the known main *C. lewisi* allergen was compared with autoradiographs of larval and pupal extracts.

Rabbit antisera

Antisera were prepared to an unfractionated adult *C. lewisi* midge extract in New Zealand white rabbits as described previously [10]. Antisera were similarly prepared to

material of approximately 25 000 dalton molecular weight obtained by Sephacryl S200 fractionation of adult *C. lewisi* extract. This was material collected between the two main allergen peaks of 32 000 and 17 000 daltons molecular weight, as indicated by RAST inhibition and rocket radioimmuno-electrophoresis [12].

5 mg dry weight of fraction material (total protein content 2.25 mg) in Freund's complete adjuvant was injected in the first immunization. This was followed by 1.5 mg of fraction material (protein 0.675 mg) in incomplete Freund's adjuvant after 5 weeks and the rabbits were boosted with this amount of fraction material in saline at intervals thereafter.

Results

Evidence that immature stages of C. lewisi are allergenic

(i) Comparable skin-prick test responses were found in sixteen Sudanese patients to adult, larval and pupal extracts of *C. lewisi* (Fig. 1). The results are plotted as the ratio of weal 'areas' of the antigen to those of histamine.

(ii) Crossed radioimmuno-electrophoresis of larval and pupal extracts of *C. lewisi*

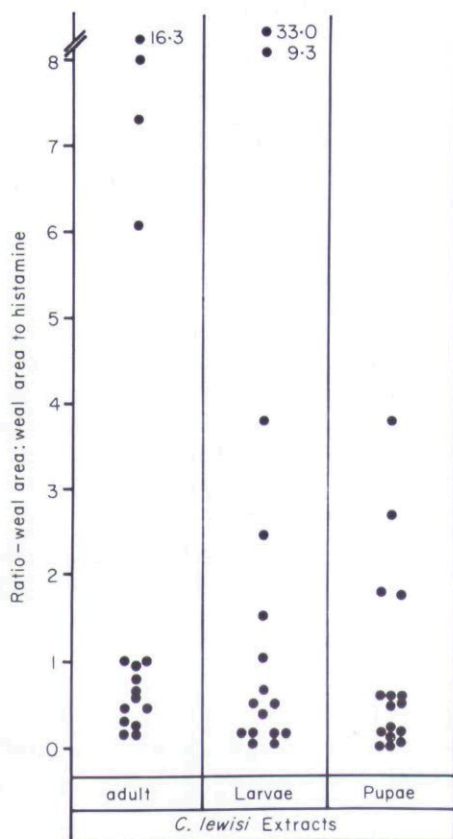


Fig. 1. Ratio of skin-prick test weal responses of adult, larval and pupal extracts of *C. lewisi* to histamine dihydrochloride in sixteen individuals.

gave similar autoradiographs to that of known allergenic fractions from Sephacryl S200 chromatography of the adult midge extract (Fig. 2). Pooled sera from *C. lewisi* hypersensitive patients produced intense diffuse radiostaining peaks of equal mobility which did not appear on the crossed immunoelectrophoresis plates stained for protein.

Evidence of common allergen determinants between C. lewisi and C. riparius

(i) *By means of skin-prick testing.* In the group of twenty-six individuals the skin-prick test responses to four extracts of *C. riparius* compared to *C. lewisi* adult extract in relation to histamine are shown in Fig. 3. The weal areas obtained were standardized by conversion to a proportion of the weal areas produced to unfractionated *C. lewisi* adult extract. When a Fisher-Yates analysis of variance test was applied to these data no statistically significant difference was found between the responses to the *C. riparius* extracts, despite the haemoglobin being a tenth of the strength.

(ii) *By means of RAST inhibition.* Dose response curves in the *C. lewisi* adult midge RAST and the *C. riparius* total haemoglobin RAST inhibition assays were obtained with the two respective antigens, both in Sudanese and German hypersensitive sera.

In the *C. lewisi* RAST there was greater inhibition of the German sera by a comparable concentration of *C. lewisi* than the Sudanese sera (37% and 19% respectively at 0.1 mg/ml), (Table 1). The German sera were greatly inhibited by the *C.*

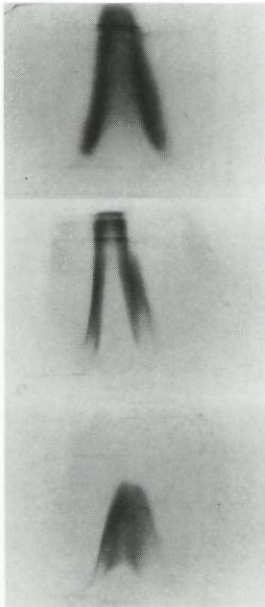


Fig. 2. Crossed radioimmuno-electrophoresis. Top frame: Autoradiograph of fraction of approximate molecular weight 25000 daltons of *C. lewisi* extract obtained by Sephacryl S200 gel filtration. In the first dimension 0.5 μ l of S200 fraction (20 mg/ml) was electrophoresed. In the second dimension, the separated antigen was electrophoresed into a 'window' containing 150 μ l of rabbit antiserum raised against this S200 fraction and 2.2 ml agarose. Middle frame: Autoradiograph of whole *C. lewisi* larval extract. One μ l of the larval extract (20 mg/ml) was electrophoresed in the first dimension. In the second dimension the 'window' contained 150 μ l of rabbit anti-unfractionated *C. lewisi* extract antiserum and 2.2 ml agarose. Bottom frame: Autoradiograph of whole *C. lewisi* pupal extract. The conditions were as for the middle frame. The overlay for all three plates contained 4 ml of pooled human serum 1:10 from patients hypersensitive to *C. lewisi*.

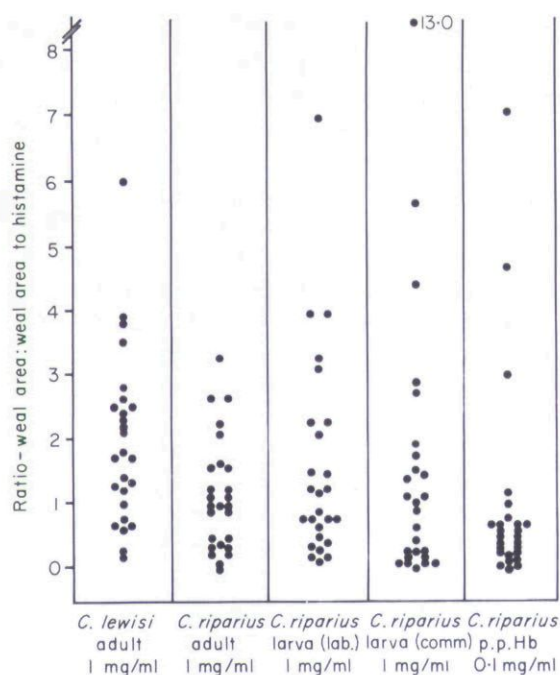


Fig. 3. Ratio of skin-prick test weal responses of extracts of *C. riparius* and whole adult *C. lewisi* to histamine dihydrochloride in twenty-six individuals hypersensitive to *C. lewisi*. Abbreviations: lab—laboratory culture; comm.—commercial 'Gamma Foods'; p.p.Hb—partially purified total haemoglobin. Haemoglobin is 1/10 dilution of other extracts.

Table 1. RAST inhibition in sera of patients hypersensitive to *C. lewisi* (Sudanese) and *C. riparius* (German)

Sera	(No.)	Inhibitor concentration (mg/ml)	Mean % inhibition	
			<i>C. riparius</i> Hb	<i>C. lewisi</i> midge
<i>C. lewisi</i> adult midge RAST:				
<i>C. lewisi</i> hypersensitive	(12)	0.1	10.5	19.4
	(12)	1.0	19.8	40.9
	(9)	10.0	ND	63.5
<i>C. riparius</i> hypersensitive	(3)	0.1	78.1	36.6
	(4)	1.0	80.7	59.5
	(2)	10.0	ND	70.3
<i>C. riparius</i> haemoglobin RAST:				
<i>C. lewisi</i> hypersensitive	(5)	0.001	23.8	0.7
	(5)	0.01	64.8	2.9
	(5)	0.1	84.2	64.0
<i>C. riparius</i> hypersensitive	(5)	0.001	34.6	7.0
	(5)	0.01	72.8	18.4
	(5)	0.1	87.4	21.2

ND=Not done.

riparius Hb antigen (78% at 0.1 mg/ml), whereas the Sudanese sera were only inhibited by 10.5% at this concentration.

In the *C. riparius* haemoglobin RAST the German and the Sudanese sera were inhibited approximately equally by the *C. riparius* haemoglobin antigen (87.4% and 84.2% respectively at 0.1 mg/ml). However, whilst *C. lewisi* produced considerable inhibition in Sudanese sera in this system (64% at 0.1 mg/ml), it was a weak inhibitor of German sera (21% at 0.1 mg/ml).

Discussion

Chironomids are amongst the few insects which contain haemoglobin in solution in the blood. Haemoglobin has been found to be maximal in the aquatic larval phase and gradually undergoes enzyme degradation as metamorphosis progresses, being minimal in the adult midge [13]. Baur [6] showed that both larvae and adults of *C. riparius* contain similar antigenic determinants, namely haemoglobins. It therefore seemed important for us to test whether the immature stages of *C. lewisi* were similarly allergenic. This was shown to be the case, since comparable skin test responses were found to extracts of larvae, pupae and adult in the group of sixteen Sudanese (Fig. 1). The presence of antigens common to all stages of this holometabolous insect is likely, since on SDS-PAGE gel the larval extract was found to have the majority of proteins in common with the adult midge extract (Tee, unpublished observations).

It is of interest that the CRIE autoradiographs of the individual larval and pupal extracts, produced using rabbit antisera raised to whole *C. lewisi* midge extract, were similar to that of the allergenic fraction using antisera raised only to this specific allergenic area (Fig. 2). CRIE autoradiographs incorporating adult midge extract and antisera raised to whole midge extract give a far more complex picture [10]. Thus, it would appear that both the larval and pupal extracts contain a higher proportion of the allergenic fractions alone, than does the adult extract.

Studies on cross-reactivity between *C. lewisi* and *C. riparius* indicate that similar allergens are present in both species. Evidence that haemoglobins are major antigens in the *C. lewisi* allergy come from the demonstration of positive skin-prick tests in twenty-six Sudanese patients to extracts of laboratory and commercial, larval and adult cultures of *C. riparius*. Despite being a tenth of the strength, the *C. riparius* haemoglobin gave comparable weals to the other extracts of the same species (Fig. 3). It is extremely unlikely that the Sudanese have been exposed to *C. riparius*, since it does not occur in Africa. Therefore their positive skin test response is probably due to the presence of similar antigenic determinants in *C. riparius* and the distantly related *C. lewisi*.

Further evidence for the close similarity between the antigens present in the two chironomids comes from RAST tests. The German patients have been occupationally sensitized to the larvae of *C. riparius*, where the principal antigens have been shown to be haemoglobins. In contrast, the Sudanese patients have been environmentally sensitized to adult *C. lewisi* where the haemoglobin content is likely to be less than in the larval and pupal stages.

In the *C. lewisi* midge RAST the high inhibition produced by *C. riparius* haemoglobin antigens in the German sera confirms that these patients' antibodies are mainly directed to haemoglobin. If the Sudanese are sensitized to haemoglobins plus an additional component(s) *x*, this latter component must be present in a high proportion since *C. riparius* is a poor inhibitor of the Sudanese sera (Table 1). That the

C. lewisi extract must contain haemoglobin antigens, however, is shown by the considerable inhibition produced by *C. lewisi* in the German sera. More *C. lewisi* antigen (Hb+x) is required to achieve better inhibition of the Sudanese sera.

Since the *C. riparius* haemoglobin RAST system is only measuring haemoglobin, high comparable inhibition is achieved both in Sudanese and German sera with *C. riparius* haemoglobin antigen. *C. lewisi* antigen contains enough haemoglobin antigen to inhibit the haemoglobin antibody in the Sudanese sera to a large extent; whereas excess haemoglobin antibody must still be present in the German sera where, when similarly inhibited with *C. lewisi*, the inhibition achieved is only 21% at 0.1 mg/ml.

Baur *et al.* [7] have recently shown that the degree of immunological cross-reactivity between fourteen chironomid species investigated (including *C. lewisi*) derives at least predominantly from haemoglobin components with common antigenic determinants in the different species and correlates with their evolutionary relationship. *C. lewisi* is very distantly related to all other species that Baur examined and has been separated from them by millions of years. With evolutionary separation there is a loss of haemoglobin congruity, so that closely related species would be expected to share more amino acid sequences than those which have diverged long ago [14]. The amino acid replacements during this period of separation could partly explain the lack of complete cross-reactivity even if haemoglobins are the respective allergens. It remains possible that x is a form of *C. lewisi* haemoglobin that has diverged from *C. riparius* haemoglobins.

Since chironomids are found world-wide and immunological cross-reactivity between them has been demonstrated, both by us here, and in fourteen species from different localities by Baur *et al.* [7], they should be seen as significant potential environmental and occupational allergens.

Acknowledgment

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