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Persistence of Hemoglobin Allergenicity and Antigenicity during Metamorphosis of Chironomidae (Insecta: Diptera)

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Abstract. Chironomid hemoglobins are potent allergens. The allergenic and antigenic activities of these hemoglobins are studied with the help of RAST, RAST inhibition and double immunodiffusion. Human as well as rabbit antisera were used. It was shown that hemoglobins are the main antigenic/allergenic components in extracts of *Camptochironomus tentans* larvae. Furthermore, immunological cross-reactivity among larvae, pupae and adult midges of this species are shown to be due to the existence of hemoglobin antigenic determinants in all developmental stages of this insect.

Introduction

Chironomidae (Insecta: Diptera), nonbiting midges, contain potent allergens [2, 3, 11, 12, 16, 19]. Clinical and immunological investigations in the FRG have shown that a significant number of people exposed to chironomid larvae through their occupation, such as insect biochemists and workers in fish food factories, as well as fish hobbyists, suffer from immediate-type hypersensitivity reactions.

Many chironomid larvae, especially those living in oxygen-poor media, are red, due to the presence of cell-free hemoglobins. The composition and number of these hemoglobins differ from one species to another [2, 3, 16, 22–24]. The antigenicity of larval chironomids has been related to these hemoglobins; [2, 3, 16], the structure of some of these has been completely defined [2, 4, 24]. In addition, cross-antigenicity between 14 chironomid species has been demonstrated and related to hemoglobin antigenic determinants in studies with human and animal sera [3, 16].

The reactions mentioned above involve larval components. Moreover, reports on respiratory diseases caused by swarms of adult chironomid midges in the Sudan [6–8, 10, 14, 19, 20, 21] and elsewhere in Africa and North America [1, 7, 8, 12] also appear important. In vivo studies indicate that similar allergens occur in larvae, pupae and adults of the chironomid species *Cladotanytarsus lewisi* which causes severe asthmatic disease in the Nile region of the Sudan [21]. Furthermore, some cross-allergenicity between this species and those involved in occupational hypersensitivity in the FRG was demonstrated [19–21]. We examined whether the known antigenicity of larval hemoglobin of chironomids persists through metamorphosis.

Because of sufficient supply, the species *Camptochironomus tentans* was used in this study as a source for allergenic/antigenic components, rather than *Chironomus thummi* which was mainly studied in previous investigations [2, 3]. *C. tentans* was also chosen because it occupies an almost intermediate position in the evolutionary tree of the chironomid family.

Materials and Methods

Larvae, Pupae, and Migdges of C. tentans

Larvae (predominantly fourth instar), pharate pupae, and adult midges of *C. tentans* were bred in laboratory cultures. Crude extracts were prepared by homogenizing the larvae, pupae or adult midges in 0.025 *M* potassium phosphate buffer, pH 6 [4]. After centrifugation for 45 min at 1,000 *g*, 4 °C, the supernatants were filtered, dialyzed and lyophilyzed.

Polyacrylamide Gel Electrophoresis (PAGE)

40-µl aliquots of crude extracts from larvae, pupae and adult *C. tentans* and larvae of *C. thummi* (= *Chironomus riparius;* concentration 19 mg/ml) were subjected to PAGE under nondenaturing conditions with a discontinuous buffer system modified according

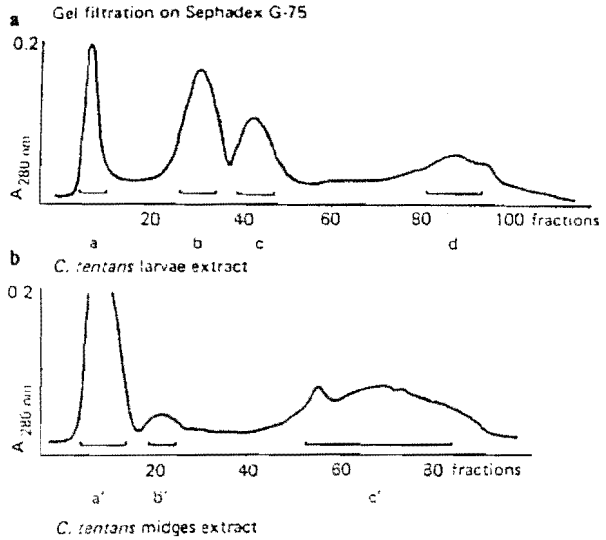

Fig. 1. Gel filtration of extracts of *C. tentans* larvae (a) and adults (b) on Sephadex G-75. Equilibration buffer: 0.025 *M* potassium phosphate, pH 6; column 96 × 2.4 cm.

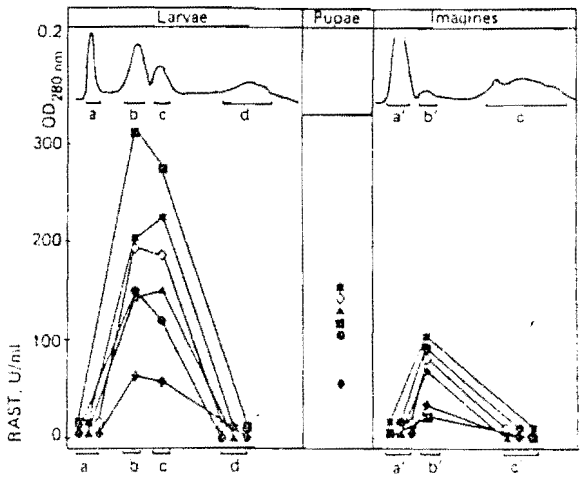

Fig. 2. Allergenicity of gel filtration fractions a–d (larvae), a'–c' (adult), and of total pupal extract of *C. tentans*. The individual fractions and total crude extracts were coupled to paper discs and studied by the RAST method. RAST results obtained with the sera of 6 clinically sensitized German patients are shown. Sera of 10 healthy controls gave results which were always below 0.35 RAST U/ml.

to *Ornstein* [15] and *Davies* [9]. A resolving gel consisting of 10% acrylamide at pH 8.9 with an acrylamide:bisacrylamide ratio of 30:0.8 was formed, and a stacking gel consisting of 3.75% acrylamide, 0.08% bisacrylamide, 0.5 *M* Tris-HCl, pH 6.8, was polymerized on top. Electrophoresis was run at 60 mA for 3 h. The gel was stained with 1% amido black 10B in 7% acetic acid.

Column Chromatography

100 mg of larvae and 75 mg of crude extracts of *C. tentans* adult midges were dissolved in 20 ml 0.025 *M* potassium phosphate buffer at pH 6 and applied onto a Sephadex G-75 column (96 × 2.4 cm) (Pharmacia Fine Chemicals). Each of the elution fractions obtained was dialyzed, lyophilyzed, and coupled to cyanogen bromide-activated paper discs for RAST studies.

RAST, RAST Inhibition

Measurement of antigen-specific IgE antibodies in human sera was carried out by the RAST method [2, 3, 5]. RAST inhibition tests were performed by preincubation of positive sera with gel filtration fraction c' of *C. tentans* adult midges. For these RAST inhibition experiments, paper discs carrying fractions b' and c' from the larvae and pupae crude extracts, respectively and fraction b' from adult midges were used. The remaining unbound amounts of specific IgE in the sera were measured by RAST and compared to those serum samples from patients which were preincubated only with 0.15 *M* phosphate buffer.

Immunological Analysis

Rabbit Antiserum. Antisera to total hemoglobin of *C. thummi* were produced by repeated subcutaneous injections of the antigen dissolved in water and mixed for the first injection with complete and for subsequent injections with incomplete Freund's adjuvant. The primary injection consisted of 15 mg of the antigen. 3 booster injections of 10 mg of the antigen were given at intervals of 2 weeks. 10 days thereafter, the animals were bled. Immunoglobulins were precipitated by addition of ammonium sulfate to 25% (w/v) and reconstituted to initial serum volumes.

Double Immunodiffusion. Double immunodiffusion experiments were performed according to *Ouchterlony* using glass slides covered with 1% buffered agarose. After gelation, 15-µl wells were aspirated according to patterns shown in figure 5. The middle well contained reconstituted rabbit antiserum against *C. thummi*, wells 1–4 contained crude extracts from larvae, pupae and adult *C. tentans*.

Results

Column chromatography of the larval extract of *C. tentans* yields 4 peaks, a–d. Peak a represents high molecular weight glycoproteins [4], peaks b and c – red-colored solutions – which by analogy with previous studies [2–4] are dimeric and monomeric hemoglobins, respectively, peak d low molecular weight components.

3 peaks, a' b' c', can be seen in the chromatogram of the adult midge extract of *C. tentans* (fig. 1b); peak b' consists of greenish-brown-colored material. Using sera from 6 German fish hobbyists known to be sensitized to chironomid larvae, the antigenicities of different chromatography elution fractions a–d (larvae),

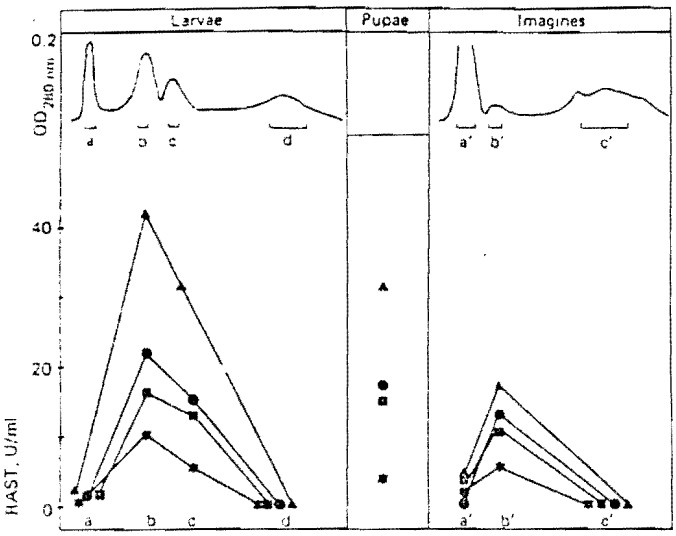

Fig. 3. Allergenicity of gel filtration fractions a–d (larvae), a′–c′ (adult), and of total pupal extract of *C. tentans*. The RAST results obtained with the sera of 4 sensitized Sudanese patients are shown. Sera of 10 healthy controls gave results which were always below 0.35 RAST U/ml.

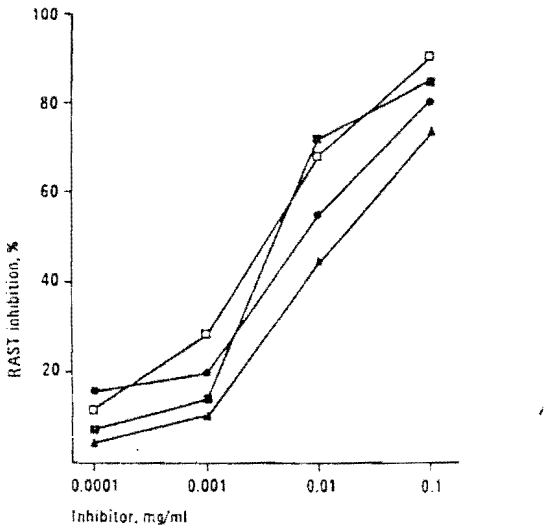

Fig. 4. Inhibition of IgE binding to solid phase fractions b (▲) and c (●) (larvae), fraction b′ (□) (adult midge), and pupal crude extract (■) of *C. tentans* by fraction b′ (*C. tentans* adult midges).

a′–c′ (adult midges) as well as that of total pupal extract were tested (fig. 2). It can be seen that the average antigenicities decline during metamorphosis, with larval fractions having the highest IgE binding activities. The individual fractions of larval and adult midge extracts, fractions b, c, and b′, exhibit the strongest IgE binding; fractions a, d, a′, and c′ gave negative RAST results.

A similar pattern of antigenicity in the RAST was obtained with 4 sera of Sudanese patients known to be sensitized to the chironomid midge *Cladotanytarsus lewisi* (fig. 3). This indicates that antigenic structures of hemoglobin are preserved during metamorphosis and represent a main or the only antigenic components of chironomids for patients in contact with adult midges.

These results suggest a possible immunological cross-reactivity between the positive fractions of different developmental stages, which could be confirmed by RAST inhibition experiments. Preincubation of one German patient's serum with low amounts of akult midge gel filtration fraction b′ resulted in dose-dependent inhibition of IgE binding to larval gel filtration fractions b and c, as well as to pupal crude extract and to adult midge fraction b′ (all in solid phase; fig. 4). This indicates that adult midges do contain components which are immunologically identical to parts of larval and pupal hemoglobins.

Cross-reactivity between larval, pupal, and adult extracts from *C. tentans* on the one hand, and other chironomids such as *C. thummi* (= *C. riparius*) on the other hand, was demonstrated by double immunodiffusion (fig. 5). Antiserum against *C. thummi* total hemoglobin (middle well) produced 2–4 precipitin lines with the crude extracts from larvae (wells 1 and 4), pupae (well 2) and imagines (well 3) from *C. tentans*. Furthermore, complete or partial identity between components of all developmental stages of *C. tentans* was obtained.

Previous studies by means of PAGE have demonstrated that nearly all bands obtained from chironomid larvae represent hemoglobins [3, 4, 16, 22–24]. As shown in figure 6 *C. tentans* pupae give a comparable pattern of bands as *C. tentans* larvae suggesting the conservation of most or all hemoglobins. On the other hand, several hemoglobin components seem to be absent in adult *C. tentans* midges.

Discussion

Using sera of sensitized patients and immunized rabbits, we have shown strong immunological cross-reactivity between larval hemoglobins and components of pupae and adult *C. tentans*. Inhibition of IgE binding to larval monomeric and dimeric hemoglobin

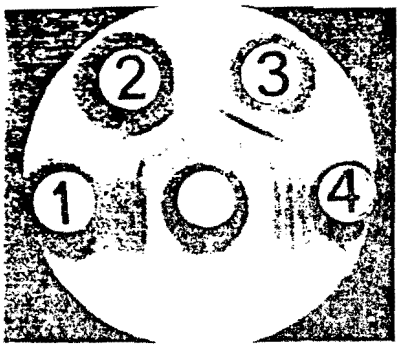

Fig. 5. Double immunodiffusion in agarose gel using crude extracts of *C. tentans* larvae (wells 1 and 4), pupae (well 2), adults (well 3), and rabbit antisera against *C. thummi* total hemoglobin (central well).

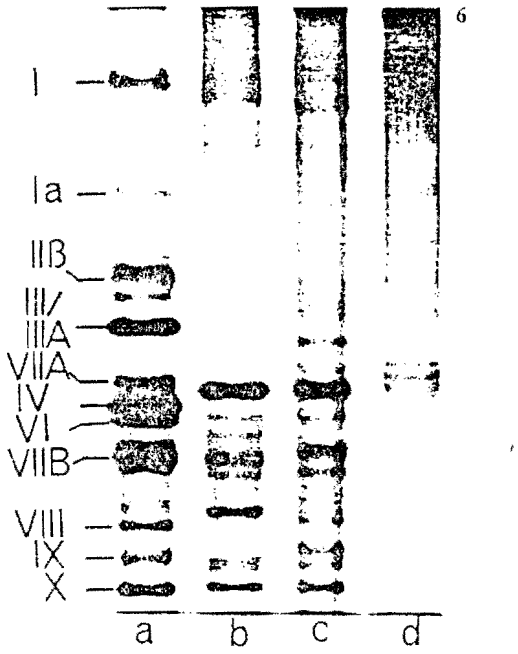

Fig. 6. PAGE patterns of crude extracts of (a) *C. thummi* larvae, (b) *C. tentans* larvae, (c) *C. tentans* pupae, and (d) *C. tentans* adults. Roman numbers indicate individual identified hemoglobins (4).

fractions (b and c) and the pupal extract by low amounts of fraction b' from adult midge extract indicates that antigenically active regions of hemoglobin are responsible for at least a major portion of this cross-antigenicity. Almost identical immunodiffusion precipitin patterns were obtained by rabbit antisera against *C. thummi* total hemoglobin and *C. tentans* larval, pupal and adult midge extracts. That hemoglobins are the components responsible for this immunological cross-reactivity is also supported by the results of PAGE analysis showing the presence of hemoglobin bands in all extracts analyzed [3]. Additional evidence for hemoglobin-based immunological cross-reactivity was obtained by the immunoblotting technique previously reported [3]. The autoradiograms thus obtained showed the binding of human antibodies to protein bands with electrophoretic mobilities identical to those of chironomid hemoglobins.

Earlier studies, using in vivo and in vitro testing, showed congruence between antigens of larvae, pupae and adults of the Sudanese *C. lewisi*, and between these adults and both larvae and adults of *C. thummi* [3, 8, 21]. The present study confirms and extends these previous reports and strengthens the observations of *Schin* et al. [17, 18] that hemoglobin is incompletely degraded through metamorphosis, although it is excreted into the meconium of the newly emerged adult [13].

Our finding that allergenic larval hemoglobin persists in the adult midge provides firm evidence of the similarity of antigens in the occupational exposure in the FRG to the larvae and the Sudanese environment exposure to chironomid adults. This confirms our view that all stages of Chironomidae must be seen as significant occupational and environmental allergen sources.

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