

Common antigenic determinants of haemoglobin as basis of immunological cross-reactivity between chironomid species (Diptera, Chironomidae): studies with human and animal sera

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SUMMARY

Chironomids, of which approximately 10,000 species exist, are reported to cause severe immediate type allergic diseases in man. In the present study, immunological cross-reactivity between 14 chironomid species from different continents was proven by RAST inhibition, double immunodiffusion and a new allergoprint technique, based upon PAGE separation of insect crude extracts. Using isolated chironomid haemoglobins and sera of sensitized persons, as well as rabbit antibodies against larval crude extract or against the haemoglobin fraction of *Chironomus thummi*, it could be proven that cross-reactivity derives at least predominantly from haemoglobin components with common antigenic determinants in the different species.

Keywords Chironomide haemoglobin antigenic determinants RAST allergoprint

INTRODUCTION

The Chironomidae are one of the largest insect families, the total number of all pertinent species being estimated at about 10,000. The juvenile stages of these midges (usually four larval instars and one pupal instar) are characteristically aquatic. They are distributed worldwide and are found in nearly all types of inland waters, some species even in marine habitats.

As reported by Freeman (1950), Lewis (1956), Kay *et al.* (1978), Cranston, Gad el Rab & Kay (1981) and S. Kagen (personal communication 1982) adult midges as well as larvae of these insects may occur in enormous numbers, even amounting to a plague, and causing severe respiratory diseases in sensitized persons.

Recently we discovered that the chironomid haemoglobin with its monomeric and homodimeric forms is a major allergen for humans (Baur *et al.*, 1982b). This paper describes a more extensive investigation of the species *Chironomus thummi*. Of 141 persons who had been exposed to airborne constituents of these insect larvae, 52 were shown to have specific IgE antibodies against highly purified chironomid haemoglobins. Moreover, at least six antigenic determinants could be identified within the polypeptide sequences of three of these haemoglobin molecules (Baur *et al.*, 1982a).

The following investigations were performed in order to determine whether there exists immunological cross-reactivity between various chironomid species of different continents.

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especially between their haemoglobin components. For these investigations sera of persons clinically sensitized to *C. thummi* as well as of immunized animals were tested.

MATERIALS AND METHODS

Larvae (predominantly fourth instar), pharate pupae and/or adult midges of 14 chironomid species were used in this study. Adults of *Cladotanytarsus lewisi*, kindly supplied by Professor Dr A. B. Kay, were collected in the Nile region in the Sudan. *C. bernensis*, *C. plumosus*, *C. moratensis*, *C. holomelas*, *C. luridus*, *C. dorsalis*, Einfeldia species 4 (undescribed) and *Glyptotendipes pallens* were reared from egg masses collected at various localities in Switzerland. *C. tepperi*, an Australian species, as well as *C. thummi* (synonym: *C. riparius*), *C. piger*, *C. annularius* and *C. tentans* (all collected in Germany) were reared in laboratory cultures. Species identification in the genus *Chironomus* was based on cytotaxonomical criteria (Keyl, 1962 and subsequent investigators, as summarized by Ryser *et al.*, 1980).

Larvae, pupae and adults were homogenized in 0.025 M potassium phosphate buffer, pH 6, using a motor driven teflon homogenizer. The homogenates were then stirred at 4°C for 1 h and centrifuged for 45 min at 10,000g, 4°C. The supernatants were passed through millipore filters (0.45 µm), dialysed and lyophilized. Supernatants were referred to as crude extracts. For isolation of the *C. thummi* total haemoglobin fraction larval crude extract of *C. thummi* was dissolved in the same buffer; then ammonium sulphate was added to a final concentration of 20%, stirred and centrifuged (12,000g, 45 min). Haemoglobins were then precipitated from the supernatant by addition of ammonium sulphate to a final concentration of 80%, followed by stirring overnight and centrifugation at 15,000g for 60 min. The pellet was dissolved in and dialysed against bidistilled water, and lyophilized.

RAST, RAST inhibition. Measurement of antigen specific IgE antibodies in human sera was performed with the RAST method (Ceska & Lunkvist, 1972; Baur *et al.*, 1982b), using paper discs which had been previously coated with the different extracts and isolated fractions of chironomids (solid phase antigens). RAST inhibition studies were performed by 16 h pre-incubation of positive sera with substances which were thought to cross-react with the solid phase antigens.

Preparation of rabbit antisera to chironomid extracts. Rabbit antisera to larval crude extract and haemoglobin fraction of *C. thummi* were obtained by repeated injection of the antigens dissolved in water and mixed for the first injection with Freund's complete adjuvant and for the subsequent ones with Freund's incomplete adjuvant. For the first immunization a total of 15 mg of the antigens (protein conc. 83–97%) was injected subcutaneously at different sites; at 2 week intervals injection of 10 mg of the antigens was repeated (at least three times). The animals were bled 10 days after the last injection. Immunoglobulins were precipitated with 25% ammonium sulphate saturation and reconstituted to initial serum volumes for use in the laboratory studies.

Double immunodiffusion in agarose gel. Glass slides were covered with 1% buffered agarose; after gelation, 20 µl wells were aspirated in the patterns shown in Figs 7 & 8. All central wells were filled with reconstituted rabbit antibodies, while the outer wells contained the various chironomid crude extracts. Incubation was carried out at room temperature and precipitation reactions were completed within 72 h. After 3 days the plates were stained with Coomassie brilliant blue R-250.

Polyacrylamide gel electrophoresis (PAGE). PAGE was carried out under non-denaturing conditions using a discontinuous buffer system modified according to Ornstein (1964) and Davies (1964). A separation gel consisting of 10% acrylamide – 0.16% Bis in 0.375 M Tris-HCl, pH 8.9, was formed; a stacking gel consisting of 3.75% acrylamide – 0.08% Bis – 0.5 M Tris-HCl pH 6.8, was polymerized on top, and 40 µl (10 mg/ml) aliquots of the protein samples were applied onto the gel. Electrophoresis was run at 40 mA for 15 min followed by 65 mA for 3 h. Gels were fixed overnight in 20% trichloroacetic acid followed by washing with 7% acetic acid, stained with Amido black 10 B and destained as described by Ornstein (1964) and Davies (1964).

Electrophoretic transfer to nitrocellulose sheets. Crude extracts of *C. thummi* and *C. tentans* larvae were subjected to PAGE as related above and the separated proteins were transferred to nitrocellulose sheets, essentially as described by Towbin, Staehelin & Gordon (1979). Briefly, after

electrophoresis, a sandwich made of the polyacrylamide gel, nitrocellulose sheets and Scotch-Brite pads was brought into the electrode cassette which was then immersed into the 'transblotting apparatus' (Bio Rad), filled with buffer consisting of 25 mM Tris–192 mM glycine–20% (vol./vol.) methanol, pH 8.3, and the transfer was carried out overnight at 30 V, 100 mA.

Allergoprints. Nitrocellulose strips carrying the separated proteins were either stained with Amido black 10 B, or processed as follows: a strip was incubated for 1 h at 40°C with 50% horse serum in 15 mM Tris-HCl, pH 7.4. After incubation and washing in saline the strip was incubated for 6 h with 300 µl patient serum diluted in 10 ml 3% BSA-saline. The washed strip was then incubated overnight with 150 µl ¹²⁵I-labelled anti-human IgE antiserum (Pharmacia, Uppsala) diluted in 10 ml 3% BSA-saline. This incubation was followed by washing, drying and autoradiography using Kodak RP films.

RESULTS

The evolutionary relationships among the chironomid species investigated are schematically illustrated in Fig. 1. Ten species which belong to three different complexes of the genus *Chironomus* were investigated, as well as one species of each of the following genera: *Camptochironomus*, *Einfeldia*, *Glyptotendipes*, and *Cladotanytarsus*, the latter belonging to the more distantly related tribe Tanytarsini.

Fig. 2 demonstrates the interspecific variations of the major protein components of larval crude extracts of 13 chironomid species as obtained by PAGE. As demonstrated in previous studies for the species *C. thummi* (Braun, Crichton & Braunitzer, 1968; Aschauer, Zaidi & Braunitzer, 1981) and *C. tentans* (Tichy, 1970, 1975; Tichy, Kleinschmidt & Braunitzer, 1981), nearly all of the visible bands represent haemoglobins, e.g. 11 bands of *C. thummi* were clearly identified as four monomeric and seven homo-dimeric haemoglobins with known primary structures. It can be clearly seen that phylogenetically closely related species—such as *C. thummi* and *C. piger*— give an almost identical electrophoretic pattern. On the other hand, considerable intergeneric and even intrageneric variations are obvious.

As shown in Figs 3 & 4, all eight sera of clinically sensitized patients exhibited antibody reactivity with larval, pupal and adult extracts of the 14 chironomid species studied. There exist only minor variations in antigenicity of the larval extracts. Extracts of adult midges produced on the average half as high RAST values as compared with the larval extracts; *Einfeldia* species 4 showed relatively high reactivity, and the distantly related *Cladotanytarsus lewisi* was only weakly positive. *C. luridus* pupae were more strongly reactive than adults of most species, but less reactive than *C.*

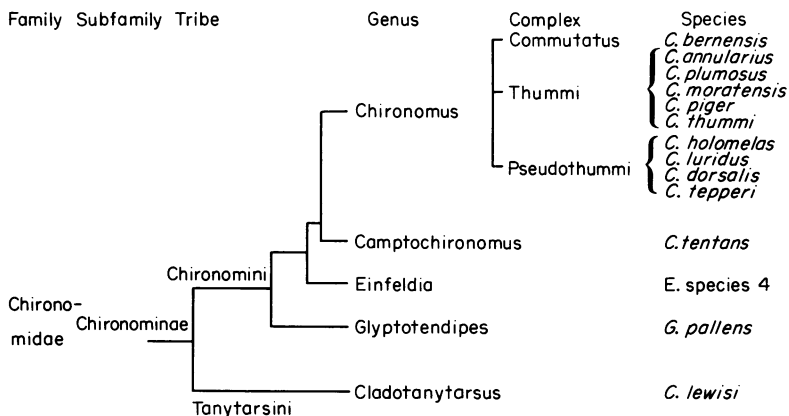


Fig. 1. Evolutionary relationships of the chironomid species investigated (according to Keyl, 1962 and subsequent investigators).

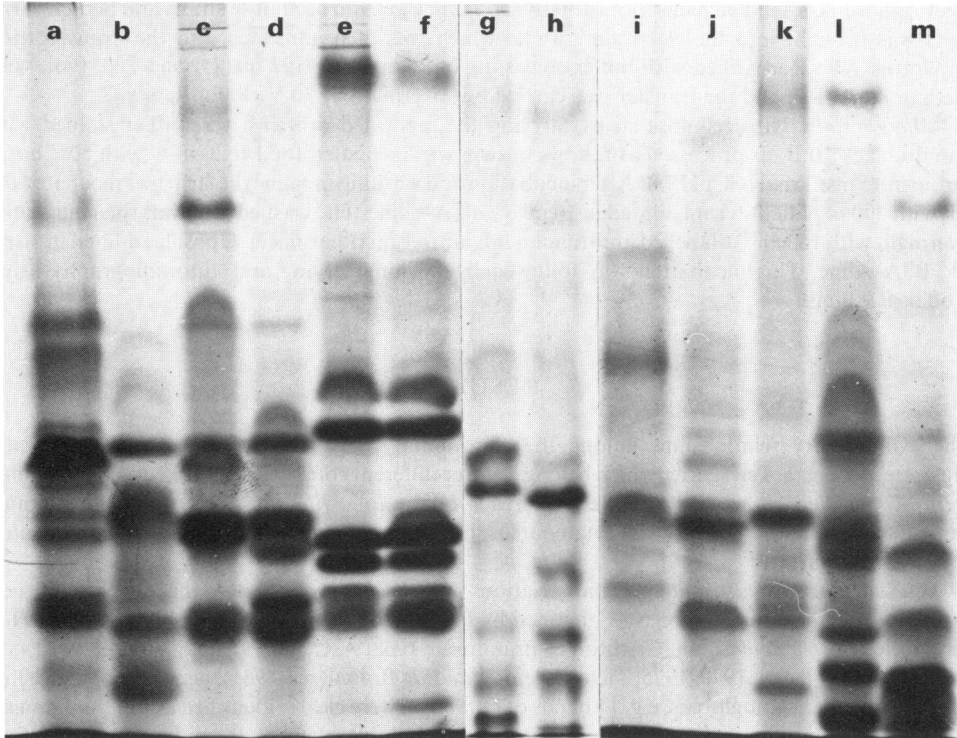


Fig. 2. PAG electrophoresis patterns of 13 chironomid species (larval extracts). (a) *C. bernensis*; (b) *C. annularius*; (c) *C. plumosus*; (d) *C. moratensis*; (e) *C. piger*; (f) *C. thummi*; (g) *C. holomelas*; (h) *C. luridus*; (i) *C. dorsalis*; (j) *C. tepperi*; (k) *C. tentans*; (l) *Einfeldia* sp. 4; (m) *G. pallens*.

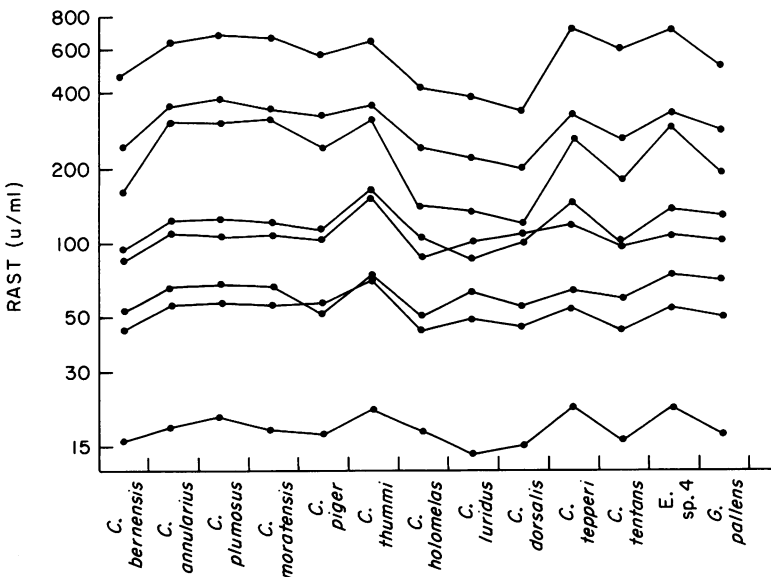


Fig. 3. Antigenic reactivities of extracts from different chironomid larvae. The RAST results obtained with the sera of eight sensitized patients are shown.

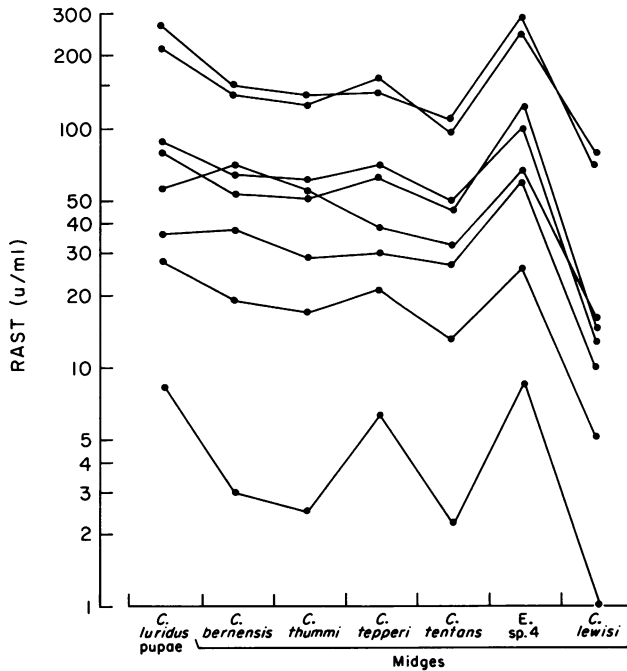


Fig. 4. Antigenic reactivities of extracts from different chironomid midges. The RAST results obtained with the sera of eight sensitized patients are shown.

luridus larvae. These results suggest immunological cross-reactivity between the different chironomid genera and even between different chironomid tribes, which is confirmed by RAST inhibition experiments.

Pre-incubation of one patient's serum with larval crude extracts of eight chironomid species resulted in dose-dependent inhibition of IgE binding to purified *C. thummi* total haemoglobin (Fig. 5). Most interesting is the observation that reactivity with purified *C. thummi* total haemoglobin could be inhibited by six extracts of adult midges in a similar manner as by the larval crude extracts (Fig. 6). Inhibition of IgE binding to *C. thummi* total haemoglobin by low amounts of different larval and midge extracts, as shown in these experiments, is evidence that haemoglobins are responsible for at least a major portion of cross-reactivity between the different chironomid species. It is noteworthy that inhibition capacities of the different extracts remarkably reflect the degree of evolutionary relationship of the different chironomid species with regard to *C. thummi*. The relatively closely related species of the thummi complex (Fig. 1) gave strong and similar degrees of cross-reactivity. Weaker cross-reactivity was obtained with species of the genera *Camptochironomus*, *Einfeldia* and *Glyptotendipes*, whereas *Cladotanytarsus lewisi*, which is phylogenetically more distant, showed a very weak degree of immunological cross-reactivity.

Cross-reactivity between different chironomid species could also be proven by double immunodiffusion with rabbit antisera against crude extract of *C. thummi* or against *C. thummi* total haemoglobin (Fig. 7 a & b). Antiserum against *C. thummi* total haemoglobin produced two to four precipitin lines with all of the species studied (one of each complex and genus respectively). Furthermore, cross-reactivity of complete and in some cases of partial identity between these species was obtained especially between the three species of the genus *Chironomus* (*C. thummi*, *C. annularius*, *C. piger*; Fig. 7a). Nearly the same pattern of reactivity was obtained when antiserum against *C. thummi* crude extract was used instead of antiserum against *C. thummi* total haemoglobin (Fig. 7b), a principal difference being that *C. piger* gave one additional line of non-identity.

As shown in Fig. 8 two of the precipitin lines found in tests with all of the chironomid species could be identified as dimeric haemoglobins and another line as monomeric haemoglobins. With

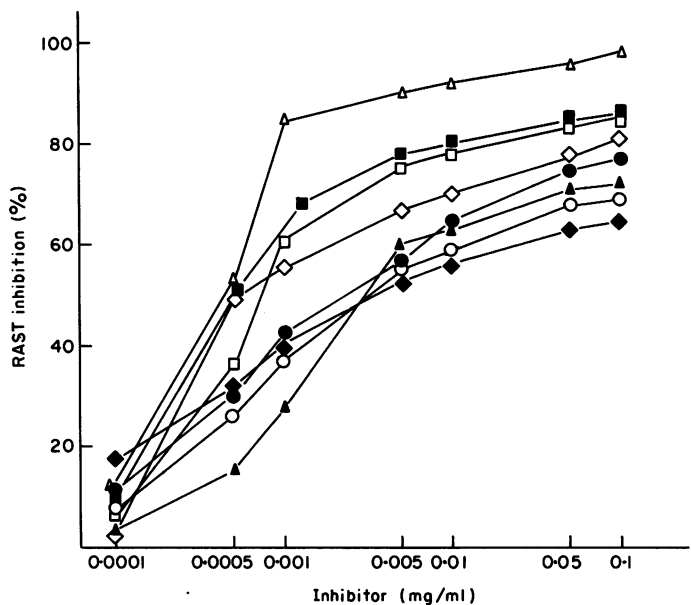


Fig. 5. Inhibition of IgE binding to solid phase CTT-Hb by crude extracts from larvae of the following chironomid species: *C. bernensis* (■); *C. annularius* (□); *C. plumosus* (◇); *C. tepperi* (●); *C. tentans* (○); *Einfeldia* sp. 4 (▲) and *Glyptotendipes pallens* (◆). CTT-Hb = Δ.

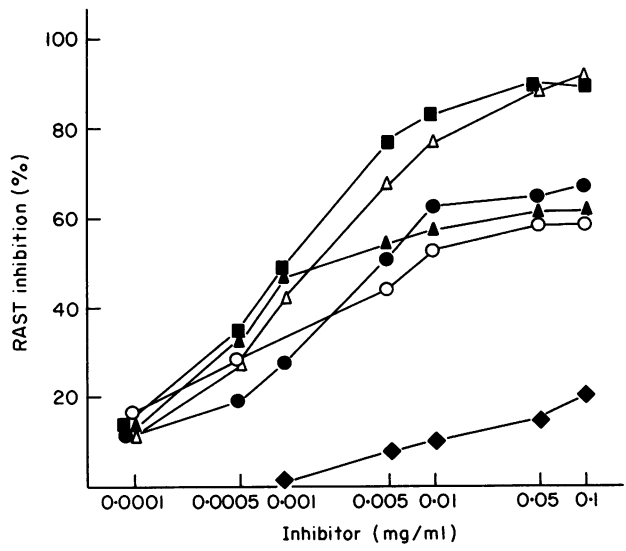


Fig. 6. Inhibition of IgE binding to solid phase CTT-Hb by crude extracts from midges of the following chironomid species: *C. thummi* (Δ); *C. luridus* pupae (■); *C. tepperi* (●); *C. tentans* (○); *Einfeldia* sp. 4 (▲); *Cladotanytarsus lewisi* (◆).

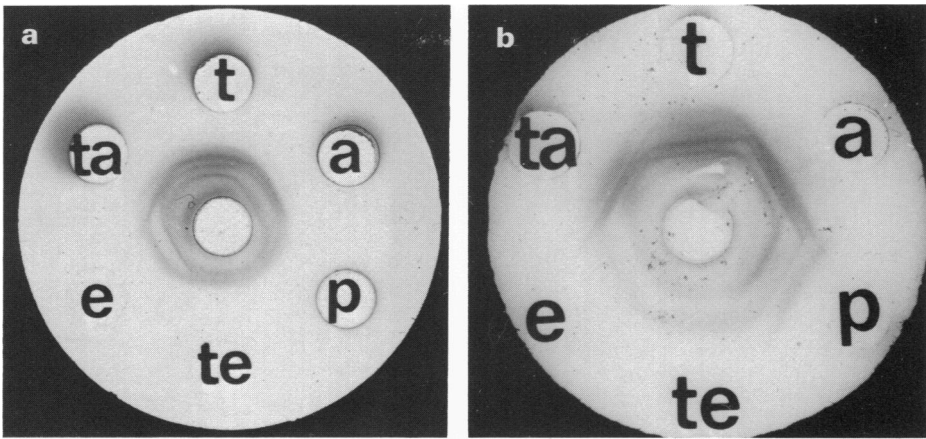


Fig. 7. Double immunodiffusion in agarose gel using larval crude extracts of different chironomid species and rabbit antibodies against *C. thummi* total haemoglobin (a) or *C. thummi* crude extract (b). Central wells were filled with rabbit antibodies; outer wells contained the following larval crude extracts: *C. thummi* (t), *C. annularius* (a), *C. piger* (p), *C. tepperi* (te), Einfeldia species 4 (e), *C. tentans* (ta).

respect to this finding it should be mentioned that monomeric and dimeric haemoglobins of the same species show only reactions of partial identity (Fig. 8, right side).

That haemoglobins are the basis for immunological cross-reactivity between different chironomid species is also demonstrated in immunological studies of components of *C. thummi* and *C. tentans* which have been separated by PAGE, clearly identified, transblotted onto nitrocellulose paper and autoradiography performed (Fig. 9). By comparing these patterns of reactivity with those obtained with clearly identified haemoglobin components (Braun *et al.*, 1968; Tichy, 1975; Tichy *et al.*, 1981; Aschauer *et al.*, 1981) it can be seen that at least most of the bands indicating reactivity of human IgE antibodies with an antigen represent haemoglobins. Nearly identical patterns of reactivity were obtained with the serum of another sensitized patient.



Fig. 8. Double immunodiffusion in agarose gel using larval crude extract of *C. thummi* (R), the homo-dimeric haemoglobin fraction of *C. thummi* (D) or the monomeric haemoglobin fraction of *C. thummi* (M) as antigen and rabbit antibodies against *C. thummi* crude extract (central well).

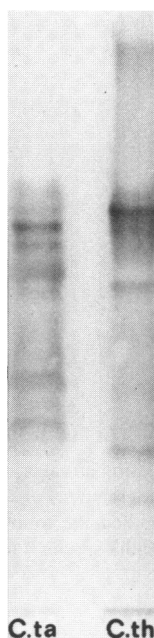


Fig. 9. Allergoprint of patient C. as evaluated by PAGE separated larval crude extracts of *C. tentans* (C.ta) and *C. thummi* (C.th). For details see text.

DISCUSSION

Using sera of clinically sensitized persons and immunized rabbits we were able to prove moderate to strong immunological cross-reactivity between 14 chironomid species. This was shown in RAST inhibition, double immunodiffusion, and immunologic studies of PAGE separated larval components.

The degree of immunological cross-reactivity between the chironomid species investigated is correlated with their evolutionary relationships. In spite of the fact that these chironomids belong to five different genera and that there exist considerable variations in the electrophoretic patterns of their larval protein components (which predominantly represent haemoglobins), this immunological cross-reactivity is mainly or exclusively due to common antigenic determinants of haemoglobins. This is documented by (a) nearly identical patterns of reactivity in double immunodiffusion with rabbit antibodies against *C. thummi* larval crude extract and with antibodies against *C. thummi* total haemoglobin, which were obtained with species of five different chironomid genera; (b) identification of nearly all precipitin lines of chironomids of five genera as components which reveal reactions of identity or of partial identity with *C. thummi* haemoglobins; (c) identification of the main allergenic components in larval crude extracts of two chironomid species as individual haemoglobins after separation by PAGE, transblotting and immunological studies with human antisera and (d) immunological cross-reactivity between individual *C. thummi* haemoglobins as well as between some haemoglobins of *C. thummi* and *C. tentans* (Tichy *et al.*, 1981; Trewitt & Bergtrom, 1981; Baur *et al.*, 1982b).

Chironomid species are distributed over all continents, especially over the holarctic region. Therefore, from the clinical point of view, the fact of cross-reactivity between antigens of 14 chironomid species of different localities means that sensitized persons worldwide may develop allergic diseases identical to those described in our previous report (Baur *et al.*, 1982b). Up to now, such illnesses have been observed in the Nile region in the Sudan and in several other water-rich areas in Africa and America (Freeman, 1950, 1973; Lewis, 1956; Grodhaus, 1963; Wülker, 1963; Bay, 1964; Satti & Ab del Nur, 1974; Kay *et al.*, 1978; Gad el Rab & Kay, 1980; Cranston *et al.*, 1981; S. Kagen, personal communication, 1982).

The ultimate goal of our current studies is the elucidation of antibody binding sites of the different *C. thummi* haemoglobins. The immunological cross-reactivity between individual *C. thummi* haemoglobins mentioned above is good evidence for structurally identical or closely related antigenic determinants.

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REFERENCES

- ASCHAUER, H., ZAIDI, Z.H. & BRAUNITZER, G. (1981) Amino acid sequence of a dimeric hemoglobin (erythrocytorin), component VI from *Chironomus thummi thummi*. *Hoppe Seyler's Z. Physiol. Chem.* **362**, 216.
- BAUR, X., ASCHAUER, H., BRAUNITZER, G., DEWAI, R. & FRUHMANN, G. (1982a) Studies on antigenic determinants of potent insect allergens (chironomid hemoglobins). *Intern. Congress Allergy Clin. Immunol. London*, 1982 (abstract 068).
- BAUR, X., DEWAI, R., FRUHMANN, G., ASCHAUER, H., PFLETSCHINGER, J. & BRAUNITZER, G. (1982b) Hypersensitivity to chironomids (non-biting midges): localization of the antigenic determinants within certain polypeptide sequences of hemoglobins (erythrocytorins) of *Chironomus thummi thummi* (Diptera). *J. Allergy Clin. Immunol.* **68**, 66.
- BAY, E.C. (1964) An analysis of the 'sayule' nuisance at San Carlos, Nicaragua, and recommendations for its alleviation. *World Health Organization/EBL Series* 20, 1.
- BRAUN, V., CRICHTON, R.R. & BRAUNITZER, G. (1968) Über monomere und dimere Insektenhämoglobine (*Chironomus thummi*). *Hoppe Seyler's Z. Physiol. Chem.* **339**, 197.
- CESKA, H.M. & LUNKVIST, H.U. (1972) A new and rapid method for the determination of IgG. *Immunochem.* **9**, 1021.
- CRANSTON, P.S., GAD EL RAB, M.O. & KAY, A.B. (1981) Chironomid midges as a cause of allergy in the Sudan. *Trans R. Soc. Trop. Med. Hyg.* **75**, 1.
- DAVIES, B.J. (1964) Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**, 404.
- FREEMAN, P. (1950) A species from the Sudan suspected of causing asthma. *Proc. R. Ent. Soc. Lond.* **19**, 58.
- FREEMAN, P. (1973) Chironomidae (non-biting midges). In *Insects and other arthropods of medical importance* (ed. by K.G.V. Smith) pp. 189–191. The Trustees of the British Museum (Natural History) London.
- GAD EL RAB, M.O. & KAY, A.B. (1980) Widespread IgE-mediated hypersensitivity in the Sudan to the 'green nimitti' midge *Cladotanytarsus lewisi* (Diptera: Chironomidae). I. Diagnosis by radioallergosorbent test. *J. Allergy clin. Immunol.* **66**, 190.
- GRODHAUS, G. (1963) Chironomid midges as a nuisance. *Calif. Vector Views*, **10**, 19 & 27.
- KAY, A.B., GAD EL RAB, M.O., STEWART, J. & ERWA, H.H. (1978) Widespread IgE-mediated hypersensitivity in northern Sudan to the chironomid *Cladotanytarsus lewisi* ('green nimitti'). *Clin. exp. Immunol.* **34**, 106.
- KEYL, H.G. (1962) Chromosomenentwicklung bei Chironomus. II. Chromosomenbauten und phylogenetische Beziehungen der Arten. *Chromosoma (Berl.)*, **13**, 464.
- LEWIS, D.J. (1956) Chironomidae as a pest in the Northern Sudan. *Acta Trop.* **13**, 142.
- ORNSTEIN, L. (1964) Disc electrophoresis. I. Background and theory. *Ann. N.Y. Acad. Sci.* **121**, 321.
- RYSER, H.M., GEIGER, H.J., SCHOLL, A. & WÜLKER, W. (1980) Untersuchungen über die Verbreitung der Zuckmücken-Gattung *Chironomus* in der Schweiz, mit besonderer Berücksichtigung von drei cytologisch nicht beschriebenen Arten. In *Chironomidae. Ecology, Systematics, Cytology, Physiology* (ed. by D. A. Murray) pp. 19–24. Pergamon Press, Oxford.
- SATTI, M.H. & AB DEL NUR, O.M. (1974) Chironomidae as a cause of nuisance and allergic manifestation. *Sudan Med. J.* **12**, 77.
- TICHY, H. (1970) Biochemische und cytogenetische Untersuchungen zur Natur des Hämoglobins Polymorphismus bei *Chironomus tentans* und *Chironomus pallidivittatus*. *Chromosoma*, **29**, 131.
- TICHY, H. (1975) Nature genetic basis and evolution of the hemoglobin polymorphism in *Chironomus*. *J. mol. Evol.* **6**, 39.
- TICHY, H., KLEINSCHMIDT, T. & BRAUNITZER, G. (1981) Studies on the evolutionary relationships between hemoglobins in *Chironomus pallidivittatus* and *C. tentans*. *J. mol. Evol.* **18**, 9.
- TOWBIN, H., STAEBELIN, T. & GORDON, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proc. Natl. Acad. Sci. USA.* **76**, 4350.
- TREWITT, P.M. & BERGTROM, G. (1981) Immunological characterization of the hemoglobins of *Chironomus thummi* (Diptera). *Insect Biochem.* **11**, 635.
- WÜLKER, W. (1963) Prospects for biological control of pest chironomidae in the Sudan. *World Health Organization/EBL Series* 11, *Vector Control*, **42**, 1.