

Partial characterization of allergens associated with hypersensitivity to the 'green nimitti' midge (*Cladotanytarsus lewisi*, Diptera: Chironomidae)

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(Received 15 November 1982; accepted for publication 30 November 1982)

Summary

Allergens in extracts of the 'green nimitti' midge, *Cladotanytarsus lewisi* Freeman (Diptera: Chironomidae), a cause of widespread hypersensitivity in the Sudan, were isolated and partially characterized by assays which depend on the binding of ^{125}I -anti-IgE to allergen-IgE complexes. These methods included RAST inhibition, crossed radioimmuno-electrophoresis (CRIE) and rocket radioimmuno-electrophoresis (RRIE). Following Sephadex G100 chromatography the 'major peak' of allergenicity as determined by RAST inhibition, RRIE and SDS-PAGE was associated with molecules of approximately 17 000 daltons. The peak eluting at V_0 contained material of molecular weight 66 000 daltons which also bound ^{125}I -anti-IgE, but had only 61% of the activity of the 'major peak' by RAST inhibition. By isoelectric focusing and RRIE of fractions obtained by chromatofocusing with polybuffer exchanger 94, the 'major peak' was associated with multiple bands with a pI range of 3.5-5.5. These results indicate that the major allergens from *C. lewisi* are a group of closely related acidic peptides.

Introduction

Since the 1920s Chironomidae (non-biting midges) have been associated with widespread hypersensitivity in people living close to the Nile in the Sudan (Lewis, 1956; Rzojka, 1964; Satti & Abdel Nur, 1974; Cranston, Gad El Rab & Kay, 1981). The dominant species is *Cladotanytarsus lewisi*, ('green nimitti') (Freeman, 1950; Kay *et al.*, 1978); and their large numbers are probably the result of increased workings of the dams which have produced lakelike conditions, optimal for breeding of the midges, on parts of the White, Blue and main Nile rivers. These increases in the numbers of

Chironomidae have been associated with a high incidence of bronchial asthma and rhinitis. Kirk (reported in Lewis, 1956) substantiated the belief that these midges were responsible for allergic symptoms by showing that crude midge extract elicited a positive skin test in Sudanese asthmatics. In previous studies, Kay *et al.* (1978) confirmed this skin-test reactivity and showed that sera from hypersensitive Sudanese passively sensitized lung fragments for the antigen-induced IgE-mediated release of histamine and SRS-A. A radioallergosorbent test (RAST) was developed in order to have a quantitative measurement of specific IgE and a relationship was demonstrated between RAST scores and severity of symptoms (Gad El Rab & Kay, 1980).

Preliminary attempts were made to chemically characterize the major allergens, and fractions obtained by gel filtration and ion exchange chromatography were assayed by direct skin-prick testing of hypersensitive individuals. These data indicated that a major proportion of the allergenic material was associated with a mol. wt 15 000–20 000 daltons and pI of 4.3. In the present study we have attempted to confirm these observations using more definitive techniques such as RAST inhibition and autoradiography with ^{125}I -anti-IgE.

Materials and methods

Preparation of antigen

Collection. Adult midges were collected by a light-trap and identified as containing over 99% *Cladotanytarsus lewisi* (Freeman). They were dried at 26°C and stored at 4°C in sealed plastic bags.

Preparation of extract

A known weight of dried midges was defatted in three changes of ether over 24 hr. Extraction was performed in Coca's solution (10% w/v) for 48 hr at room temperature after which the midges were removed by filtration and the filtrate centrifuged at 18 000 g for 40 min. The supernatant was passed through a 0.45 microlitre Millipore filter (Millipore Ltd, Bedford, U.S.A.), dialysed against six changes of distilled water over two days and lyophilized.

Radioallergosorbent test (RAST)

Preparation of allergen polymer complex (APC)

Ten mg of lyophilized crude extract of *C. lewisi* was coupled per gram of CNBr-activated Sepharose 4B (Pharmacia).

Radioallergosorbent-test assay

Optimum conditions for the assay were established with two 16-hr incubations with 50 μl of serum and 100 μl APC (6.25% concentration) in the first and 50 μl ^{125}I -anti-IgE (Pharmacia) in the second.

Cord blood sera containing no demonstrable total IgE were used in all assays as negative controls.

RAST inhibition with sera from patients hypersensitive to C. lewisi

(a) *Unfractionated extract.* One hundred microlitre aliquots of a suitable dilution of sera were agitated for 16 hr with 100 μl of unfractionated *C. lewisi* extract solutions

containing 0.01, 0.1, 1, 10 and 40 mg/ml respectively. The RAST was then performed as described and percentage binding compared with that of unabsorbed sera.

(b) *G100 fractions of C. lewisi extract.* Nine triads of freeze-dried G100 fractions (55-7, 67-9, 76-8, 87-9, 96-8, 107-9, 113-5, 125-7 and 135-7) were each reconstituted in 1 ml of normal saline ($\times 12$ concentration). Then RAST inhibition was performed as for the unfractionated extract with preincubation of equal volumes of reconstituted fractions with sera from four hypersensitive patients.

Gel filtration

Sephadex G100. A column of Sephadex G100 (90 $\times$ 5 cm) (Pharmacia, Uppsala, Sweden) was equilibrated with 0.05 M NH_4HCO_3 (pH 8.1) at 4°C. One hundred mg of lyophilized *C. lewisi* extract was applied to the column and 4-ml fractions collected. The column was calibrated with blue dextran (mol. wt 10^6K), bovine serum albumin (67K), ovalbumin (43K), chymotrypsinogen (25K), cytochrome C (12.4K) and vitamin B₁₂ (1.4K). Rocket radioimmuno-electrophoresis was performed in order to ascertain which of the G100 fractions contained allergens and a 'score' attributed to them based on density of tracer uptake as shown in Fig. 4.

Isoelectric focusing

LKB Ampholine® PAG plates pH 3.5-9.5 were used according to the instructions. Twenty μl of the G100 fractions at 100 mg/ml were focused in parallel with 10 μl of low pI protein markers (pH 2.5-6.5, Pharmacia). The pH gradient was also determined by placing a surface pH electrode at 1-cm points across the gel at the end of the experiment. The plate was stained for protein with 0.1% Coomassie blue R250.

Chromatofocusing

Polybuffer™ exchanger 94 gel (PBE94) was equilibrated with 0.05 M imidazole/HCl buffer pH 7.4 in a column (30 cm $\times$ 1.6 cm) and 2-cm swollen Sephadex G25 packed on top of it to ensure even sample application. The sample consisted of 6 ml of the most allergenic fractions of a G100 column (fractions 76-98, concentrated $\times 12$). The column was eluted with Polybuffer™ 74, 1:8 at pH 4 and 120 fractions of 4 ml were collected and pooled, such that the pH difference of the contents was not greater than 0.1 of a unit. Following dialysis against 0.05 M NH_4HCO_3 and lyophilization, rocket radioimmuno-electrophoresis was performed on the pooled fractions (concentrated $\times 40$).

Crossed radioimmuno-electrophoresis (CRIE)

These tests were performed by the method of Lowenstein (1978) with the following modifications regarding the preparation of plates: 7.5 ml of molten 1% agarose in 25% veronal buffer, pH 8.6, was pipetted on to 8-cm² glass slides which had been coated with 0.1% agarose. Antigen was electrophoresed at 5 V/cm for 90 min in the first dimension, and at 1.4 V/cm for 20 hr in the second dimension. The 5 $\times$ 3.5 cm 'window' contained a rabbit antiserum to an unfractionated extract of *C. lewisi* as described by Longbottom *et al.* (1976).

The washed plates were dried down under filter paper, first with weights and then cool air. Four ml of a 1:10 dilution of pooled sera from patients hypersensitive to *C. lewisi* was allowed to react over the window area for 16 hr. After three washes this was followed by a further 16-hr incubation with 4 ml of the tracer, ¹²⁵I-anti-IgE

(Pharmacia) (100 μ l contained approximately 12 000 CPM). The washed, dried plates were then exposed to Kodak NS-2T x-ray film for 7 days. Films were developed for 4 min in Kodak DX80 developer (1:5) and fixed for 6 min in Kodak FX 40 liquid fixer (1:5) with 25 ml/l Kodak HX40 hardener added. The glass plate was stained with 0.5% Coomassie blue R250. Negative control plates were always processed in parallel. In these, sera from individuals not sensitive to *C. lewisi* replaced the 'allergic' sera.

Rocket radioimmuno-electrophoresis (RRIE)

Diffusion was allowed to take place for 45 min before the antigens (G100 fractions) were electrophoresed directly into the rabbit *anti-C. lewisi* antiserum at 1.4 V/cm for 20 hr. One microlitre crude *C. lewisi* extract was run in parallel as a positive control. One microlitre of fractions 76–100 instead of 5 microlitres was electrophoresed into the antiserum to enable the rocket reactions to take place in the 'window'. The plates were then processed as for CRIE.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

12.5% SDS-PAGE gels were prepared by the method of Laemmli (1970), between two clamped glass plates (10 $\times$ 8 $\times$ 0.2 cm), separated by plastic spacers and the edges sealed with molten vaseline. A 3% acrylamide stacking gel was added to improve banding of the proteins.

Lyophilized samples were solubilized in 0.5 M Tris/HCl buffer pH 6.8 containing 3% SDS and 5% mercaptoethanol at 60°C for 30 min. The prepared samples, coloured with 0.01% bromophenol blue, were electrophoresed at 15 mA until they entered the lower gel. The current was then raised to 25 mA and electrophoresis continued for a total of 4.25 hr. After fixing in 25% isopropanol/10% acetic acid for 1 hr the gel was stained in 0.1% Coomassie Blue R250 for 16 hr and then destained in 8% acetic acid until the background was colourless.

Rabbit antisera

Antisera were prepared to an unfractionated extract of *C. lewisi* in New Zealand white rabbits by immunization with 35-mg dry weight of extract (total protein content 14.7 mg) in incomplete Freund's adjuvant via two intramuscular and two subcutaneous injections per rabbit. The rabbits were boosted with 20 mg *C. lewisi* extract in incomplete Freund's adjuvant 5 weeks later and with 4 ml of extract in normal saline into two thigh muscles at intervals thereafter. Pooled sera from several bleeds were used for CIE.

Results

Radioallergosorbent tests (RAST) and RAST inhibition

Preliminary experiments were undertaken to determine the optimal conditions for RAST and RAST inhibition. Maximal binding of IgE was achieved following a 16-hr incubation with APC (100 μ l at 6.25% concentration) and 50 μ l of the patient's serum. The percentage binding of the isotope was proportional to the dilution of serum.

When various dilutions of serum from hypersensitive individuals were preincubated with increasing concentrations of an unfractionated extract of *C. lewisi* there was a dose-dependent inhibition of RAST (Fig. 1). When the same experiments were performed with an extract of an unrelated Sudanese seasonal allergen (Neem tree

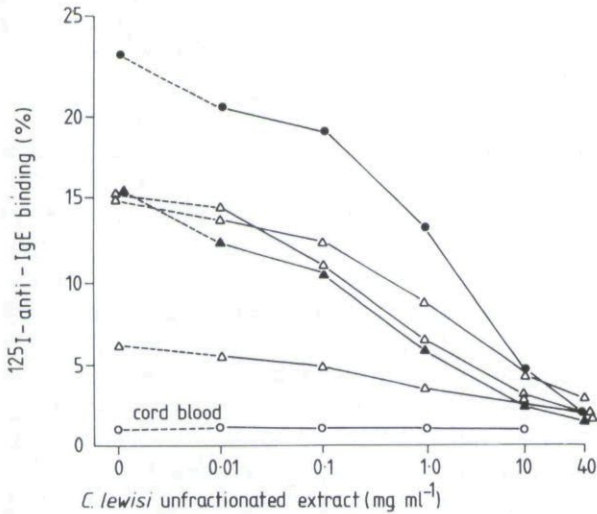


Fig. 1. *C. lewisi* RAST inhibition with an unfractionated extract of *C. lewisi*. Sera were obtained from five patients hypersensitive to green nimiti. Sera were diluted 1 in 16 (●), 1 in 32 (▲); undiluted (△); cord blood (○). A total of 22 500 cpm were added to each assay tube.

pollen, *Azadirachta indica*) there was no appreciable inhibition of RAST over a concentration range comparable to that used for *C. lewisi*.

The relationship of skin-prick-test reactivity to isotope binding in the RAST is shown in Fig. 2. Twenty-four U.K. skin-test-negative controls, sixteen of whom were atopic, gave between 0.6 and 2.6% binding. In contrast, sensitive subjects with weal 'areas' of 1–20 mm² gave values between 4 and 21% and the patients giving a more

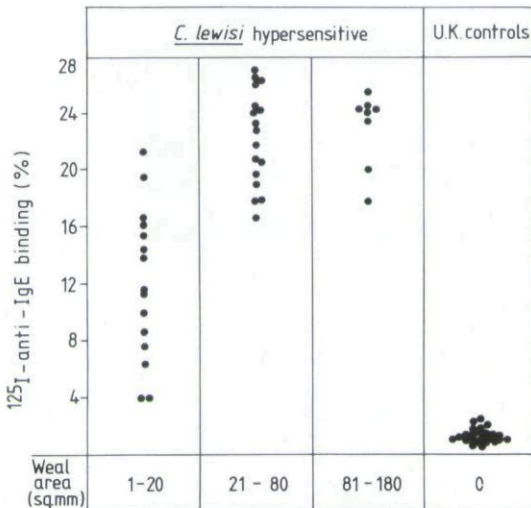


Fig. 2. Comparison of *C. lewisi* RAST and skin-prick test. The skin-testing solution was prepared by dissolving the unfractionated *C. lewisi* extract at 1 mg/ml in Coca's solution glycol (1:1), and passing it through a 0.45 micron Millipore membrane filter before use. A control skin test was performed with Coca's solution glycerol at the same time as the *C. lewisi* skin tests, the weal 'area' of this (max. × min. diameter) was deducted from that produced by the test solution.

vigorous skin-prick-test response, i.e. 21–180 mm², had values in the RAST of between 17 and 27%. These values were statistically highly significant (1–20-mm² weals vs 0-mm² weals ($P < 0.005$), 1–20-mm² weals vs 21–80-mm² weals ($P < 0.025$), 21–80-mm² weals vs 81–180-mm² weals (not significant)).

The results of CRIE using antisera to an unfractionated extract of *C. lewisi* raised in rabbits and sera from *C. lewisi* hypersensitive patients and a non-sensitive U.K. control are shown in Fig. 3. Sera from the allergic individuals produced an intense diffuse radiostaining peak which did not appear on the CIE, i.e. without autoradiography. These differences were more marked when the allergen was diluted 3-fold. With the diluted allergen at least four diffuse broad bands reacting with the ¹²⁵I-anti-IgE were observed. These were not visible in the CIE. Radioactive uptake was also observed in association with the major precipitin peaks, i.e. those giving a clear protein stain on CIE. The negative control gave negligible uptake of the isotope. This procedure was performed routinely in all further autoradiographic studies and gave similar results.

Extracts of *C. lewisi* were applied to columns of Sephadex G100 and a representative optical density profile at 280 nm, together with the degree of RAST inhibition and RRIE 'scores' are shown in Fig. 4. There were two principal protein peaks, one at V_0 and a larger, broader one associated with molecules of < 10 000 daltons. Maximum RAST inhibition activity was found with material having a mol. wt of approximately 20 000 daltons. This area also corresponded to the maximum uptake of ¹²⁵I-anti-IgE using the RRIE technique (Fig. 5). The degree of RAST inhibition in the material eluting at V_0 was approximately 40% compared with 65% for the major allergenic area and 16% for the low (i.e. < 10 000 daltons) mol. wt material. This gel filtration experiment was repeated on three occasions and gave virtually identical results. When the Sephadex G100 procedure was performed in the presence of 2 M

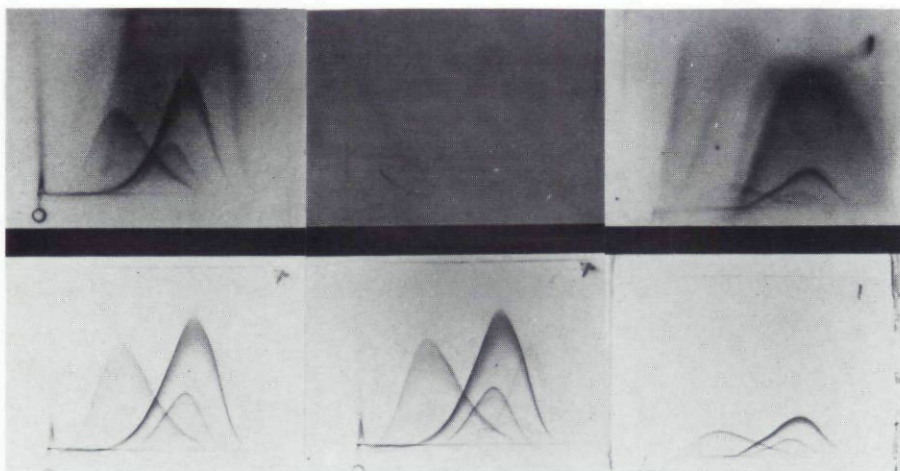


Fig. 3. Crossed radioimmuno-electrophoresis (CRIE) of unfractionated *C. lewisi* extract. Bottom row: plates stained for protein. Top row: autoradiographs from the same plates. In the 1st dimension 3.4 μ l of unfractionated *C. lewisi* extract (20 mg/ml) were electrophoresed in the left and central plates, and 1 μ l of same in the right plate. In the second dimension, the separated antigen was electrophoresed into 'windows' containing 200 μ l of rabbit anti-unfractionated *C. lewisi* extract antiserum and 2.2 ml agarose in all plates. In the left and right plates the overlay contained 4-ml human serum (1:10) from patients hypersensitive to *C. lewisi*. The overlay of the central plate contained 4-ml human serum (1:10) from a non-allergic subject.

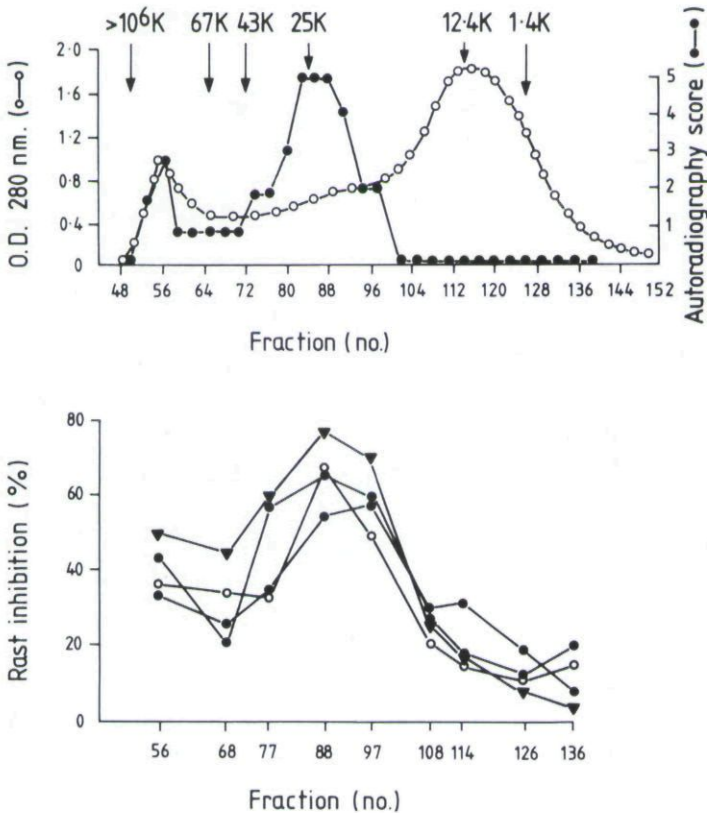


Fig. 4. Sephadex G100 chromatography of *C. lewisi* extract. The column was calibrated with markers of known mol. wt as indicated. The autoradiography score was compiled on the basis of the uptake of radiolabelled anti-IgE as judged from the intensity of exposure. *C. lewisi* RAST inhibition was performed with sera from four hypersensitive patients using triads of fractions concentrated 12-fold.

NaCl and the fractions subsequently dialysed against water the same profile of RAST inhibition was observed.

The mol. wt of the major allergen area, i.e. fractions 86–88 in Fig. 4, were determined by SDS-PAGE and estimated to be 13 000–18 000 daltons (Fig. 6). The pI of this major allergenic material was estimated by isoelectric focusing. Fourteen protein bands were observed which were confined to the pH range 3.5–5.5. Confirmation of these pI values was obtained by subjecting the same G100 fractions to chromatofocusing on Polybuffer exchanger 94 and the fractions eluted were then screened for allergens by RRIE (Fig. 7). No uptake of the isotope was observed between pH 6.6 and 5.3. From pH 5.2 to 3.9, approximately, there were two broad bands visible by autoradiography. The more intense lower band was observed across this pH range, whereas the upper band had a slightly narrower pH range of 4.8 to 3.9.

Discussion

In the present investigation, as in earlier studies, a whole-body midge extract, rather than a homogenized one, was used since hypersensitive individuals develop symptoms within minutes of being exposed to live midges. In this study, the RAST has been slightly modified, compared to the conditions previously reported (Gad El Rab & Kay,

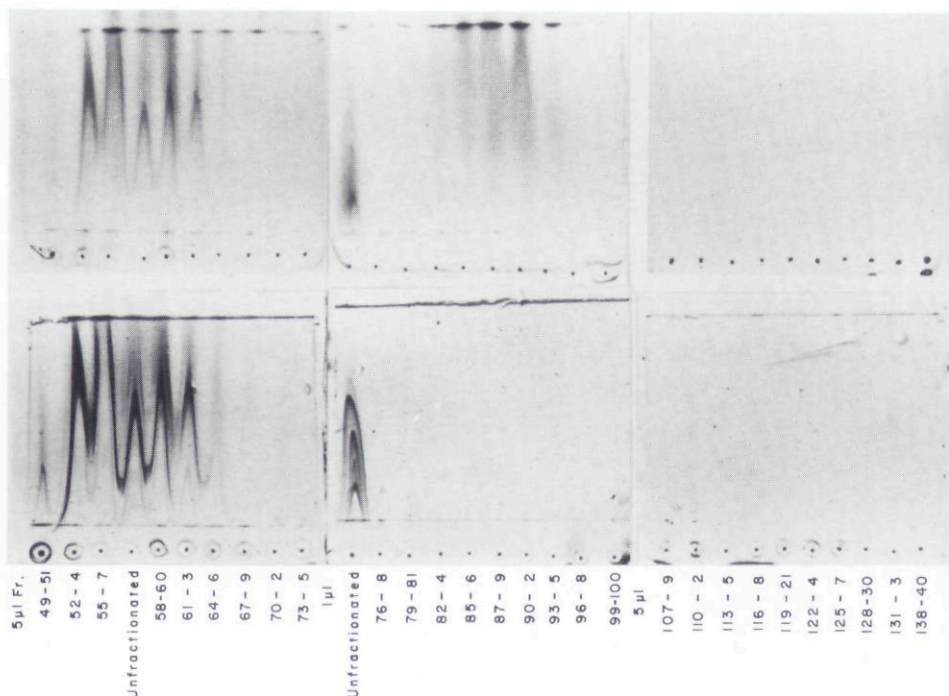


Fig. 5. Rocket radioimmuno-electrophoresis (RRIE) of fractions of *C. lewisi* extract from Sephadex G100. Bottom row: plates stained for protein. Top row: autoradiographs from the same plates.

1980) and there is now minimal binding of the isotope using sera from UK skin-test-negative controls (i.e. between 0.6% and 2.6%). This has been achieved by altering the conditions of allergen extraction and by lowering the percentage of allergen polymer complex from 25% to 6.25%. A satisfactory dose-response curve was obtained in RAST inhibition with unfractionated *C. lewisi* extract (Fig. 1) indicating that this technique was suitable for allergen characterization. The improved RAST assay also enabled us to appreciate clearer separation between controls and the hypersensitive individuals (Fig. 2). Thus there was a clear statistical difference between the skin-prick-test response and the RAST scores when patients with differing degrees of hypersensitivity to the green-nimitti allergen were compared, e.g. RAST values in those with 1–20-mm² weals *vs* those with 21–80-mm² weals ($P < 0.005$). The specificity of the RAST was further confirmed by showing that the Neem pollen allergen (*Azadirachta indica*), also known to be a cause of immediate-type symptoms in the Sudan (R. D. Tee & A. B. Kay, manuscript in preparation) did not cross-react in the *C. lewisi* RAST.

Crossed radioimmuno-electrophoresis, a recognized technique for the identification of allergens, was used in the present study. Throughout the study the CRIE and rocket precipitin peaks (Figs 3 and 5) were always associated with uptake of ¹²⁵I-anti-IgE. Whilst some of this may be non-specific uptake due to co-precipitation, this higher mol. wt material (fractions 52–60 in Fig. 5) was shown to be allergenic by inducing 40% inhibition of the *C. lewisi* RAST (Fig. 4). In order to determine whether the higher mol. wt allergen was derived from aggregation of the lower mol. wt material,

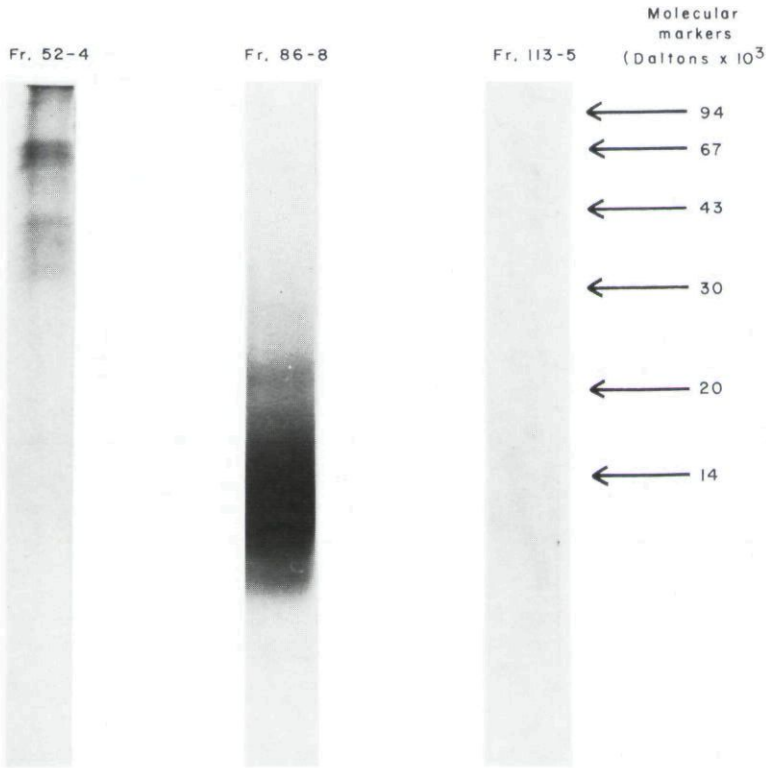


Fig. 6. Sodium dodecyl sulphate/polyacrylamide gel electrophoresis of fractions of *C. lewisi* extract from Sephadex G100 column. Twenty-microlitre samples of reconstituted lyophilized material at 100 mg/ml were applied to the gels. A reference sample contained proteins of known mol. wt (phosphorylase B, 94K); albumin, 67K; ovalbumin, 43K; carbonic anhydrase, 30K; trypsin inhibitor, 20.1K; and α -lactalbumin, 14.4K).

the gel filtration columns were eluted with 2 M NaCl, but on testing the fractions by RAST inhibition, no appreciable alteration in allergenicity had occurred.

The main allergen area (fractions 76–97) gave diffuse radiostaining, characteristic of high IgE binding but little or no protein precipitation (Fig. 5). This apparent lack of protein precipitate may be due in part to the poor sensitivity of Coomassie blue and to the fact that glycoproteins, especially those of low mol. wt, tend to give diffuse precipitin peaks (Lowenstein, 1978). The maximum allergen activity was shown by RAST inhibition (fractions 87–9) to have a molecular size of approximately 17 000 daltons (Fig. 4).

Isoelectric focusing indicated that the major allergens were a group of closely related proteins with pIs ranging from 3.5 to 5.5. These values were confirmed by chromatofocusing where fractions tested by RRIE bound the radiolabelled anti-IgE between pH 5.2 and 3.9 only (Fig. 7). The present studies confirm the findings of Gad El Rab, Thatcher & Kay (1980), who used skin-prick-test reactivity as the biological assay. In addition to the 15–20 000 daltons acidic peptides, we have shown that there was allergenic material of higher mol. wt. The RAST inhibition gives a more quantitative measure of allergenicity than testing fractions by direct skin-prick tests and correlated well with the autoradiographic findings.

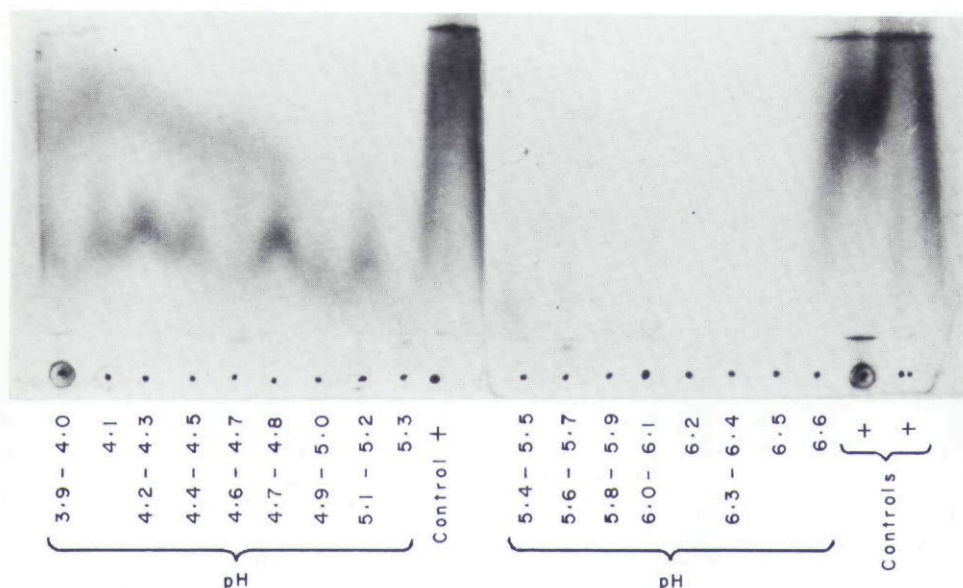


Fig. 7. Autoradiographs of rocket radioimmuno-electrophoresis of fractions eluting from a chromatofocusing column between pH 6.6 and 3.9. The chromatofocusing column was loaded with fractions 76-98 from the Sephadex G100 separation.

Similar studies have recently been undertaken with sera from subjects hypersensitive to *Chironomus thummi thummi* (CTT) (Baur *et al.*, 1982). Allergic symptoms in individuals who handle freeze-dried larvae of this chironomid are well recognized since the material is widely used in Europe as pet fish food. Chironomids are known to possess low mol. wt haemoglobins which show a high degree of polymorphism. Eleven haemoglobins were previously identified from CTT and their primary structures have been completely elucidated (summarized in Baur *et al.*, 1982). By RAST and RAST inhibition it was shown that the haemoglobins were allergenic and that specific antibody against them accounted for a proportion of the 'total' IgE in individuals hypersensitive to CTT larvae (Baur *et al.*, 1982). Preliminary observations (R.D. Tee, unpublished) indicate that there was variable inhibition of the *C. lewisi* RAST by CTT haemoglobin (kindly supplied by Dr X. Baur). This suggests that haemoglobins are unlikely to account for the total allergenicity in all hypersensitive individuals. Further studies on the relationship between *C. lewisi* allergens and the CTT haemoglobins are in progress.

Acknowledgment

This study was supported by the Wellcome Trust.

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