

Further characterisation of allergens associated with hypersensitivity to the "green nimitti" midge (*Cladotanytarsus lewisi*, Diptera: Chironomidae)

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Chironomid midges are small (2-15 mm) non-biting flies, characteristically seen swarming by water at dusk. Allergens of the "green nimitti" midge, *Cladotanytarsus lewisi* (Freeman) (Diptera: Chironomidae), a cause of widespread hypersensitivity in the Sudan, were isolated and partially characterized by Sephadryl S200 chromatography. The allergenicity of the fractions was identified by "rocket" autoradiography, RAST inhibition, skin "prick" tests, and the immunoblot technique. The fractions were further analysed by isoelectric focusing and SDS-PAGE. Two major allergens with pI's ranging from 4.3 to 6.0 were identified and had molecular weights of approximately 17,000 and 32,000 daltons, sizes compatible with their being monomeric and dimeric haemoglobins. Since chironomids occur in nuisance numbers worldwide and their haemoglobins have been shown to produce severe hypersensitivity reactions in man, they should be seen as an important potential cause of environmental and occupational allergy.

Key words: antigenic determinants; Chironomidae; haemoglobin; IgE; immunoblot; RAST.

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Allergens of the non-biting midge *Cladotanytarsus lewisi* (Freeman) (Diptera: Chironomidae) (Plate I), are an important cause of IgE-mediated hypersensitivity in an appreciable proportion of the Nilotic Sudanese population (4, 5, 7, 8). Preliminary attempts to chemically characterise the major allergen(s) (using skin "prick" tests) indicated that these were acidic glycoproteins in the molecular weight range 15,000-20,000 daltons, having a pI of approximately 4.3 (3). In a further study, employing more definitive techniques such as RAST inhibition, fused rocket radioimmuno-electrophoresis (FRRIE) and crossed radio-immunoelectrophoresis (CRIE), it was confirmed that allergenicity was associated with molecules of approximately 17,000 daltons (9).

By *in vivo* and *in vitro* studies the present paper confirms and extends these observations with evidence of an additional major allergen of approximately 32,000 daltons. Skin prick testing and

assays depending on the binding of ¹²⁵I-anti-IgE to allergen IgE complexes, including the immunoblot, were used to determine the relative allergenicities and molecular sizes of four pertinent fractions produced by Sephadryl S200 gel filtration.

MATERIAL AND METHODS

Preparation of antigen. Adult midges were collected and an aqueous extract made as described previously (10).

Human sera. Sudanese patients who attributed their symptoms of bronchial asthma and/or allergic rhinitis to *C. lewisi*, and who also had a positive skin "prick" test to *C. lewisi* extract, were bled for antibody studies.

Rabbit antisera. Antisera were prepared to an

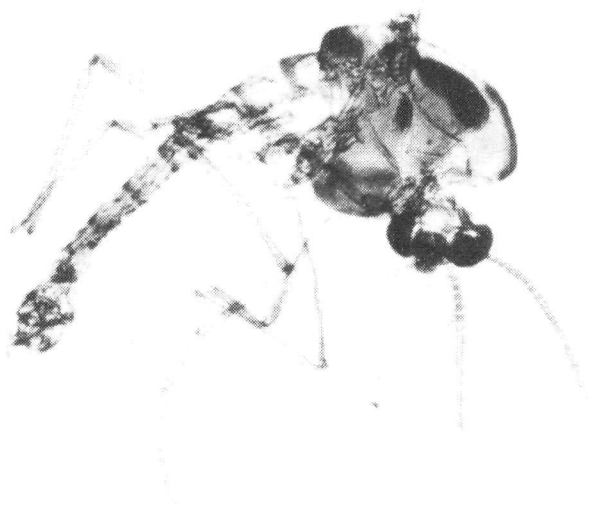


Plate 1. *Cladotanytarsus lewisi* adult midge, body length 2 mm.

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 FRRIE.

Gel filtration. A column of Sephacryl S200 (90 × 2.6 cm) (Pharmacia, Uppsala, Sweden) was equilibrated with 0.05 M NH_4HCO_3 at 29.5 ml Hr^{-1} . The column was calibrated with blue dextran ($> 10^6\text{K}$), Aldolase (158K), bovine serum albumin (67K), ovalbumin (43K), chymotrypsinogen (25K), ribonuclease (13.7K) and vitamin B_{12} (1.4K). One hundred mg of lyophilised adult *C. lewisi* extract was applied to

the column and 4 ml fractions collected. Fused rocket radioimmuno-electrophoresis was performed to test which of the fractions contained allergens (Fig. 1). When a greater quantity of *C. lewisi* extract was applied to the column, two additional areas of increased protein content, 2 and 3, were resolved. Fig. 2a represents the OD profile at 280 nm of the gel filtration of 200 mg of *C. lewisi* extract. To obtain a requisite amount of study material of approximate molecular weight 32 and 17K daltons (maximum RAST inhibition found with these fractions) this chromatography was repeated four times. Fraction areas marked 1, 2, 3 and 4 from the four fractionations of 200 mg of extract were each pooled individually and purified further by rechromatography on the S200 column as shown in b, c, d and e respectively. These fractions were then lyophilised and used for skin prick testing and further studies.

Radioallergosorbent test (RAST)

1. Preparation of allergen polymer complex (APC)

Ten mg of lyophilised, unfractionated adult *C. lewisi* extract were coupled per gram of CNBr-activated Sepharose 4B (Pharmacia). Lyophilised fractions 1, 2, 3 and 4 from the Sephacryl S200 filtrations were coupled similarly.

2. Assay

Optimum conditions for the RAST assays were determined as two 16 h incubations with 50 μl of serum and 100 μl of APC (6.25 vol % concentration) in the first and 50 μl ^{125}I -anti-IgE (Pharmacia) in the second. Cord blood sera with no demonstrable IgE were used in all assays as negative controls.

RAST inhibition with sera from patients hypersensitive to C. lewisi

Each alternate fraction (50) from a Sephacryl S200 fractionation of 100 mg *C. lewisi* extract was lyophilised and reconstituted in distilled water ($\times 8$ concentration). One hundred μl of each fraction was agitated for 16 h with an equal volume of sera pooled from seven patients hypersensitive to *C.*

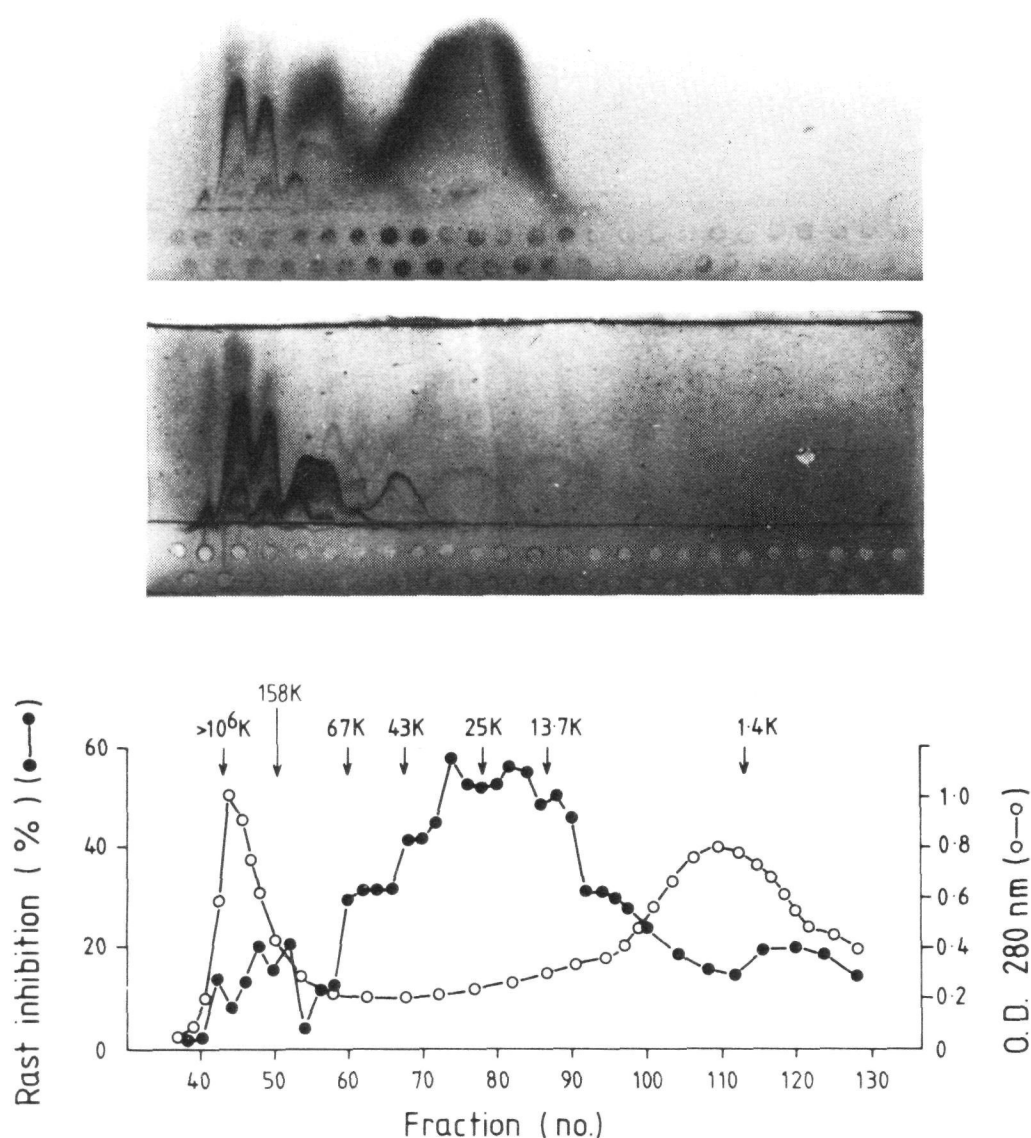


Fig. 1. Sephacryl S200 chromatography of an extract of *C. lewisi*. Bottom frame: The column was calibrated with markers of known molecular weight as indicated. *C. lewisi* RAST inhibition with pooled sera from seven hypersensitive patients preincubated with fractions concentrated 8-fold. Fused rocket radioimmuno-electrophoresis (FRRIE) of fractions. Middle frame: Platelets stained for protein. Top frame: Autoradiographs from the same plate.

lewisi. The RAST with the unfractionated *C. lewisi* extract was then performed in triplicate, and percentage binding of the ¹²⁵I-anti-IgE compared with that of the unabsorbed serum pool.

Skin "prick" testing

Twenty-six Sudanese patients previously shown to be hypersensitive to whole body *C. lewisi* were skin "prick" tested with an unfractionated extract of *C. lewisi* and the four Sephacryl S200 fractions 1, 2, 3 and 4. The skin-testing solutions were prepared by dissolving the antigens at 1 mg/ml in Coca's solution and glycerol (1:1) and

passing them through a 0.45 μ filter before use. Histamine dihydrogen chloride at 1 mg/ml was used as a positive control solution and the diluent as a negative control. Tests were performed in duplicate and positioned in reverse order on the second arm. The weal area (maximum $\times$ minimum diameter) of resultant weal was measured after 15 min and any reaction provoked by the negative control deducted from that caused by the test solution. The data obtained was extremely non-normal and therefore distribution-free methods were employed in analysis: in particular the Fisher-Yates analysis of variance by ranks was used to consider weal sizes.

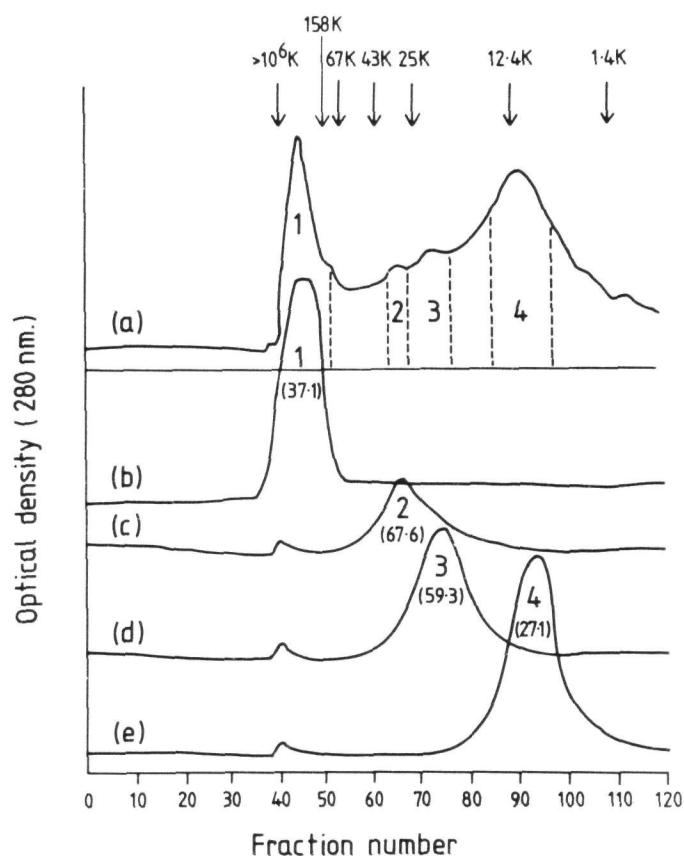


Fig. 2. Sephacryl S200 gel filtration of an extract of adult *C. lewisi*. The column was calibrated with markers of known molecular weight from 1.4K to $> 10^6$ K daltons. (a) 200 mg of unfractionated *C. lewisi* extract; (b), (c), (d) and (e) rechromatography of fractions 1, 2, 3 and 4 respectively from four fractionations of (a). Numbers in parenthesis indicate relative magnitude of the skin test weal responses indicated in text.

Fused rocket radioimmuno-electrophoresis (FRRIE)

Rocket electrophoresis was performed in 1% agarose in 25% Veronal buffer pH 8.6 on gel bond. Diffusion was allowed to take place for 45 min before the alternate Sephacryl fractions (50) were electrophoresed directly into 9% rabbit anti-whole *C. lewisi* extract antiserum in agarose at 1.4 v/cm for 20 h. The plate was then dried down and processed for autoradiography. A 1:10 dilution of pooled sera from patients hypersensitive to *C. lewisi* was allowed to react over the dried gel for 16 h. After three washes this was followed by a further 16 h incubation with the tracer, ^{125} I-anti-IgE (Pharmacia) (100 μ l contained approximately 12,000 CPM). The washed, dried plate was exposed to Kodak NS-2T X-ray film for 7

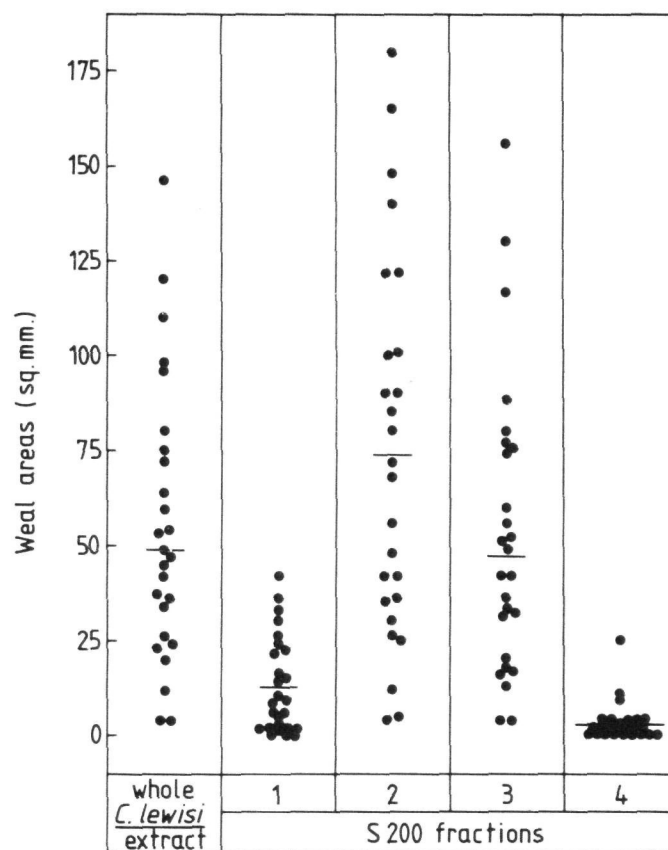


Fig. 3. Skin "prick" test weal responses to whole *C. lewisi* extract and four Sephacryl S200 rechromatographed fractions 1, 2, 3 and 4 (Fig. 2b, c, d and e).

days and then developed. The gel bond plate was stained with 0.5% Coomassie Blue R250. A negative control plate was processed in parallel where sera from individuals not sensitive to *C. lewisi* replaced the "allergic" sera.

Isoelectric focusing

LKB Ampholine® PAG plates pH 3.5–9.5 were used according to the instructions. Ten microlitres of the Sephacryl S200 fractions at 10 mg/ml were focused in parallel with 10 μ l of low pI protein markers (pH 2.5–6.5, Pharmacia) and the plate stained for protein with 0.1% Coomassie Blue R250.

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

A resolving 12.5% SDS-PAGE gel with a 3% acrylamide upper stacking gel was cast by the method of Laemmli (6) in a vertical Protean™ Slab Cell (Biorad). Lyophilised samples of

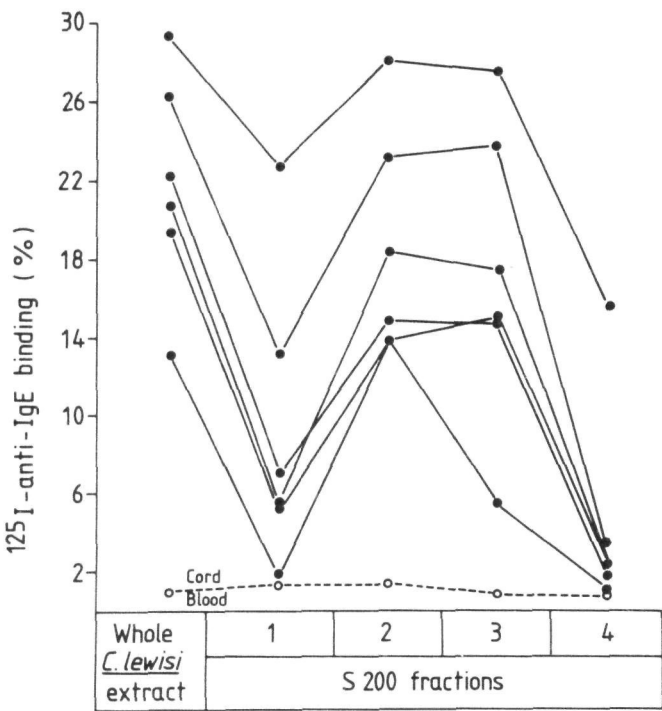


Fig. 4. Comparison of RAST ¹²⁵I-anti-IgE binding in sera from seven patients to whole *C. lewisi* extract and the four Sephacryl S200 of *C. lewisi* extract. Cord blood sera in the same assays indicate the background percentage binding.

whole *C. lewisi* extract and S200 fractions were solubilized in 0.5 M Tris/HCl buffer pH 6.8 containing 3% SDS at 100°C for 15 min. Equal quantities of the prepared samples and standard high and low molecular weight protein solutions (Pharmacia), solubilized at 100°C for 5 min, were electrophoresed in duplicate at 18 mA for 1 h, followed by 3 h at 30 mA. The gel was cut in two, one half processed by the “immunoblot” technique and the duplicate half fixed in 25 vol % propan-2-ol/10 vol % acetic acid for 1 h, followed by staining in 0.1 g % Coomassie Blue R250 for 16 h and destaining in 8 vol % acetic acid.

Immunoblot

Proteins from the duplicate SDS gel were electrophoretically transferred on to a nitrocellulose sheet as described by Towbin (11). A Trans-Blot™ apparatus (Biorad) was used and the transfer carried out at 0.22 A for 2 h in 25 mM Tris - 192 mM glycine buffer pH 8.3 containing 20% w/v methanol. Any additional protein binding sites were blocked by immersing

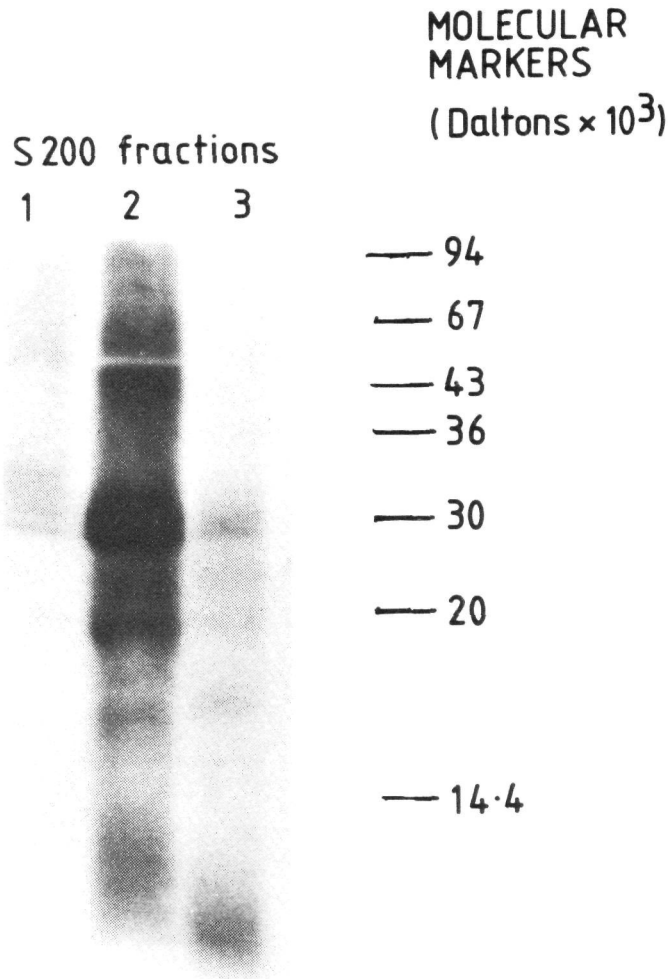


Fig. 5. Immunoblot of SDS-PAGE of Sephacryl S200 fractions 1, 2 and 3 of *C. lewisi* extract.

the nitrocellulose sheet in 2% bovine serum albumin in PBS for 1 h at 39°C. After washing the sheet was processed for autoradiography as for FRIE.

RESULTS

Extracts of adult *C. lewisi* were applied to columns of Sephacryl S200, and a representative optical density profile at 280 nm together with the degree of RAST inhibition produced by the fractions are shown in Fig. 1. Maximum RAST inhibition activity was found with material having molecular weights of approximately 32,000 and 17,000 daltons. RAST inhibition was also produced by higher molecular weight material (> 67K) but maximally was only one third (20%) of that produced by the major allergenic area (60%). These results were in agreement

with those found by FRRIE (Fig. 1) where the maximum uptake of ^{125}I -anti-IgE was by fractions 67-87 (approximate molecular weight 43K-14K) in an area of relatively low protein concentration. The negative control FRRIE plate gave negligible uptake of the isotope.

When a greater weight of *C. lewisi* extract (200 mg) was applied to the Sephacryl S200 column, four elevated protein areas 1, 2, 3, 4 were differentiated (Fig. 2a). These were pooled individually from four columns and rechromatographed (Fig. 2b, c, d, e), when areas 2 and 3 became better defined, despite their low protein content. They had molecular weights of approximately 32,000 and 17,000 daltons respectively and corresponded to the two peaks found previously by RAST inhibition.

The skin "prick" test responses to the S200 fractions 1, 2, 3, 4 in 26 Sudanese subjects hypersensitive to *C. lewisi* were compared with their responses to the whole extract and are shown in Fig. 3, where the bars represent the mean response. The weal data obtained was ranked and the Fisher-Yates analysis of variance test applied after the mean "scores" were rescaled to a mean value of 50. The scores of the S200 fractions 1, 2, 3, 4 were 37.1, 67.6, 59.3 and 27.1 respectively and are shown in Fig. 2 in parentheses under each area. The score for unfractionated *C. lewisi* was 62.3, thus the weal response to fraction 2 was significantly greater ($P < 0.05$) than that to either the whole extract or fraction 3 (which were not significantly different from each other). These were both significantly greater ($P < 0.001$) than the high molecular weight fraction 1; and this in turn was significantly greater than the low molecular weight fraction 4 ($P < 0.001$).

Relative specific IgE antibody levels to the four S200 fractions were compared in six subjects hypersensitive to *C. lewisi* by coupling equivalent dried weight quantities of the four fractions and a whole extract to CNBr activated Sepharose 4B. ^{125}I -anti-IgE binding was highest in the fraction 2 and fraction 3 RAST assays (mean 18.8 and 17.4% binding respectively of total counts of isotope added), lower in the high molecular weight fraction RAST (mean 9.3% binding) and lowest in the low molecular weight

fraction 4 assay (mean 4.4% binding) (Fig. 4). Cord blood sera, present as negative control gave comparable binding in all the assays (mean 1.1% binding).

With isoelectric focusing all bands of the four rechromatographed S200 fractions were below pI 6.3. Those of fraction 1 ranged from pI 5.2 to 6.1 and those of the major allergenic fractions 2 and 3 (15 bands) from pI 4.3 to 6.0. An equal weight of fraction 4 (0.1 mg) was focused, but was insufficient for any bands to stain.

The immunoprint from a SDS-PAGE polyacrylamide gel of the non-reduced S200 fractions is shown in Fig. 5. By referring to the known molecular weight standards in the duplicate gel, the high molecular weight fraction 1 was found to have allergen bands at 30,000 to 32,000 daltons. Whilst the strongest allergen bands in fraction 2 were of these molecular weights, additional allergen bands between 43 and 67K and at 20,000 and 17,000 daltons were also found, due to incomplete size separation by the gel filtration. Fraction 3 had allergen bands of 30,000 to 32,000 and 17,000 daltons, whilst the low molecular weight fraction 4 showed no allergen bands. Material of approximately 14,000 daltons molecular weight, possibly degradation product(s), was also shown to be allergenic. The negative control blot, where sera from individuals not sensitive to *C. lewisi* replaced the allergic sera, gave negligible uptake of the isotope.

DISCUSSION

Fused rocket radioimmuno-electrophoresis (FRRIE) was used as a method to ascertain which of the Sephacryl S200 fractions contained allergens. Some uptake of the ^{125}I -anti-IgE did occur with the high molecular weight fractions ($> 67\text{K}$), but the intense diffuse radiostaining, typical of high IgE binding, was seen in the area of material of molecular weight 43K-14K. RAST inhibition, a more definitive technique to estimate relative allergenicity of fractions, indicated two peaks of maximum activity at approximately 32,000 and 17,000 molecular weight within the allergen area depicted by FRRIE. RAST inhi-

bition also verified that the higher molecular weight material ($> 67K$) did contain allergen, but only a third of that of the major allergen.

Loading more *C. lewisi* midge extract on the Sephacryl S200 column differentiated four main protein areas. The four areas were further purified and concentrated by rechromatography and, after lyophilisation, various *in vitro* procedures were performed on them and compared with *in vivo* skin "prick" testing.

The allergen area 2, with an approximate molecular weight of 32,000 daltons elicited a significantly greater skin test response in 26 Sudanese patients than did unfractionated *C. lewisi* midge extract. The allergen area 3, with an approximate molecular weight of 17,000 provoked a response equivalent to that of the whole extract. The higher molecular weight fraction 1 and the lower molecular weight 4 each elicited much smaller skin test weal areas, none higher than the response to the whole extract.

Confirmation of the relative allergenicity of the fractions was shown in the comparison of ^{125}I -anti-IgE binding in RAST assays where equivalent dry weights of the individual fractions were coupled to the solid phase. The mean percentage binding of the isotope was highest and comparable in the fractions 2 and 3 RAST assays (18.8% and 17.4% respectively of total counts added), with much lower binding in the high molecular weight fraction RAST (9.3%) and still lower in the fraction 4 RAST (4.4%). These assays further corroborate that the high molecular weight fraction 1 is much less allergenic than fractions 2 and 3.

The immunoblot technique confirmed the main allergen in fraction 2 to be between 30,000 and 32,000 daltons molecular weight, whilst fraction 3 contained allergen of 17,000 daltons. Allergen of 32,000 daltons was also found in the high molecular weight fraction but no allergen was demonstrated in the low molecular weight fraction 4 (not shown).

By use of these high resolution techniques the major allergenic activity is clearly associated with fractions 2 and 3, 32,000 and 17,000 daltons molecular weight respectively. Whilst fraction 4 is shown to have some allergenic activity

by RAST, RAST inhibition and skin "prick" test, more fraction material is required to demonstrate allergenicity by FRIE and the immunoblot. This may be partly accounted for by the use of different "allergic sera" in the various methods, but the main dissimilarity is probably due to the greater sensitivity of the RAST and skin test techniques.

Baur and his colleagues (1) have shown that the degree of immunological cross-reactivity between 14 chironomid species investigated (including *C. lewisi*) derives at least predominantly from haemoglobin components with common antigenic determinants in the different species. In collaboration with our German colleagues we have demonstrated cross-reactivity by RAST inhibition and skin "prick" testing between *C. lewisi* and *Chironomus riparius* (10), a chironomid whose larvae is widely used in Europe as pet fish food and which causes allergic symptoms in individuals occupationally exposed to them.

It is significant that the molecular weights of the allergens reported here coincide closely with those reported for dimeric and monomeric haemoglobin and our results strengthen the view that the *C. lewisi* allergens are haemoglobins (2, 9).

Due to the ubiquity of chironomids in the world and the evidence of extensive immunological cross-reactivity between species, they should be seen as sources of significant potential environmental and occupational allergens.

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