

Widespread IgE-mediated hypersensitivity in the Sudan to the 'green nimitti' midge, *Cladotanytarsus lewisi* (Diptera: Chironomidae)

II. IDENTIFICATION OF A MAJOR ALLERGEN

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SUMMARY

A major allergen has been identified in an aqueous extract of the 'green nimitti' midge, *Cladotanytarsus lewisi* (Diptera: Chironomidae). Following chromatography on Sephadex G-100 allergenic activity, as assessed by skin ('prick') testing, eluted as two closely related peaks (pools I and II) at about 50% bed volume. When these pools were applied separately to columns of CM-cellulose, activity in each eluted with 0.05 M NaCl. Isoelectric focusing of the unfractionated allergen gave a single peak of activity at pI 4.3. By SDS-PAGE, biological activity in the whole 'green nimitti' extract and the material eluting from both pools I and II of the Sephadex G-100 column migrated to the same positions and were associated with a molecular size of 15,000–20,000 daltons. Skin test reactivity of the unfractionated material and the Sephadex G-100 pool I and II eluates were all destroyed following incubation with trypsin, chymotrypsin, thermolysin and neuraminidase. These experiments indicate that a major allergen derived from the 'green nimitti' midge, a cause of widespread and severe immediate-type allergy in the Sudan, is an acidic glycoprotein of 15,000–20,000 molecular weight.

INTRODUCTION

In a previous report we described a radioallergosorbent test (RAST) for identifying individuals hypersensitive to the 'green nimitti' midge, *Cladotanytarsus lewisi* (Diptera: Chironomidae) (Gad El Rab & Kay, 1980). This important and widespread hypersensitivity in the Sudan has been recognized since about 1925 and is probably due to the working of dams which has produced lake-like conditions on the White, Blue and main Niles where breeding of the midges has evidently increased (Lewis, 1956; Lewis, Henry & Grindley, 1954). Because of the magnitude of this problem it seemed important to obtain information on the chemical characteristics of the allergic principle in the midge extract. In the present study we show that a major allergen appears to be an acidic glycoprotein of molecular size 15,000 to 20,000 daltons.

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MATERIALS AND METHODS

Preparation of the antigen

Adult midges were collected by a light trap sample and identified by Drs D. J. Lewis and P. S. Cranston, Department of Entomology, British Museum (Natural History), as *Cladotanytarsus lewisi*, less than 1% of over 500 of the insects counted belonging to different species of chironomids (Gad El Rab & Kay, 1980). They were dried at room temperature and kept in plastic bags at -20°C until use.

Twenty grams of midges (1 g approx. 18,000 midges) were defatted by vigorously shaking with ether (Gad El Rab & Kay, 1980). The material was then allowed to settle and the remaining ether removed by evaporation. Extraction was performed with 200 ml of Coca's solution and the midges allowed to soak for 48 hr at room temperature with occasional agitation. The extract was concentrated to one-tenth of the original volume by dialysis in air before passage through Millipore filter units (Swinnex 25, Millipore UK Ltd). The final material had a dry weight of $111\text{ mg}\cdot\text{ml}^{-1}$ and a protein content of $5\text{ mg}\cdot\text{ml}^{-1}$ as assessed by the Coomassie blue method. The extract was stored at 4°C until use.

Gel filtration

A column of Sephadex G-100 ($100\times 5\text{ cm}$) (Pharmacia, Uppsala, Sweden) was equilibrated with phosphate-buffered saline (PBS), pH 7.2, at 4°C . Four millilitres of the 'green nimitti' extract ($6\text{ mg}\cdot\text{ml}^{-1}$) were then applied and eluted with PBS. Two-millilitre fractions were collected at a flow rate of $60\text{ ml}\cdot\text{hr}^{-1}$. The column was calibrated with blue dextran and vitamin B₁₂ (mol. wt 1,500). The chromatographic procedure was performed three times at 4°C . Every tenth tube was tested for biological activity. The activity was measured by skin ('prick') testing of a 'green nimitti' sensitive individual (M.O.G.) although ten other hypersensitive individuals were tested in the final purification steps (see Results section). The skin response was assessed as the weal 'area' by multiplying the longest and shortest diameters of the weals. The relative protein concentrations were measured by optical density at 280 nm. Material giving positive skin tests were pooled (as shown in Fig. 1), dialysed against distilled water, lyophilized and reconstituted in distilled water to the original volume applied to the column.

Carboxyl-methyl (CM) cellulose chromatography

A 2-ml syringe was packed with CM-52 cellulose (Pharmacia, Uppsala, Sweden) and equilibrated with 0.1 M acetate buffer in 0.025 M NaCl (pH 5.5). Two microlitres of either pool I or II from the G-100 fractionation step were dialysed against this starting buffer, applied to separate columns and eluted in a stepwise fashion with increasing molarities of NaCl. Four tubes of 0.25 ml were collected after each change of buffer. Each tube was tested for allergenic activity and those giving positive skin reactions were pooled, lyophilized and reconstituted in 2 ml of distilled water.

Isoelectric focusing

Isoelectric focusing (IEF) gels were prepared by boiling 2.6 g of LKB 1802 IEF agarose with 3.3 g of D-sorbitol in 30 ml of distilled water. On clarification the gel solution was cooled to 60°C and 1 ml of LKB ampholines (range pH 3–9) and 1 ml of Pharmacia pharmalytes (range 3–10) were added. The solution was then poured on to a $10\times 20\text{ cm}$ clean glass plate which had been previously warmed at 60°C . Crude nimitti stock extract (1 ml) was desalted into 0.1 M Tris-Cl buffer, pH 8.0, using a $1\times 5\text{ cm}$ column of Sephadex G-25 SF and 20 μl applied to a 4-mm-square glass-fibre pad on the surface of the blotted gel. Visible marker proteins (Pseudomonas C-551 cytochrome, pI 4.7, and azurin, pI 5.4, and horse heart cytochrome c, pI 10.0) were added to the gel surface in a similar manner. Isoelectric focusing occurred after 90 min at 4 W constant power, using 0.5 M NaOH on the cathodic wick and 0.5 M L-aspartic acid on the anode. When the coloured proteins had focused the current was discontinued and the pH gradient determined directly using a 3-mm-diameter flat-ended pH electrode (Russel pH Ltd, Auchtermuchty, Scotland). The gel was then sliced into 1-cm strips and extracted in Coca's solution prior to skin testing.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

It was first established that the crude and G-100 'green nimitti' extract did not lose biological activity following heating at 80°C for 1 hr with 0.1% SDS buffer. Sodium dodecyl sulphate/polyacrylamide gel disc electrophoresis was performed in a slab-gel apparatus (Raven Scientific, Haverhill, Suffolk) using the discontinuous Tris/glycine buffer system described by King & Laemmli (1971). Each gel contained a linear gradient of acrylamide polymer from 30% (w/v) at the bottom to 10% at the top. Samples of the G-100-fractionated 'green nimitti' allergens were exposed to SDS buffer for 15 min at 105°C prior to application to the gel. Electrophoresis was terminated when the bromophenol blue marker dye reached the bottom of the gel. The gel was then removed from the apparatus and sliced. Part of the gel was wrapped in commercial cling film and stored at -15°C until required for skin testing. The remainder of the gel was fixed in methanol/acetic acid/water (5:1:5 by vol.) and stained with 0.1% (w/v) Coomassie blue. The gels were then destained with 10% (v/v) acetic acid and excess dye scavenged with plastic foam test-tube plugs.

Enzyme treatment

The Sephadex G-100 and CM-cellulose fractions (pools I and II) were treated with various proteolytic or glycolytic enzymes as follows. For trypsin, chymotrypsin or thermolysin, 20 µl of enzyme containing 1 mg·ml⁻¹ was incubated with 0.1 ml of allergen at 37°C for 1 hr. With neuraminidase and α-amylase, 10 µl (1 mg·ml⁻¹) was incubated with 0.05 ml of antigen at 37°C and 25°C respectively for 1 hr. Allergenic activity was tested immediately after the addition of enzyme and at 15-min intervals for 1 hr thereafter. Neuraminidase and α-amylase were purchased from the Sigma Chemical Co., St Louis, Missouri. Trypsin (TRSF-6188) and chymotrypsin (1608-9) were purchased from Worthington Biochemical Co., Freehold, New Jersey. Thermolysin was purchased from Chugai Boyaki Co., PO Box 106, Higashi, Osaka, Japan. All the enzymes were homogeneous on SDS-PAGE.

RESULTS

It was first established that the 'green nimitti' extract produced negative skin reactions in individuals who had not been exposed to *Cladotanytarsus lewisi*. Twenty-three atopic and twelve non-atopic British subjects gave entirely negative skin ('prick') tests to the same batch of 'green nimitti' allergen which was used in the previous report (Gad El Rab & Kay, 1980).

In hypersensitive individuals allergenicity was retained for 2 hr after heating at 56°C, after which time it slowly declined, but was still demonstrable at 5 hr.

The aqueous defatted extract of 'green nimitti' had a deep-brown colour before fractionation. When this was applied to a column of Sephadex G-100, the brown material was retarded on the column and eluted after the vit. B₁₂ molecular weight marker (mol. wt 1,500). It had no allergenic activity. All the tubes having skin test reactivity were located in two closely related peaks which eluted at about 50% bed volume. This G-100 Sephadex procedure was performed three times and on each occasion gave virtually identical results. The two peaks of activity were pooled separately as indicated (Fig. 1) and designated pool I and pool II.

Each G-100 pool was then applied separately to columns of CM-cellulose. The activity was eluted by increasing the molarity of NaCl in a stepwise manner (Fig. 2). Skin test activity was detected only with samples eluting with molarity of 0.050 mol·l⁻¹ and to a lesser extent with 0.100 mol·l⁻¹. The pattern of elution with pool I and pool II was virtually identical in three separate experiments.

Whole 'green nimitti' extract was subjected to isoelectric focusing over the pH range 4.2 to 8.5. Four hypersensitive individuals all of whom gave positive skin reactions and a history of asthma following exposure to the midge were tested with the fractions. Gel slices eluting at pH 4.2, 4.3 and 4.4 gave 'weal areas' of more than 10 mm in each subject. The other eleven fractions, prepared by increasing pH up to 8.5, were negative. The strongest weal, in each individual, was at pH 4.3.

Pools I and II and a mixture of these pools (pool I + II) were then subjected to SDS-PAGE. The gels were cut into slices and tested for allergenic activity. With either pool I or pool II only one peak

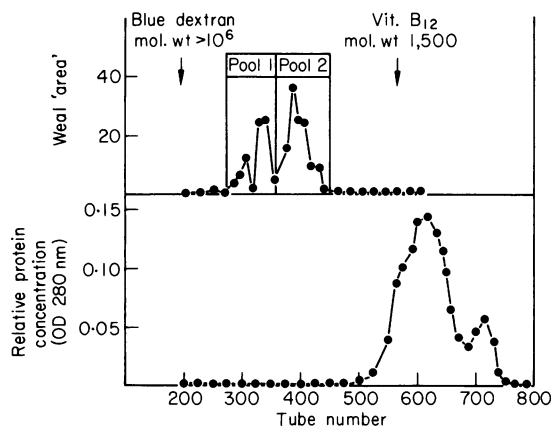


Fig. 1. Sephadex G-100 chromatography of the 'green nimitti' extract. Four millilitres of the 'green nimitti' extract containing $6 \text{ mg} \cdot \text{ml}^{-1}$ was applied to the column. The protein concentrations of pools I and II (following lyophilization and reconstitution—see Materials and Methods section) were 1.5 and $2.0 \text{ mg} \cdot \text{ml}^{-1}$ respectively. Representative of three experiments using one hypersensitive individual for the skin tests. The weal 'area' was obtained by multiplying the longest and shortest diameters.

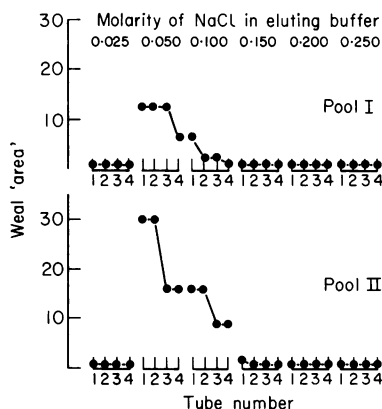


Fig. 2. Carboxyl-methyl (CM) cellulose chromatography of the allergenic activities (pool I or II) from Sephadex G-100 fractionation of the 'green nimitti' extract. Pool I and II fractions from Fig. 1 were used. Representative of three experiments using one hypersensitive individual. The weal 'area' was estimated as in Fig. 1.

of activity was detected (Fig. 3). The material recovered from gel slice nos 19, 20 and 21 were pooled, reconcentrated with lyophilization and reapplied to a gel slab of SDS-PAGE. Following electrophoresis part of the gel was sliced and tested on the skin. The rest of the gel was stained with 0.1% (w/v) Coomassie blue. As before, the single peak of activity was located at gel slice no. 20 but with a small amount of activity at slice no. 19. The material giving a positive skin test appeared to be associated either with a single band (no. 3) or two closely related bands (nos 2 and 3). The SDS-PAGE was repeated three times with the G-100 pool I and pool II, and with pools I and II which had been further purified by CM-cellulose chromatography (Fig. 2). On each occasion the results were virtually identical with a single peak of activity maximal with slice no. 20. In order to exclude the possibility that allergenic material might have been adsorbed on to the Sephadex G-100 column, whole unfractionated 'green nimitti' extract was also applied to SDS-PAGE, cut into slices, tested for allergenic activity and the findings compared with those obtained with pools I and

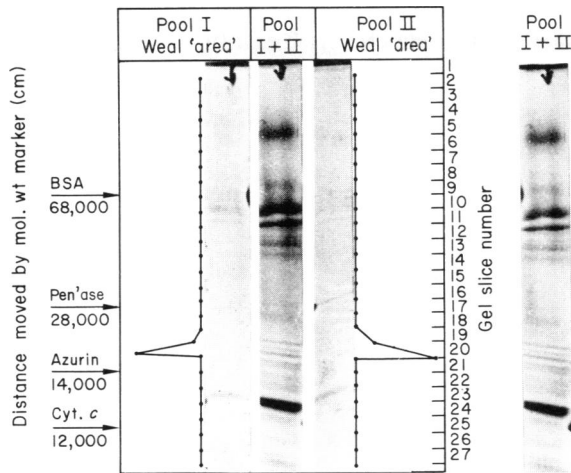


Fig. 3. Sodium dodecyl sulphate/polyacrylamide gel disc electrophoresis of the allergenic activities (pools I and II) from Sephadex G-100 fractionation of the 'green nimitti' extract. Pool I and II fractions from Fig. 1 were used and 50 μ l was applied to the gels. The weal 'area' was estimated as in Fig. 1.

II. The results, on five different occasions, were virtually identical to those shown in Fig. 3, i.e. a single peak of activity in the 15,000–20,000 molecular weight range (see below).

The SDS-PAGE slices were then tested on a total of ten individuals sensitive to 'green nimitti', five of whom had bronchial asthma on exposure to the midge (Table 1). With each subject skin test activity was elicited with slice no. 20, with a smaller reaction at slice no. 19. The rest of the gel slices were entirely negative.

The molecular size of the allergic principle was estimated by comparing the distance moved by the molecular weight markers with the slice giving a positive skin test (Fig. 4) and appeared to be between 15,000 to 20,000 daltons.

When 'green nimitti' extracts were treated with various enzymes and tested at time intervals over a 1-hr period, activity was rapidly and totally destroyed by trypsin, chymotrypsin and thermolysin (Fig. 5). Total abrogation was also achieved with neuraminidase after 45 min of incubation whereas partial inhibition of skin test reactivity was achieved with α -amylase under the same conditions. The crude extract and pools I and II from Sephadex G-100 (Fig. 1) and CM-cellulose (Fig. 2) when similarly tested all gave virtually identical patterns of inhibition.

DISCUSSION

The importance of IgE-mediated hypersensitivity to the 'green nimitti' midge in the Sudan was discussed in the previous study in which a radioallergosorbent test was described and shown to be of potential value in diagnosis (Gad El Rab & Kay, 1980). Since it is estimated that several tens of thousands of individuals suffer from bronchial asthma and/or rhinitis in association with exposure to 'green nimitti' (Gad El Rab & Kay, 1980), it seemed important to acquire as much information as possible about the chemical properties of the allergen(s) with a view to preparing a refined extract for immunotherapy.

The sample of midges used to prepare the allergen extract was virtually uncontaminated with other species, 99% being *Cladotanytarsus lewisi*. The extract had a deep-brown coloration but this material was not associated with allergic activity and eluted at the end of the Sephadex G-100 column along with other small molecules (Fig. 1). It is possible that this brown material is a lymph-like substance, or an as yet unidentified pigment.

By skin testing thirty-five UK subjects (twenty-three atopic, twelve non-atopic) we established

Table 1. Skin test reactivity to the pooled (pool I+pool II) SDS-PAGE fractions of several individuals hypersensitive to 'green nimitti'

Sensitive individual	Gel slice number			
	1-18 inclusive	19	20	21-27 inclusive
1. Weal	0	2 × 2	3 × 4	0
Itch		+	++	
2. Weal	0	1 × 2	2 × 3	0
Itch		+	+++	
3. Weal	0	1 × 2	2 × 3	0
Itch		+	+++	
4. Weal	0	1 × 1	2 × 2	0
Itch		+	++	
5. Weal	0	1 × 1	3 × 2	0
Itch		+	+	
6. Weal	0	1 × 2	2 × 4	0
Itch		+	++	
7. Weal	0	1 × 2	2 × 3	0
Itch		+	++	
8. Weal	0	1 × 2	2 × 3	0
Itch		+	+	
9. Weal	0	0	0	0
Itch		+	++	
10. Weal	0	0	0	0
Itch		+	+	

All subjects had lived for several years in areas affected by 'green nimitti'. Subjects 1-5 had a past history of asthma on exposure to 'green nimitti', no. 6 had rhinitis and nos 7-10 were asymptomatic but gave a positive skin reaction to the crude extract. For weal 'area' see the Materials and Methods section.

that only those individuals who had been exposed to the midge (i.e. Sudanese), gave a positive skin prick test. A similar finding, in terms of positive RAST tests between UK and Sudanese subjects was described in the previous report (Gad El Rab & Kay, 1980). For this reason the various fractions, prepared by the protein separation techniques described (Figs 1-3, Table 1), were only tested on those Sudanese who reacted to the whole, crude, starting material. However, a few UK subjects were tested with the various active fractions in order to exclude the possibility that the separation procedure had 'unmasked' non-specific 'histamine releasers', but these tests were invariably negative.

Two closely related peaks of 'allergic' activity were consistently observed following Sephadex G-100 chromatography. However, both peaks of activity (designated pool I and pool II) eluted in an identical fashion from CM-cellulose and migrated to exactly the same point following SDS-PAGE as did the unfractionated material. This suggests that we were probably detecting a single allergen which gave two peaks of activity on Sephadex G-100 possibly as a result of dimerization. An alternative explanation is that the allergic activity is contained in a single glycoprotein which may have some heterogeneity in its tertiary structure either *per se* or as a result of the extraction procedure. This might have caused slight retardation of some of the molecules during their passage through the Sephadex beads. Although the amino acid composition of pools I and II might have provided evidence for or against their isoallergenicity, both pools gave multiple bands on SDS-

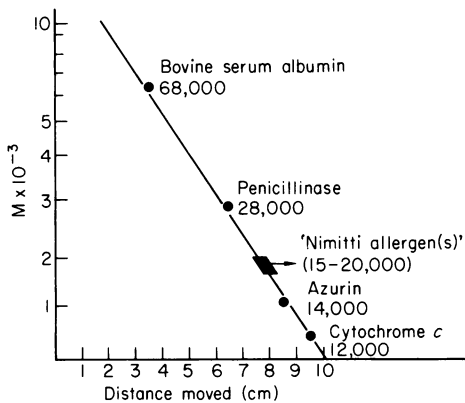


Fig. 4

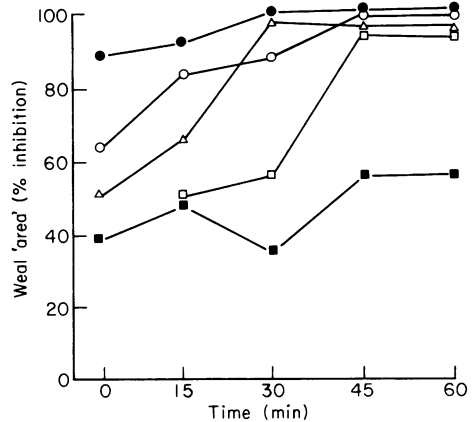


Fig. 5

Fig. 4. Estimation of the molecular size of the 'green nimitti' allergen. The molecular size ($M \times 10^{-3}$) is indicated on the ordinate.

Fig. 5. The effect of treatment with enzymes on the allergenic activity in 'green nimitti' extract. The results are the mean values of six experiments: pool I and pool II from Sephadex G-100 was performed twice and pool I and pool II from CM-cellulose was performed once. One hypersensitive individual was used and the weal 'area' expressed as in Fig. 1. (●—●) Trypsin, (○—○) chymotrypsin, (△—△) thermolysin, (□—□) neuraminidase, (■—■) α -amylase.

PAGE, most of which were unrelated to biological activity (Fig. 3). Further purification procedures are required in order to determine the precise relationship between pool I and pool II.

It is unlikely that major allergenic material absorbed to the Sephadex G-100 column since (1) further purification by SDS-PAGE gave a peak which migrated to the same position as that observed with the crude material and (2) isoelectric focusing of the whole allergen also gave a single peak.

The CM-cellulose chromatography results indicated that the allergen was acidic and this was confirmed by isoelectric focusing in which the allergenic principle was shown to have a pI of 4.3.

The enzyme studies indicated that the allergenic material was probably a glycoprotein. Although the enzyme gave a single band following SDS-PAGE, one cannot completely exclude the possibility that other enzymatic activities directed against different substrates may have been present in trace amounts.

The results of the SDS-PAGE suggested that the two peaks of allergenic activity eluting from the Sephadex G-100 column (pools I and II) were probably identical. Although with the Sephadex G-100 and CM-cellulose fractions only one individual was used for skin testing, ten individuals were used with SDS-PAGE. All gave peak activities with slice no. 20, which corresponded to band no. 3 (Fig. 3 and Table 1). A weaker skin reaction was given with slice no. 19 (which corresponded to band no. 2; Fig. 3) and which may represent a second closely associated antigen.

There are many difficulties in using skin tests as an assay for allergenicity. These include variations in reactivity at different skin sites and the requirement for appropriate hypersensitive volunteers. Other screening methods for detecting allergens such as RAST inhibition and histamine release from leucocytes of sensitive individuals are indicated. We have in fact shown recently that the Sephadex G-100 pool I and II fractions can be used for the preparation of the allergen-particle complex in the RAST and that these pools inhibit the RAST when whole allergen is coupled to the Sepharose beads (Gad El Rab & Kay, 1980). These studies will be reported later (in preparation). The major difficulty with RAST inhibition experiments has been the recovery of sufficient quantities of material during the various separation procedures. For instance, we calculate that a positive skin

test can be achieved with less than $10 \mu\text{g} \cdot \text{ml}^{-1}$ of protein indicating that there is a high amount of biological activity with a relatively small mass.

The allergenic activity in the 'green nimitti' extract appears to be very homogeneous but we cannot exclude the presence of other potential allergens until much larger numbers of hypersensitive individuals, with varying periods of exposure to the midge, have been tested.

In conclusion, it seems reasonable to believe that we have identified a major allergen from 'green nimitti' but that additional studies are required (a) to provide detailed information on its physico-chemical nature, and (b) to determine whether this is the only allergen of significance in this important cause of immediate-type hypersensitivity in the Sudan.

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