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Fractionation and Characterization of Urinary Filarial Antigen in Bancroftian Filariasis

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SUMMARY Urine samples from microfilaraemic patients were concentrated and fractionated by gel chromatography on Ultrogel AcA 44. Four protein fractions labelled as UFA C1, UFA C2, UFA C3 and UFA C4 were tested for filarial antigenicity by sandwich ELISA. UFA C1 and UFA C2 showed antigenic activity. On further analysis by SDS – PAGE, UFA C1 and UFA C2 showed antigenic components with MW ranging from 10.4 K to 123 K. UFA C1 – 1 and UFA C2 – 2 showed high antigen titre in ELISA. Urinary albumin was observed as a major component in UFA C2. Absorption of albumin from UFA C2 enhanced its antigenic activity considerably. As little as 0.01 pg antigenic protein per test was found to be sufficient for the detection of filarial antibody in ELISA. Biochemical characterization indicated a glycoprotein nature of UFA C2.

Parasite antigens in human urine were demonstrated in tropical diseases like schistosomiasis,[1,2] filariasis,[3-6] leprosy,[7] malaria[8] and typhoid.[9] Urine is a convenient source of antigen especially in *Wuchereria bancrofti* infection, where availability of parasite material is scarce due to the lack of a suitable laboratory animal model. To understand the nature of these antigens, separation and characterization of different antigen fractions from crude antigen extracts may be helpful, and it also enhances the specificity of the antigen for use in the diagnosis of active infection.[10-12] This communication reports the fractionation and characterization of urinary filarial antigens by gel filtration on Ultrogel AcA 44 followed by SDS-PAGE.

MATERIALS AND METHODS

Urinary filarial antigen (UFA)

Twenty-four hour urine samples were collected from parasitologically confirmed filarial cases after administration of diethylcarbamazine (6 mg/kg body wt). The samples were pooled, concentrated > 100 fold by ultrafiltration and dialysed for 72 hrs against sodium phosphate buffer (SPB) 0.01 M pH 7.2. The supernatant was separated by centrifugation and protein was estimated by Lowry method.[13]

Control UFA

Twenty-four hour urine samples were collected from healthy endemic normals after administration of diethylcarbamazine (6 mg/kg body wt). The samples were processed as above and labelled as control UFA.

Filarial serum immunoglobulin G (FSIgG)

Filarial serum immunoglobulin (FSIg) was isolated from pooled clinical filarial sera by 33 % ammonium sulphate precipitation and the IgG fraction (FSIgG) was isolated from it by DEAE cellulose (Whatman DE 52) column chromatography as described by Reddy *et al.*[14]

Microfilarial excretory and secretory antigen (Mf ES antigen)

Culture fluid containing mf ES antigen was obtained by *in vitro* maintenance of about 5000 *W. bancrofti* microfilariae in medium 199 supplemented with organic acids and sugars of Grace's medium for 3 to 4 days. The medium was separated every 24 hours. The culture fluid was dialysed and concentrated by freeze-drying. The protein content was estimated by

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Lowry method,[13] after reconstituting in one ml of 0.05 M SPB pH 7.2.

Sera

Venous blood was collected from filarial patients (microfilaraemic and clinical filariasis cases with clinical manifestations such as hydrocoele and elephantiasis) and healthy normals (non-infected individuals from endemic and non-endemic regions). Serum was separated in cold and stored at $-20°C$ with addition of 0.1 % of sodium azide as preservative.

Gel filtration on AcA 44

Gel filtration was carried out on Ultrogel AcA 44 (LKB Sweden) with bed dimensions of 1.6×82 cm. Equilibration and elution were done with SPB 0.05 M pH 7.2 at a constant flow-rate of 8 ml/hr and 2.66 ml (20 min) fractions were collected using a LKB 2112 Radirac Fraction collector under continuous monitoring at 280 nm (LKB 2238 Uvicord SII with LKB 2210-011 automatic 1-channel recorder). The calibration of the column was done using alcohol dehydrogenase (MW 150 K), human albumin (MW 66 K), carbonic anhydrase (MW 29 K) and cytochrome C (MW 12.4 K) (Sigma Kit No., MW GF 200) as molecular weight markers. Four protein fractions, namely, UFA Cl (48-61 ml), UFA C2 (83-99 ml), UFA C3 (107-125 ml) and UFA C4 (144-171 ml) were collected (Fig 1), pooled separately and concentrated by ultrafiltration. The fractions were tested for antigenicity by sandwich ELISA, after estimation of protein by Lowry method.[13] The same procedure was followed for control UFA fractionation.

ELISA

Conjugation of penicillinase (Sigma) and FSIgG and anti-human IgG was achieved by the method of Avrameas.[15] The substrate in ELISA consisted of soluble starch (150 mg) in 27.5 ml of 0.25 M SPB pH 7 containing 10.64 mg of penicillin V and 100 μl of 0.08 M iodine in 3.2 M potassium iodide solution. The solution was prepared fresh before use.

The sandwich ELISA was carried out as described by Voller[16] and Malhotra *et al.*[4] Hundred microlitre volumes of FSIgG (25 μg/ml), urine fractions (10 μg/ml) and FSIgG-penicillinase conjugate (1:200) were used in the assay system.

The indirect ELISA was carried out as described by Kharat *et al.*[17] Hundred microlitre volumes of absorbed UFA C2 (0.1 pg protein/ml) and *Wb* mf ES antigen (3.5 ng protein/ml), sera samples (1:300), penicillinase labelled anti-human IgG conjugate (1:1200) and starch-iodine penicillin substrate were used in the assay.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Concentrated urine samples from microfilaraemic patients (UFA) and its antigenic fractions UFA Cl and UFA C2 were reduced with mercaptoethanol and analysed by SDS-PAGE, according to the instructions in the LKB manual (LKB 2117 Multiphor, Sweden) in a horizontal 10 % polyacrylamide gel (12×24 cm) at a constant current of 190 mA for 5 hrs. A portion of the gel was cut and stained with silver stain; the unstained portion of the gel was cut at 1 cm intervals (7 slices designated UFA C1-1 to UFA C1-7 or UFA C2-1 to UFA C2-7) and the protein was eluted from each slice into 5 ml of 0.05 M SPB after grinding the gel in a mortar and pestle and leaving overnight at 4°C in the same buffer for complete elution.[18,19] The supernatant was dialysed against 0.05 M SPB. The protein was estimated by Lowry method.[13] Detection of antigenic components in the eluant of each slice was done by sandwich ELISA. The marker proteins used were β-galactosidase (116 K), phosphorylase B (97.4 K), bovine albumin (66 K), egg albumin (45 K) and carbonic anhydrase (29 K) (Sigma Kit No. MW SDS 200).

Absorption of albumin from UFA C2

Eight mg of IgG fraction of rabbit antihuman albumin (Cappel, USA) was coupled to 2 ml of CNBr activated Sepharose 4B beads[20] (Pharmacia Fine Chemicals, Sweden). Protein was estimated in the supernatant before and after coupling. The percentage of binding was about 80%. UFA C2 (3 mg in 2 ml) was absorbed with 2 ml of anti-albumin coupled beads. To confirm the complete absorption of albumin from UFA C2, double diffusion was performed with absorbed UFA C2 and anti-human albumin.

Biochemical characterization

Four mg of each enzyme, α-amylase (1355 units/mg, Sigma), chymotrypsin (CSIR Biochemicals) and lipase (595 units/mg, Sigma) was coupled separately to 1 ml CNBr activated Sepharose 4B beads. Protein in the supernatant was estimated before and after coupling and the binding was about 80-85% in all the cases. The enzyme-coupled beads were tested for enzyme activity before use. Heat inactivated enzymes were also coupled simultaneously.

Enzyme treatment

UFA Cl and UFA C2 fractions (0.5 mg in 0.5 ml) were treated separately with 0.5 ml of different enzyme-coupled beads and the mixture was kept at 37°C for 24 hrs. The supernatant was separated by centrifugation at 4°C and diluted optimally (10 μg/ml) to use in sand-

wich ELISA.

Heat inactivation

Heating of antigens was carried out at 100°C for 45 min in a water bath. After cooling, fractions were centrifuged and the supernatants were used in ELISA.

Periodate treatment

Both antigens were treated separately with 0.1 M sodium metaperiodate (BDH, England) at 37°C for 24 hrs. The reaction was stopped by dialysis against 0.01 SPB pH 7.2.

RESULTS

Concentrated urine samples (4 mg protein) from parasitologically confirmed cases were fractionated into 4 protein peaks labelled UFA C1, UFA C2, UFA C3 and UFA C4 by Ultrogel AcA 44 gel filtration (Fig 1). Pooled and concentrated endemic normal urines were fractionated as above into Cont.UFA C1, Cont.UFA C2, Cont.UFA C3 and Cont.UFA C4. Two of the four frations, UFA C1 and UFA C2, showed antigenic activity in sandwich ELISA. The reciprocal antigen titres of UFA C1 and UFA C2 (10 μg protein/ml) were 1:200 and 1:2000. Analysis of UFA and the fractions UFA C1 and UFA C2 by SDS-PAGE followed by silver stain showed 11, 6 and 8 protein bands respectively in the MW range of 10.4 K to 123 K. Antigenicity of the protein bands was detected by sandwich ELISA (Table 1).

Absorption of albumin from UFA C2 using rabbit anti-human albumin further enhanced its antigenic activity requiring as little as 0.01 pg protein/test for detection in sandwich ELISA. Albumin-absorbed Cont.UFA C2 did not react with FSIgG. Using the absorbed UFA C2 and mf ES antigen, a total number of 30 human sera from patients belonging to different groups were analysed for the presence of IgG antibody by indirect ELISA. Seven of 10 microfilaraemia, 8/8 clinical filariasis, 1/6 endemic normals and none of the 6 nonendemic normals showed the presence of filarial antibody for both antigens (Table 2). Biochemical characterization (Table 3) shows the effects of heating, amylase, chymotrypsin, periodate and lipase action on UFA C1 and UFA C2. UFA C1 was not affected either by enzyme digestion or by heat or periodate oxidation. Though UFA C2 was stable to heat, it lost its complete antigenic activity on treatment with amylase, chymotrypsin and periodate. Lipase has no action on both antigens. No change in antigenic activity was observed in control experiments, where treatment was done with heat inactivated enzymes.

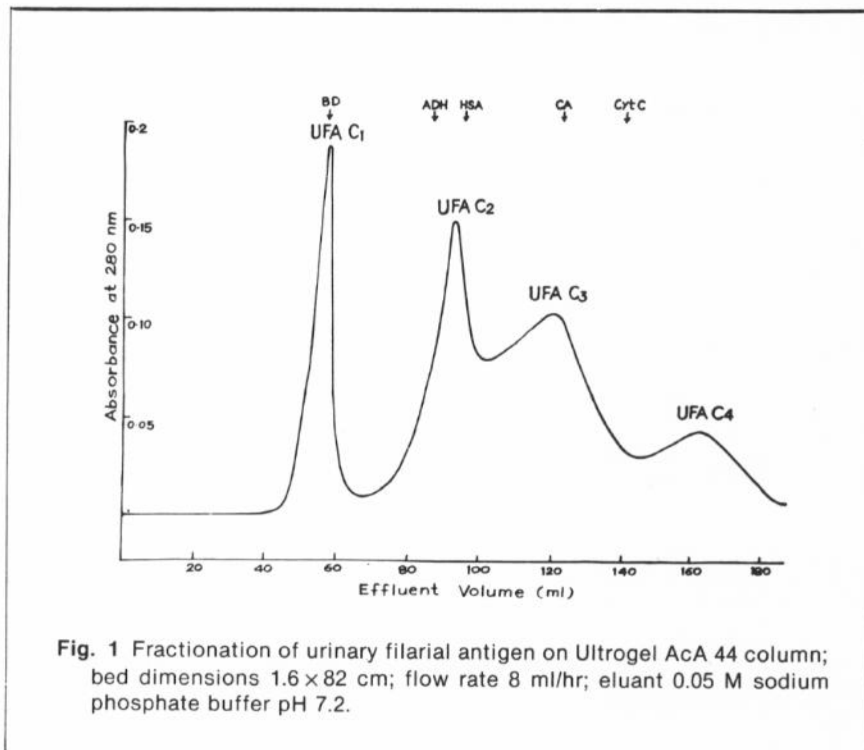

Fig. 1 Fractionation of urinary filarial antigen on Ultrogel AcA 44 column; bed dimensions 1.6 × 82 cm; flow rate 8 ml/hr; eluant 0.05 M sodium phosphate buffer pH 7.2.

DISCUSSION

Antigens from human urine have been isolated in different parasitic diseases, but studies on the characterization of these antigens are scanty. This study reports the separation of crude urinary filarial antigen into 4 fractions and their immunological and biochemical characterization. Out of 4 fractions UFA C1, UFA C2, UFA C3 and UFA C4 separated on Ultrogel AcA 44, only 2 fractions, UFA C1 (130 K; eluted in void volume) and UFA C2 (66 K), were observed to be antigenic. Antigenic titres showed that UFA C2 was 10 fold more active than UFA C1.

SDS-PAGE analysis of UFA showed 11 bands in the MW range of 10.4 K to 123 K, whereas UFA C1 and UFA C2 showed 6 (MW 31.6 K to 123 K) and 8 (MW 10.4 K to 69 K) bands respectively. Antigenic analysis revealed the polydispersed nature of antigens in several PAGE fractions. However, UFA C1-1 (83 K-123 K) and UFA C2-4 (25 K-36 K) showed reciprocal antigen titres of 1:4 and 1:32 respectively. UFA C2-2 (55 K-83 K) showed antigen titre of 1:64 after absorption of albumin. The very high antigenic

Table 1 Detection of antigen in SDS-PAGE fractions of UFA C1 and UFA C2 by sandwich ELISA

	PAGE fraction showing antigen	No.of bands	Antigen titre	Approximate molecular weight
UFA C1	1	3	1:4	83 K – 123 K
	3	1	N	36 K – 55 K
UFA C2	2*	2	1:64	55 K – 83 K
	4	3	1:32	25 K – 36 K
	5	—**	N	15.8 K – 25 K
	7	1	N	10.4 K

* UFA C2 – 2 showed antigenic activity after absorption of albumin.
** No protein band could be observed; N, Neat (10 μg protein/ml).

Table 2 Diagnostic utility of UFA C2 in comparison with mf ES antigen

Sera	Number screened	UFA C2 albumin absorbed (0.1 pg/ml)	ES antigen (3.5 ng/ml)
Microfilaraemia	10	7	7
Clinical filariasis	8	8	8
Endemic normal	6	1	1
Nonendemic normal	6	0	0

Table 3 Chemical characterization of antigens

Treatment	Antigenic titre in sandwich ELISA (starting dil 10 μg protein/ml)	
	UFA C1	UFA C2*
Untreated	1:200	$1{:}10^8$
Heat inactivation	1:200	$1{:}10^8$
Amylase	1:200	0
Chymotrypsin	1:200	0
Periodate	1:200	0
Lipase	1:200	$1{:}10^8$

* Absorbed with rabbit anti – human albumin – Sepharose 4B

activity was not found in any of the SDS-PAGE fractions. Irreversible destruction of antigenicity under SDS-PAGE conditions was observed by Gottstein[21] in an attempt to determine the MW of the specific antigen component of *E.multilocularis*. SDS-PAGE studies with filarial excretory-secretory antigens showed 27 bands in the MW range of 18 K to 145 K (unpublished observation). Dissanayake *et al.*[5] has reported an antigen of MW 67 K in urine from *W.bancrofti* infected patients by SDS-PAGE, using a monoclonal antibody raised against *O.gibsoni*. After removal of albumin, a major urinary component of this fraction, the sensitivity of UFA C2 was increased by 50,000-fold. Host albumin was observed to be a major component on the surface of *W.bancrofti* microfilariae.[22] The increase in antigen titre after absorption of host albumin may be because of albumin interference with the antigen. Preliminary studies with addition of albumin to the purified antigen fraction in sandwich ELISA showed inhibition of antigen-antibody reaction. Packer *et al.*[23] have reported the blocking nature of albumin on merozoite surface antigens which may be involved in antibody-mediated clearance of parasites. As little as 0.01 pg protein per test was found to be sufficient for the detection of urinary antigen or filarial antibody in penicillinase ELISA. Harris and co-workers[24] could detect 0.1 femtogram (10^{-16} gram) or approximately 600 molecules of toxin and rotavirus in an ultrasensitive enzymatic radioimmunoassay (USERIA). The enzyme penicillinase has high turnover number (160000) and has been found to be more sensitive than alkaline phosphatase or peroxidase in enzyme immunoassays. Studies from this laboratory with penicillinase ELISA showed high reciprocal antibody titres of 5-20 million in filarial sera.[17] Studies on chemical characterization showed a complete loss of antigenic activity of UFA C2 on treatment with amylase, chymotrypsin or periodate, suggesting its glycoprotein nature. Heat stability of both the antigens UFA C1 and UFA C2 confirmed our earlier studies on detection of antigen by IRMA from this laboratory[25] Preliminary studies on the diagnostic potential of albumin-absorbed UFA C2 antigen fraction showed increased sensitivity requiring as little as 0.01 pg per test compared to *W.bancrofti* ES antigen (0.35 ng).

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