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Analysis, characterization and diagnostic use of circulating filarial antigen in bancroftian filariasis

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Abstract. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of circulating filarial antigen fraction-2 isolated from plasma of microfilaraemic patients with *Wuchereria bancrofti* infection has shown 21 bands with molecular weights ranging from 12 to ⩾120 kDa. The gel (12 cm) was sliced at an interval of one cm and the eluates of all the gel slices *viz.,* CFA2–1 to CFA2–12 showed the presence of filarial antigen by sandwich enzyme-linked immunosorbent assay. The low molecular weight circulating filarial antigen fractions were found to share a common epitope with *Wuchereria bancrofti* microfilariae excretory-secretory antigen and urinary filarial antigen. The 3 antigen fractions CFA2-1, CFA2–9 and CFA2–12 showed higher sensitivity in detecting filarial immunoglobulin M antibodies than immunoglobulin G antibodies. However CFA2–9 fraction was found useful in serological differentiation of microfilaraemics from those with disease manifestations when filarial immunoglobulin G antibodies were detected. The antigenic epitope of CFA2–1 appears to be a carbohydrate, whereas CFA2–9 appears to be protein in nature.

Keywords. Enzyme linked immunosorbent assay; filarial serum immunoglobulin G; circulating filarial antigen; sodium dodecyl sulphate; Polyacrylamide gel electrophoresis.

Introduction

Antigens released *in vitro* or *in vivo* have been of considerable interest in immunodiagnosis (Harinath, 1986). Analysis and characterization of the filarial antigens may be useful in identifying a suitable antigen for detection of filarial infection. Circulating filarial antigens (CFA) have been detected in humans infected with *Wuchereria bancrofti* (Au *et al.,* 1981; Kaliraj *et al.,* 1981; Dasgupta *et al.,* 1984; Hamilton *et al.,* 1984; Dissanayake *et al.,* 1984; Forsyth *et al.,* 1985; Weil *et al.,* 1986), *Brugia malayi* (Au-*et al.,* 1981) and *Onchocerca volvulus* (Ouaissi *et al.,* 1981; Desmoutis *et al.,* 1983) and in animals infected with *Dirofilaria immitis* (Weil *et al.,* 1985), *Brugia pahangi* (Au *et al.,* 1981) and *Litomosoides carinii* (Dasgupta and Bala, 1978). In the previous studies from our laboratory an active CFA fraction-2 (CFA2) was isolated from microfilaraemic plasma and it was shown to be sensitive in detecting filarial immunoglobulin M (IgM) antibodies in patient's sera (Reddy *et al.,* 1986). This paper reports analysis of CFA2 by sodium dodecyl sulphate (SDS)

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Abbreviations used: CFA, Circulating filarial antigen; IgM, immunoglobulin M; SDS, sodium dodecyl sulphate; PAGE, Polyacrylamide gel electrophoresis; *Wb* mf ES Ag, *Wuchereria bancrofti* excretory-secretory antigen; UFA, urinary filarial antigen; DEC, diethylcarbamazine; IgG, immunoglobulin G; FSIgG, filarial serum IgG, SPB, sodium phosphate buffer; ELISA, enzyme linked immunosorbent assay; TCA, trichloroacetic acid; GMRT, geometric mean reciprocal of antibody titre.

polyacrylamide gel electrophoresis (PAGE), detection of antigen levels in different SDS-PAGE fractions of CFA2, their characterization and diagnostic importance in detection of filariasis.

Materials and methods

Sera

Blood samples were collected from filarial patients (both microfilariae positive individuals and patients with clinical manifestations like elephantiasis, hydrocoele etc.) living in Sevagram and its surrounding villages which are endemic for filariasis. Blood samples were also collected from healthy individuals living in endemic region without any history of filariasis (endemic normal) and from healthy individuals living in regions non-endemic for filariasis (non-endemic normals) such as Punjab and Kashmir. Sera were separated and stored at – 20°C with the addition of 0·1% sodium azide as preservative.

Circulating filarial antigen

Circulating filarial antigen was prepared as described by Reddy *et al.* (1986). Microfilariae were removed from citrated blood samples of microfilarial patients by nucleopore membrane (5 μm) filtration. The blood cells were pelleted at 13,000 *g* for 30 min and the CFA was isolated from the plasma by 36–75% ammonium sulphate precipitation. CFA was fractionated on ultrogel AcA 34 gel column (LKB, France) The pooled fractions of the second protein peak showing filarial antigens were concentrated and designated as CFA2.

W. bancrofti microfilariae excretory-secretory antigen

W. bancrofti microfilariae excretory-secretory antigen (*Wb* mf ES Ag) was obtained by maintenance of microfilariae in medium 199 supplemented with organic acids and sugars of Grace's medium as described by Kharat *et al.* (1982). The culture supernatent was dialysed with a 12,000 molecular weight cut-off membrane and concentrated 200-fold by freeze drying.

Urinary filarial antigen

Urinary filarial antigen (UFA) was isolated from pooled 24 h microfilaraemic urine samples, after diethylcarbamazine (DEC) treatment and concentrated by ultrafiltration. UFA was fractionated on ultrogel AcA 44 gel column (LKB, France). The pooled fractions of second protein peak showing filarial antigen were concentrated and labelled as UFAC2. Albumin in UFAC2 was removed by absorbing with rabbit antihuman albumin immunoglobulin G (IgG) coupled CNBr Sepharose 4B beads (Ramaprasad and Harinath, 1987). Albumin absorbed UFAC2 was designated as UFAC2-A.

Filarial serum IgG

Filarial serum IgG (FSIgG) was prepared from pooled clinical filarial sera by ammonium sulphate precipitation followed by DEAE cellulose chromatography as previously described (Reddy *et al.,* 1984a)

SDS-PAGE of CFA2

CFA2 (400 μg protein in 20 μl) was diluted 10 times with SDS-sample buffer containing 3% SDS, 1% glycerol, 0·1% bromophenol blue and 5% mercaptoethanol (v/v) in 0·5 M Tris-HCl buffer, pH 6·8 heated for 3 min in a boiling waterbath and analysed by SDS-PAGE as described by Laemmli (1970) with slight modifications. Electrophoresis was carried out on vertical 10% acrylamide homogenous slab gels (9×13 cm) with 3·5% stacking gel. The molecular weight marker proteins (Sigma Chemical Co., USA) lysozyme (14·3 kDa), carbonic anhydrase (29 kDa), albumin egg (45 kDa), Phosphorylase b (97·4 kDa) were used for calibration of the gel. The samples were stacked at a constant current of 20 mA and separated at 25 mA to a length of 12 cm. Proteins in a strip of the gel were visualized by staining with Coomassie brilliant blue (R-250). The remaining part of the gel was cut into 12 horizontal slices at 1 cm intervals and each slice was subjected to mechanical grinding to. facilitate the elution of the protein into 5 ml of 0·05 M sodium phosphate buffer (SPB), pH 7·2 as described by Guellaen *et al.* (1984). The eluates of the 12 fractions were dialysed against 0·05 M SPB, concentrated by ultrafiltration and tested for filarial antigen in sandwich enzyme linked immunosorbent assay (ELISA).

ELISA

For the ELISA, conjugation of *Wb* mf ES Ag, UFAC2-A, FSIgG, antihuman IgG and anti human IgM with penicillinase was achieved by the method of Avrameas (1969) using glutaraldehyde.

The substrate in ELISA consisted of soluble starch (150 mg) in 27·5 ml of 0·25 M SPB (pH 7·2) containing 10·64 mg of penicillin ‘V and 100 μl of 0·08 M iodine in 3·2 M potassium iodide solution. The substrate was prepared fresh before use.

Sandwich ELISA

Sandwich ELISA was carried out as described by Reddy *et al.* (1984a). FSIgG (25 μg/ml), SDS-polyacrylamide gel eluates of CFA2 (starting dilution 10 μg/ml and serially diluted 10-fold) and FSIgG penicillinase conjugate (1:400) were used in the assay.

Inhibition ELISA

Inhibition ELISA was carried out as described by Malhotra and Harinath (1984). FSIgG (25 μg/ml), SDS-polyacrylamide gel eluates of CFA2 (starting dilution

10 μg/ml and serially diluted 10-fold) and *Wb* mf ES Ag penicillinase conjugate (1:100) or UFAC2-A penicillinase (1:50) were used in the assay.

Indirect ELISA

Stick ELISA for antibody detection was carried out as described by Parkhe *et al.* (1986). Five μl of optimally diluted antigen fractions (0·1 μg/ml each of CFA2-1 and CFA2-9 and 1 μg/ml of CFA2-12) applied on cellulose acetate membrane attached to plastic strip, sera (1:300 diluted and serially diluted 4-fold up to 1:19,200 dilution), antihuman IgG penicillinase conjugate (1:4000) or antihuman IgM penicillinase conjugate (1:1000) were used in the assay.

Characterization of CF A2-1 and CF A2-9 fractions

Four mg of the enzymes chymotrypsin (CSIR, Biochemicals) was coupled to 1 ml CNBr Sepharose 4B beads as described earlier (Ramprasad and Harinath, 1987). The protein in the supernatant was estimated before and after coupling. The binding was 82–85%.

Treatment with enzymes

The antigen fractions CFA2-1 and CFA2-9 (250 μg in 0·5 ml of 0·05 M SPB, pH 7·2) were treated separately with an equal volume of enzyme coupled beads for 24 h at 37°C with gentle shaking (Rolfe and Fingold, 1979). The supernatant was separated by centrifugation (600 *g,* 15 min) at 4°C and diluted (l0 μg/ml) to use in sandwich ELISA.

Periodate treatment

The 2 antigen fractions (250 μg in 0·5 ml of 0·05 M SPB, pH 7·2) were treated separately with 0·1 M sodium metaperiodate (BDH, England) at 37°C for 24 h with gentle shaking (Mak *et al.,* 1977). The reaction was stopped by dialysis against 0·01 M SPB, pH 7·2 and used in sandwich ELISA.

Heat inactivation

The 2 antigen fractions were heated at 100°C for 45 min in a boiling water bath (Reddy *et al.,* 1984b). The fractions were centrifuged and the supernatants used in sandwich ELISA.

Trichloroacetic acid treatment

The 2 antigen fractions were treated with equal volume of trichloroacetic acid (TCA, 30%) as described by Weil (1987). The fractions were then centrifuged (12,000 *g,* 20 min) and supernatants dialysed against 0·01 M SPB, pH 7·2. The

supernatants were then heated in a boiling water bath for 30 min, again centrifuged (16,000 *g* for 10 min) and used in sandwich ELISA.

Statistical analysis was carried out by Z test.

Results

Analysis of CFA2 by SDS-PAGE showed 21 bands with molecular weights ranging from 12kDa to ⩾ 120 kDa (figure 1). Eluates of all the 12 gel slices were positive for filarial antigen in FSIgG sandwich ELISA (table 1) with the reciprocal of filarial antigen titres ranging from 10 to 100,000, suggesting polydispersed nature of this antigen. Earlier studies from our laboratory have shown the polydispersed nature of antigen activity in *Wb* mf ES Ag (Ramaprasad *et al.,* 1988). However, CFA2-1 and

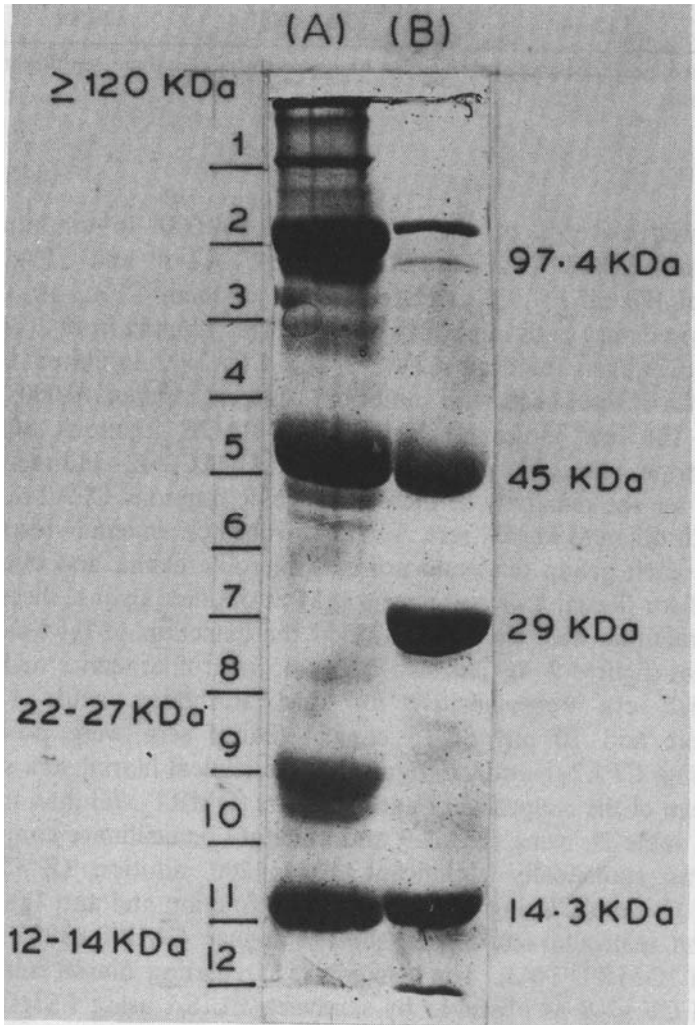

Figure 1. SDS-PAGE analysis of CFA2. The proteins in the gels were stained by Coomassie brilliant blue (R-250). (A), CFA2; (B), molecular weight markers: Lysozyme (14·3 kDa), carbonic anhydrase (29 kDa), albumin egg (45 kDa), Phosphorylase b (97·4 kDa).

Table 1. Detection of circulating and excretory-secretory antigens by sandwich and inhibition ELISA·

CFA2 SDS-poly acrylamide gel fractions	Approximate molecular weight ($\times 10^3$)	Reciprocal of filarial antigen titre* by sandwich ELISA using FSIgG	Reciprocal of filarial antigen titre* by inhibition ELISA for detection	
			Wb mf ES Ag	UFAC2-A
CFA2–1	≥ 120	10,000 (76×10^4)	100	—
CFA2–2	100–120	1,000 (6×10^3)	—	—
CFA2–3	80–100	10 (48×10)	100	—
CFA2–4	64–80	Neat (6×10)	—	—
CFA2–5	50–64	10 (48×10)	—	—
CFA2–6	42–50	10 (6×10^2)	—	—
CFA2–7	35–42	Neat (48×1)	—	—
CFA2–8	27–35	1,000 (48×10^3)	—	—
CFA2–9	22–27	100,000 (64×10^5)	10	10,000
CFA2–10	18–22	100 (8×10^2)	10,000	—
CFA2–11	14–18	10 (44×10)	100,000	—
CFA2–12	12–14	10 (44×10)	100,000	10,000

*Starting dilution of Polyacrylamide gel fractions 10 μg protein/ml. Total antigen titre in each fraction is given in parenthesis.

CFA2-9 showed elevated titres of 10,000 and 100,000. In inhibition ELISA, 5 antigen fractions CFA2-1, CFA2-3, CFA2-10, CFA2-11 and CFA2-12 inhibited the binding of *Wb* mf ES Ag to FSIgG and 3 of them CFA2-10, CFA2-11 and CFA2-12 showed very high inhibitory antigen titres ranging from 10,000 to 100,000 (table 1). Two antigen fractions CFA2-9 and CFA2-12 inhibited the binding of UFAC2-A with FSIgG each with inhibitory antigen titres of 10,000. These studies suggest that the low molecular weight SDS-PAGE fractions of CFA2 share common epitope with *Wb* mf ES Ag and UFAC2–A. Indirect ELISA was performed to see the reactivity of these 3 antigenic fractions CFA2–1, CFA2–9 and CFA2–12 with different filarial sera. Ten sera from non endemic normal group and 15 sera from each group (endemic normal, microfilaraemia and clinical filariasis) were screened for filarial IgG and filarial IgM antibodies against these antigens. All 3 antigenic fractions were more sensitive in the detection of IgM antibodies than IgG antibodies (figures 2–4). Thirteen out of 15 microfilaraemics and 14 out of 15 clinical filarial sera were positive for IgM antibodies while 12 out of 15 microfilaraemic and 10 out of 15 clinical filarial sera were positive for IgG antibodies using CFA2–1 antigen (figure 2). The clinical filarial sera showed higher geometric mean of the reciprocal of antibody titre (GMRT 396) than microfilaraemic (GMRT 172, table 2), using CFA2–9 and anti IgG penicillinase conjugate and the difference was statistically significant at 1:1200 dilution ($Z > 2{\cdot}58$, $P < 0{\cdot}01$, significant at 1% level). Using CFA2–12 antigen fraction and anti IgM penicillinase conjugate, the microfilaraemic sera showed higher GMRT (3971) than clinical filariasis sera (GMRT 1443). The two active circulating filarial antigen fractions CFA2-1 and CFA2-9 as observed by sandwich ELISA using FSIgG were further characterized and reported in table 3. CFA2-1, the high molecular weight antigen was found to be sensitive to periodate treatment but was not affected by chymotrypsin, heat and TCA treatment. In contrast, CFA2–9 was sensitive to heat, TCA and chymotrypsin treatment but was stable to periodate treatment (table 3).

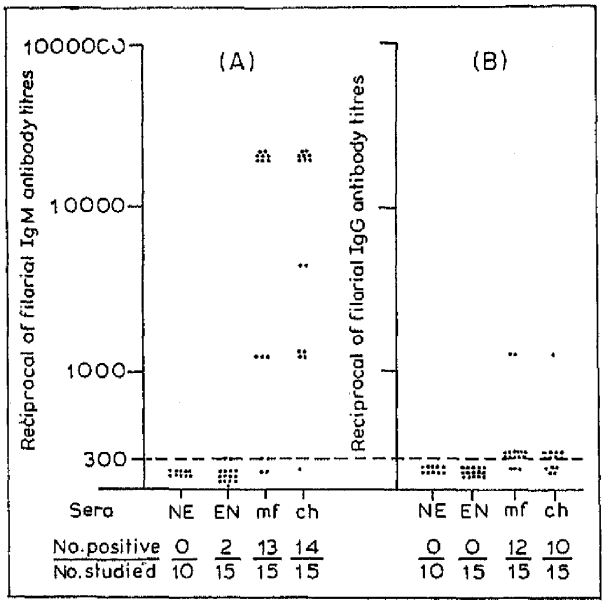

Figure 2. Detection of antibody using CFA2-1 antigen by indirect ELISA. (A), IgM antibody; (B), IgG antibody.

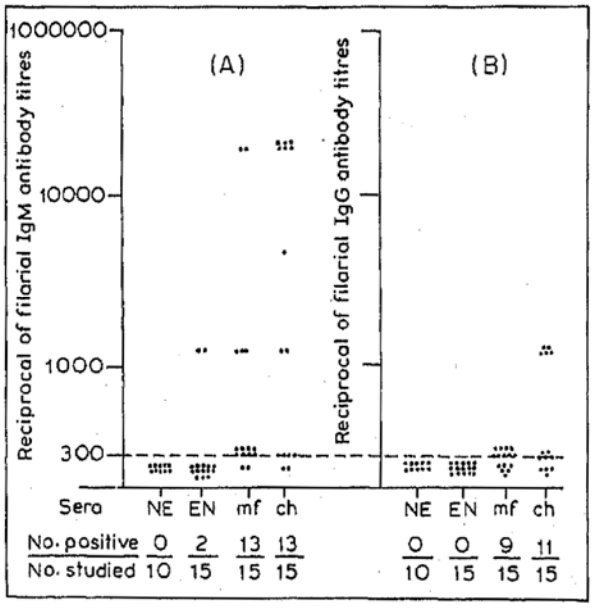

Figure 3. Detection of antibody using CFA2–9 antigen by indirect ELISA. (A), IgM antibody; (B), IgG antibody.

Discussion

The parasite antigenemia in bancroftian filariasis was first demonstrated by Franks (1946) by passive cutaneous anaphylaxis. Later on with a variety of immunoassay

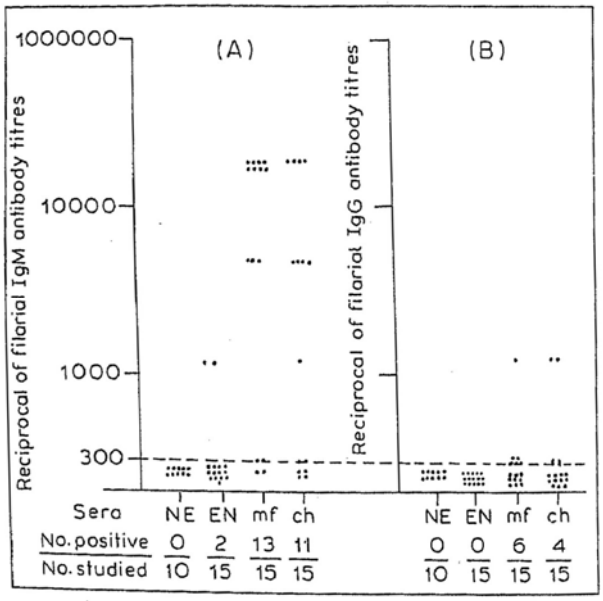

Figure 4. Detection of antibody using CFA2–12 antigen by indirect ELISA. (A), IgM antibody; (B), IgG antibody.

techniques, circulating antigens were detected in humans infected with *W. bancrofti* (Kaliraj *et al.,* 1979), *B. malayi* (Au *et al.,* 1981) and *O. volvulus* (Desmoutis *et al.,* 1983) but relatively little has been reported on the analysis, characterization and diagnostic use of circulating antigens in the human blood.

The low molecular weight SDS-PAGE fractions of CFA–2, CFA2–9 and CFA 2–12, share common epitope with *Wb* mf ES Ag and UFAC2–A. Both *Wb* mf ES Ag and UFAC2A have been shown to be highly sensitive and useful in the diagnosis of filariasis (Ramaprasad and Harinath, 1987; Ramaprasad *et al.,* 1988). Hence their identical fractions in CFA2 and particularly those with high inhibitory antigen titres may be useful as candidate antigens in the diagnosis of filariasis. Moreover, CFA2–1 and CFA2–9 showed very high reciprocal of filarial antigen titre (table 1). Weil and Liftis (1987) and Lal *et al.* (1987) have reported detection of high molecular weight (≅ 200 kDa) circulating antigen in bancroftian filariasis

Table 2. Geometric mean of the reciprocal of filarial IgM and IgG antibody titres in different sera using CFA2 SDS-polyacrylamide gel antigenic fractions (CFA2–1, CFA2–9 and CFA2–12) by indirect ELISA.

	GMRT					
	CFA2–1		CFA2–9		CFA2–12	
Group	IgM	IgG	IgM	IgG	IgM	IgG
Non endemic normal	—	—	—	—	—	—
Endemic normal	90	75	109	75	109	75
Microfilaraemia	3023	273	573	172	3972	143
Clinical filariasis	5264	249	1904	396	1443	131

Table 3. Effect of different biochemical agents on the antigenic activity of CFA2–1 and CFA2–9 reactions as determined by sandwich ELISA.

	Reciprocal of filarial antigen titres of CFA2 fractions following treatment	
Biochemical agents	CFA2–1*	CFA2–9*
Sodium phosphate buffer	10,000	100,000
Heat (100°C)	10,000	0
TCA/heat treatment	10,000	100
Periodate	0	100,000
Chymotrypsin	10,000	100

*Starting dilution of CFA2–1 and CFA2–9 fractions was 10 μg protein/ml.

using monoclonal antibodies AD 12·1, DH 6·5 and CA 101 respectively which may be equivalent to CFA2–1 based on the size of the molecule. The diagnostic use of 3 circulating filarial antigen fractions was studied by indirect ELISA. The results (figures 2–4) indicate that all the 3 antigen fractions CFA2–1, CFA2–9 and CFA2–12 were found to be more sensitive in the detection of IgM antibodies in filarial sera, confirming earlier reports from this laboratory (Reddy *et al.,* 1986) showing that CFA2 fraction as a whole was more sensitive in detecting IgM antibodies than IgG antibodies in filarial sera. The higher GMRT of clinical filarial sera than GMRT of microfilaraemics using CFA2–9 antigen in detecting IgG antibody is in agreement with the studies of Prasad and Harinath (1988) who showed immunecomplex SDS-PAGE fraction–9 (IC–9) to be more useful in differentiating microfilaraemic and clinical filarial cases based on IgG antibody detection. Furthermore, CFA2–12 was shown to be more useful in detection of microfilaraemic carriers than clinical filarial cases by detecting IgM antibodies. Ramaprasad *et al.* (1988) also showed *Wb* mf ES Ag SDS-PAGE fraction–12 (ESA–12) to be useful in detecting elevated IgM antibody in active filarial infection.

Dissanayake *et al.* (1982) reported evidence in favour of circulating glycoprotein antigen in immunecomplexes from *W. bancrofti* patients followed by demonstration of immunoblot of parasitic antigens reactive with monoclonal antibody Gib 13 in *W. bancrofti* sera (Dissanayake *et al.,* 1984). Weil *et al.* (1986) showed that the immunoreactivity of circulating antigen was preserved after treatment with heat and TCA but destroyed by metaperiodate. Paranjape *et al.* (1986) reported that the circulating filarial antigen was resistant to heat and TCA treatment but destroyed by pronase and sodium periodate. Our present study shows the high molecular weight antigen (CFA2–1) to be sensitive to periodate treatment but not affected by heat and TCA treatment. In contrast CFA2–9 was found to be sensitive to heat, chymotrypsin, treatment but stable to periodate treatment. Thus antigenic epitope of CFA2–1 appears to be a carbohydrate whereas that of CFA2–9 is protein in nature. The present study reveals that host immune response to the CFA2–9 fraction can be used for serological differentiation of active infection from clinical infection whereas CFA2–1 and CFA2–12 may be used to detect IgM antibodies for diagnosis of filarial infection. Thus, the serum derived circulating antigens from

microfilaraemic patients can be used for the detection of filarial antibodies. The study further reveals that CFA2–9 which is observed in increased levels in microfilaraemic plasma may be target antigen for confirming active infection using specific polyclonal antibody.

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