Biological activity and diagnostic use of detergent soluble antigens from *Setaria digitata*

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Abstract. Filarial parasite, responsible for filariasis is known to remain in the host for long periods of time. A 29 kDa protein isolated from *Setaria digitata* by gel electrophoresis and electroelution of detergent soluble surface antigens showed a 70% inhibition of the cell mediated immune response. On evaluation of its diagnostic application, the same protein was found to be very sensitive in detecting antibody at an antigenic protein concentration as low as 1 ng per μl. The cross reactivity of surface antigen with bancroftian filarial patient's sera was tested by Dot-ELISA and ELISA. Both the antigen as well as antibody detection tests showed 100% positivity with all types of filariasis cases. It did not produce any positive reaction with non-endemic control sera. However, a proportion of endemic normal subjects showed positivity and this is attributed to the fact that people in endemic areas are exposed to infective mosquito bites. The biological property of inhibition and 100% positivity of filariasis cases in both antibody and antigen detection tests point towards the bifunctional nature of the surface proteins before and after release. The same may be happening under normal conditions also, perhaps at a much lower rate.

Keywords. Filariasis; surface antigens; immunosuppression; cross reactivity.

1. Introduction

Lymphatic filariasis, caused by *Wuchereria bancrofti* and *Brugia malayi*, is a major public health problem in the tropical and subtropical countries, affecting more than 100 million people all over the world (WHO 1990). A cure for this disease by any means has been elusive. The general paucity of knowledge on the causals, lack of early diagnostic methods, and lack of effective drugs or vaccines are the bottlenecks in combating the disease (Goodwin 1984; Ottesen 1984; WHO 1987, 1989).

The ability of parasites to survive in the immunized host, depends upon a variety of escape mechanisms (Capron and Camus 1979). One of these is the inhibition or the suppression of the immune response of their hosts. Several possible explanations have been put forward for this suppression such as antigenic competition, acquired tolerance as well as the blocking role of soluble antigens or the circulating immune complexes (Ramalho-Pinto et al 1976; Smithers and Terry 1976; Capron et al 1977).

There is evidence that human as well as animal filarial infections are associated with decreased lymphocyte reactivity to parasite related antigens (Prasad et al 1991).

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Filarial parasite proteins or soluble parasite products released under in vivo conditions could interfere with various cellular functions, thus playing a role in regulating immune reactivity in filarial infections (Forsyth et al 1985; Lal et al 1987; Maizels et al 1987).

*Setaria digitata* (Linstove 1906), a filarial nematode dwelling in the peritoneal cavity of cattle *Bos indicus*, has immunological determinants of filarial infection (Sheng 1958). It closely resembles the human filarial parasite, *W. bancrofti* in its morphology, histology (William Decruse and Kaleysa Raj 1990), response to drugs (Hawking 1978) and antigenicity (Dissanayake and Ismail 1980). Hence it has been used as a model system in the present study. Some of the biological properties of *S. digitata* and an analysis of the cross-reactivity of its purified surface antigens form the subject matter of this paper.

2. Materials and methods

2.1 Isolation and purification of surface antigen

Live adult *S. digitata* were collected from freshly slaughtered cattle in Tyrode medium. The surface antigens from whole worm, isolated cuticle or 125I-labelled *S. digitata* were isolated using a non-ionic detergent Triton X-100 (TX100) as reported earlier (Bright and Kaleysa Raj 1991). Thoroughly washed worms were treated with TX100 in PBS and incubated at 0–4°C for 10 min. The suspension was vortexed for 5 min, the worms removed by hand picking and the medium centrifuged at 300 g for 10 min to remove larger particles and microfilariae (mf). The supernatant obtained was extensively dialysed against PBS and then against distilled water to obtain the surface antigen preparation (SAP). This antigen was purified by PAGE separation followed by electroelution as reported elsewhere (Bright and Kaleysa Raj 1993). The SAP containing 1 mg protein was separated by electrophoresis on a 10% Polyacrylamide slab gel. After electrophoresis, a reference strip was cut out from the gel and stained with Coommassie blue. Gel slices corresponding to the protein bands were cut out and electroeluted separately in Tris EDTA buffer, in dialysis bags at 0–4°C, by applying 100 V for 3 h and then the polarity of the current was reversed for 30 s. Gel slices were removed and the eluate dialysed against PBS. Total protein content was estimated according to the method of Lowry et al (1951).

2.2 Immunization of animals with antigens

Balb/c mice 4–6 weeks old were used for the study. The antigens were detergent soluble materials from whole worm and isolated cuticle, and purified materials (Bright and Kaleysa Raj 1993). Mice were immunized with antigens in PBS. Five daily injections were given intraperitoneally. The amounts used were crude antigen-150 μg/mouse and purified antigens-50 μg/mouse. Controls received PBS. Blood was collected by eye puncture.

2.3 Total lymphocyte count

Lymphocytes from heparinized blood were separated by density gradient separation
with Histopaque. The separated cells were washed and counted using WBC hemocytometer and Neubauer counting chamber.

2.4 B-lymphocyte assay

Two weeks old SRBC and 1 : 1000 rabbit anti SRBC in PBS were mixed and incubated at 37°C for 30 min. Antibody coated erythrocytes (EAC) were washed and incubated further at 37°C for 30 min after mixing with 1:10 mouse complement. The complement treated EAC was then washed and mixed with lymphocytes to give a ratio of 100 : 1. These cells were incubated at 37°C for 15 min and centrifuged at 35 g for 10 min. The percentage of rosette forming cells was determined by counting about 500 lymphocytes.

2.5 Lymphocyte proliferation assay

Lymphocytes were separated and aliquots counted to get the total cell number. Concanavalin A (Con A) (15 μg/ml) was added to these cells with RPMI containing 10% fetal calf serum and incubated at 37°C in a 5% CO₂ atmosphere. [³H]thymidine was added to the culture after 48 h. Cells were harvested after 18 h of incorporation and washed in 5% TCA in cold isotonic saline and methanol. They were then suspended in scintillation mixture and radioactivity was measured in a LKB β-liquid scintillation counter.

Per cent of suppression was calculated by the formula

\[
\text{% of suppression} = 100 - \frac{\text{count/min of test cultures}}{\text{count/min of control cultures}} \times 100.
\]

2.6 Radioiodination of parasite surface

The radioiodination of S. digitata was achieved by using chloramine-T technique of Hunter and Greenwood (1962), with some modifications. Adult female worms were suspended in 0·05 % (w/v) chloramine-T and 10 μCi carrier free radioactive iodine ¹²⁵I and incubated at 25–30°C for 2 min. The reaction was stopped by the addition of 0·6 ml of saturated tyrosine. The worms were washed and ¹²⁵I-labelled surface antigen was isolated by TX100 technique (Bright and Kaleysa Raj 1991). The incorporation was measured by counting the antigen preparation in a LKB gamma counter.

2.7 Preparation and purification of surface antibodies

Antiserum to surface antigen was raised in albino rabbits. A total of 5 mg surface protein was administrated in 5 subcutaneous injections within a period of 15 days. The hyperimmune rabbit was bled after 10 days, the sera separated and preserved frozen. The control serum was collected by bleeding the rabbit prior to immunization. The antibodies specific to purified surface antigen fractions were purified from polyclonal antiserum by immunoaffinity techniques as described earlier (Bright and Kaleysa Raj 1993).

2.8 Human sera

The blood samples were collected from volunteers with visible symptoms of filariasis.
and asymptomatic mf positive. The human cord blood was obtained from the Obstetrics department of Medical College, Trivandrum. Endemic normal sera were collected from volunteers residing in endemic zone where the incidence of filariasis was high. Non endemic control blood samples were collected from Marayur, Iddukki district (more than 80 km away from any endemic belt). Particular care was taken to collect control sera from people of the same age, sex and socioeconomic group. The sera were separated from blood samples and preserved frozen until use.

2.9 SDS-PAGE analysis of purified antigens

The purified surface antigens were analysed on a discontinuous gel system containing 10% acrylamide as separating gel and 3% acrylamide as stacking gel at reduced state and silver stained.

2.10 Indirect fluorescence antibody test

Localization of surface antigens was effected by indirect fluorescence antibody test (IFAT) (Jaton et al 1979). Fresh worms as well as those treated with 0·5% TX 100 for 5 and 10 min were washed in PBS, suspended in 1:50 diluted detergent soluble surface antiserum, kept at 4°C for 30 min and then washed in PBS containing 2% BSA. 1 : 1000 diluted anti rabbit immunoglobulin G (IgG) FITC was added, incubated at 4°C for 30 min washed, recovered and suspended in phosphate buffered glycerol. They were then mounted on glass slides and viewed under fluorescent microscope.

2.11 Counter immuno electrophoresis

Counter immuno electrophoresis (CIEP) was performed on 1% agarose to test cross-reactivity against human antisera. The gels were stained with Coomassie blue after thorough washing in PBS.

2.12 Radioimmuno precipitation

The $^{125}$ I-labelled surface antigen was mixed with antiserum to surface antigen or human sera of patients and normal subjects. It was kept at 0–4°C for 12 h. The immune complexes formed were centrifuged, washed and radioactivity measured using a LKB gamma counter.

2.13 Dot-ELISA

Antigen solution of varying protein concentrations was spotted on nitrocellulose paper (NCP) discs and immobilized by keeping them at 37°C for 2 h in microtitre plates. It was quenched with 2% BSA, washed and treated with anti-human IgG peroxidase conjugate at 37°C for 10 min followed by dianaminobenzidene to get the visible reaction spots.

2.14 Dot-IFAT

Circulating filarial antigens in the patients blood was detected by Dot-IFAT. Sera
(0.25 μl) samples were spotted on NCP, immobilized, quenched and reacted with IgG antibodies to surface antigen followed by anti IgG rabbit FITC. The observations were made under fluorescent microscope.

2.15 ELISA

The principle of the experiment was the same as in § 2.13. However 96 well plates were used for efficient comparison of the different groups.

3. Results and discussion

In an adult parasite weighing about 30 ± 5 mg, the dissected cuticle was 6 ± 1 mg (20%). The total protein content of the whole worm was estimated to be 79.3 ± 0.05 mg/g body weight of which 15.3 ± 0.03 mg (19%) was cuticular proteins. The surface antigens from whole worms and isolated cuticle by TX 100 technique was 67 ± 17 μg and 93 ± 5 μg/g body weight of worm respectively.

Increased concentrations of TX 100 caused increased release of protein (table 1). Incubation of worms with different concentrations resulted in paralysis, within seconds. After treatment with detergent, the worms were incubated in normal medium and recovery was noted over a period of 6 h. Within this period recovery was observed up to a maximum concentration of 0.5% TX100. All the recovered worms showed only sluggish movement. As the concentration of detergent increased, the time required for recovery increased and mf release decreased. CIEP with antisera to surface proteins and protein released using different concentration of detergent showed similar pattern of precipitin bands. The mf release was insignificant.

The surface antigens of *S. digitata* isolated by TX 100 technique, when analysed on PAGE, resolved into three major and two minor protein bands as shown in figure 1. The three major protein bands were 36, 29 and 17 kDa. Of these, the 29 kDa protein was found to be the major one.
The total lymphocyte count showed initial decrease followed by increase in all the experiments in comparison with controls (6.48 × 10^5 ± 0.47/ml). Crude detergent soluble protein showed a suppression of 55% (figure 2). Electroeluted 29 kDa showed the maximum suppression of 70% and the low and high molecular weight proteins which were also electroeluted showed suppressions of 25% and 22% respectively (figure 2). The test B-lymphocyte count did not show any significant change from that of control (8.54 × 10^4 ± 0.39/ml). However blastogenesis with the electroeluted samples also showed similar levels of suppression as in the case of total lymphocyte count (figure 2).

The mechanism of immunosuppression in filariasis is not well understood. Immunosuppression due to T lymphocytes has been implicated in filariasis by Ottesen et al (1977) and Piessens et al (1982). Prasad et al (1991) observed reduced lymphocyte transformation to *W. bancrofti* mf excretory secretory antigen and Con A in clinical filarial patients. Non-specific immunosuppression by CD8+ T-cells in *B. pahangi* infected Lewis rats was investigated by Owhasi et al (1990). The present study clearly shows that surface proteins released from adult worms also produces a similar biological effect. Compared to other fractions 29 kDa protein electroeluted from the detergent soluble preparation showed maximum suppression. The 29 kDa antigen is a common constituent present in all species of lymphatic filariae and is recognized early by the host immune system (Maizels et al 1985; Morgan et al 1986; Philipp et al 1986). It appears from the present study that 29 kDa is a multifunctional protein, which on release may undergo some transition so that it is able to cause immunosuppression. An antigen with an approximate
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**Figure 2.** Effect of surface materials on the lymphocyte level.

1. Total count of cuticular surface protein; 2. Total count of whole worm surface protein.

Total count of electroeluted fraction of 2—(a) 29kDa; (b) low molecular weight; (c) high molecular weight.

Blaslogencsis of electroeluted fractions of 2—(d) 29 kDa; (e) low molecular weight; (f) high molecular weight.

Size of 29 kDa has been previously described from a number of sources (Kaushal et al 1982; Maizels et al 1983, 1985; Sutanto et al 1985; Morgan et al 1986; Selkirk 1986; Devaney 1987) and shown to be on the exterior face by antibody binding and absorption studies (Devaney 1987). The fact that the intensity of fluorescence increased in response to exposure time of the worm in TX 100 and the absence of fluorescence in the normal worm (figure 3) confirms the fact that antigenic
Figure 3. IFAT showing difference in intensities of fluorescence of (a) 0.5% TX 100 for 5 min and (b) 0.5% TX 100 for 10 min (c) Treated adult female *S. digatata* (× 250)
epitopes are normally kept hidden and are exposed during detergent treatment thus warranting further detailed study of the surface of the parasite.

Visible precipitin band was formed in CIEP when the whole worm homogenate was tested against sera from *W. bancrofti* infected microfilaremic cases (figure 4a). The presence of cross reacting antigens in *S. digitata* was then tested. The cuticle and surface antigen preparations also produced visible precipitin bands in CIEP (figure 4b). The detergent soluble surface protein showed denser bands in CIEP than the whole worm homogenate or whole cuticle suggesting the high antigenicity of the former preparation. Because of greater ease, the CIEP method was used in selecting the most active antigenic fraction.

The human sera from all the groups of bancroftian filarial patients precipitated significant quantities of radioiodinated surface antigens of *S. digitata* (table 2). The values were low in the case of endemic normals and considerable high in the case of microfilaremic cases. The symptomatic patients showed much less counts whereas no significant counts were obtained with non endemic control sera.

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**Table 2.** Radioiodinated surface antigens of *S. digitata* precipitated with bancroftian filarial patient's serum.

<table>
<thead>
<tr>
<th>Serum samples</th>
<th>Radioactivity in immunoprecipitate (CPM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antisurface antiserum (<em>n</em> = 6)</td>
<td>7460 ± 130</td>
</tr>
<tr>
<td>Non endemic control (<em>n</em> = 32)</td>
<td>70 ± 20</td>
</tr>
<tr>
<td>Endemic normal (<em>n</em> = 20)</td>
<td>2064 ± 1542</td>
</tr>
<tr>
<td>Mf + ve asymptomatic (<em>n</em> = 55)</td>
<td>3302 ± 452</td>
</tr>
<tr>
<td>Symptomatic patients (<em>n</em> = 25)</td>
<td>3071 ± 146</td>
</tr>
</tbody>
</table>

Values are mean ± SD of the total counts in the precipitates when incubated 10 lig antigen (2290 CPM/p4 protein) with 50 111 serum samples.
The cross reactivity of the three purified surface antigen fractions was tested against immune sera from *W. bancrofti* infected patients using Dot-ELISA. All the fractions produced visible reaction spots up to a concentration of 100 ng protein per spot whereas the 29 kDa fraction produced visible spot with 1 ng protein indicating its high sensitivity (data not shown). The result on the evaluation of surface antigens for the diagnosis of bancroftian filariasis by Dot-ELISA are shown in tables 3 and 4. Table 3 shows the percentage positivity of surface antigen fractions (SAFs) (100 ng protein per spot) when challenged against different human serum samples (100 times diluted) in the antibody detection tests. A cent per cent positivity was observed in all the SAFs against both mf +ve, asymptomatic as well as symptomatic subjects. None of these SAFs produced visible reactions against non-endemic control sera indicating the absence of filarial antibodies in them. But a significant proportion of the endemic normals gave positive reactions with all the antigens tested.

The results of Dot-IFAT experiments conducted to evaluate the activity of IgG antibodies to surface antigens specific to the three surface antigen fractions for the detection of circulating filarial antigens in the *W. bancrofti* infected patients are shown in table 4. Hundred per cent positivity was observed in all the IgG fractions with mf +ve as well as symptomatic patients. No visible reactions were observed against the IgG fractions specific to SAF6.

### Table 3. Positivity (%) of surface antigen fractions of *S. digitata* in the antibody detection of *W. bancrofti* infected human serum samples by Dot-ELISA.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Non endemic control (n = 32)</th>
<th>Endemic normal (n = 20)</th>
<th>Mf +ve (n = 55)</th>
<th>Symptomatic (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAP2</td>
<td>ND</td>
<td>65</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SAF1</td>
<td>ND</td>
<td>50</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SAF3</td>
<td>ND</td>
<td>50</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SAF6</td>
<td>ND</td>
<td>40</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Values are in percentage.
ND, not detected; SAP2, surface antigen preparation; SAF, surface antigen fraction.

### Table 4. Positivity (%) of surface immunoglobulin fractions of *S. digitata* in the antigen detection of *W. bancrofti* infected human serum samples by Dot-IFAT.

<table>
<thead>
<tr>
<th>SlgGF</th>
<th>Non endemic control (n = 32)</th>
<th>Endemic normal (n = 20)</th>
<th>Mf +ve (n = 55)</th>
<th>Symptomatic (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SlgG</td>
<td>ND</td>
<td>55</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SlgGF1</td>
<td>ND</td>
<td>45</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SlgGF3</td>
<td>ND</td>
<td>45</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SlgGF6</td>
<td>ND</td>
<td>35</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Values are in percentage.
ND, not-detected; SlgGF, IgG ab to surface antigens.
Diagnostic use of detergent soluble antigen from *S. digitata* was carried out using PVC microtiter plate ELISA. The different antibody source such as mf + ve and endemic control showed significant responses (figure 5). As in the case of screening for AIDS, setting a suitable baseline valve is necessary, and in this case OD 0.9 clearly showed high reactivity in the case of mf + ve and negativity in the case of non endemic controls. However endemic controls showed moderate reactivity and symptomatic and cord blood showed least reactivities.

In the present study, *S. digitata* surface antigen based Dot-ELISA to detect the antibodies of *W. bancrofti* seems to be a simple and sensitive technique. The antigen detection tests by Dot-IFAT using antiserum of surface antigens to *S. digitata* also gave positive reaction with all forms of *W. bancrofti* patients. In both antibody and antigen detection tests, the observation of 100% positivity with all forms of patients and practically nil reaction with non endemic control sera suggest the utility of surface antigens in general and 29 kDa in particular in the immunodiagnosis of bancroftian filariasis. Further analysis is however required to
establish the true identity of the material.

Many of the vaccination schemes consider the surface of the parasite as the primary target of immune attack. In most of the parasitic infections the protective antibody was predominantly raised against surface antigens (Maizels et al 1983; Kaushal et al 1984; Cabrera and Parkhouse 1986). The failure in all such cases appears to be due to the non-separation of immunogenic and immunosuppressive materials (Linda John and R Kalaysa Raj, under preparation). The present paper gives a clear indication of the presence of two types of surface proteins. The exposed surface showed antigenicity as is evidenced by IFAT and the proteins released by the same treatment showed immunosuppression as shown earlier.

The biological function of immunosuppression and potential diagnostic use of 29 kDa protein, the most reactive of the surface proteins may appear a contradiction. But in reality it is not so. The severe immune reaction and raised level of antibodies observed following provocation of the parasite especially following DEC treatment has to be due to release of antigens other than 29 kDa or 29 kDa itself with a different configuration. The argument that these are two forms of 29 kDa (Kwan Lim et al 1989) is also probably true. The one that is bound to the surface is in the native form where reactive epitopes are masked, while the one that is released may be in a denatured form and produces general suppression of immune response, thereby helping the parasite to survive for long periods of time in the host.

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