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Identification of circulating parasite antigen in patients with bancroftian filariasis

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SUMMARY

Because many cases of lymphatic filariasis cannot be diagnosed either clinically or by immunodiagnostic test based on antibody detection, recent efforts have been more directed towards developing methods for detecting parasite antigen in the blood or urine. Using a solid phase (Sepharose 4B) two-site immunoradiometric assay (IRMA) employing hyperimmune rabbit antifilarial antisera, we have previously shown (Hamilton *et al.*, 1984) that essentially all cases of patent (ie. microfilaremic) infection in patients with bancroftian filariasis can be detected by this semi-quantitative assay as well as some individuals with amicrofilaremic (i.e., 'cryptic') infection. The present communication reports the results of studies that identify a prominent circulating antigen detected by this IRMA in sera from patients with microfilaremia. The antigen was eluted from Sepharose-bound rabbit polyclonal antiserum that had been reacted with known antigen positive sera. It was run in SDS-PAGE, blotted to nitrocellulose paper and identified autoradiographically using ^{125}I-labelled rabbit antifilarial antiserum. Its high molecular weight ($\sim$200 kD), stability to acid and boiling, and sensitivity to pronase and periodate suggest its being a glycoprotein. Isolation of this antigen will permit the development of specific reagents (such as monoclonal antibodies) which should enhance both the sensitivity and utility of the currently available antigen detection systems.

Keywords filariasis circulating antigen

INTRODUCTION

Direct demonstration of the parasite or of parasite products in the body of the host is the only means by which to diagnose filarial infection with certainty. In lymphatic filariasis, however, making a parasitological diagnosis often can be extremely difficult or, indeed, impossible. Adult worms of *Wuchereria bancrofti*, *Brugia malayi* or *Brugia timori* are lodged in lymphatic tissue sites that are generally not accessible; and even in those patients with circulating microfilaremia the microfilariae usually appear in the blood only periodically, often nocturnally. While immunodiagnostic techniques such as skin tests, complement fixation, haemagglutination inhibition and various other immunoassays have been employed to try to demonstrate the presence of anti-filarial antibodies as an indicator of filarial infection (Kagan, 1980), in the absence of a parasitological diagnosis all of

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these antibody-based assays have shared the major drawback of not being able to discriminate between past exposure and current infection. Furthermore, the usefulness of these antibody assays is limited by the problem of extensive cross-reactivity among the antigens of helminthic parasites, by the absence of a relationship between the magnitude of host response and the parasite burden or clinical manifestation of disease, and by the lack of common standardized reagents.

Because of these and other limitations in such serological techniques, recent attention has focused on the detection of parasite antigens in infected patients' blood and other body fluids. A number of such antigen detection assays have been reported in human schistosome and onchocercal infections (eg. Deelder *et al.*, 1980; Des Moutis *et al.*, 1983; Ouassi *et al.*, 1981; Santoro, van dem Eulbrouke & Capron, A., 1978) and more recently even in the sera from some patients with lymphatic filarial infection (Dissanayke, Galahitiyawa & Ismail, 1982; Prasad, Reddy & Harinath, 1983; Hamilton, Hussain & Ottesen, 1984). However, because of practical limitations of these assays (such as antigenic specificity, technical difficulty and, especially, limited reagent availability), none has yet been fully exploited for practical application to the diagnosis of filarial infections.

Of the different antigen detection systems thus far developed for use in lymphatic filariasis, the two-site immunoassays (Prasad *et al.*, 1983; Hamilton *et al.*, 1984; Forsyth, Mitchell & Copeman, 1984; Dissanayake *et al.*, 1984) appear to be the most promising because of ease of performance and the potential for extreme assay sensitivity. Using such an assay Dissanayake *et al.* (1984) exploited the antigenic cross-reactivity of a monoclonal antibody directed against the eggs of the cattle filarid *Onchocerca gibsoni* (Dissanayake *et al.*, 1984) to detect *W. bancrofti* antigen in sera from infected humans. Our own previous study utilized polyclonal antibody reagents raised against the closely related human parasite *B. malayi* to detect antigen in the serum of patients with *W. bancrofti* (Hamilton *et al.*, 1984). In these assays, however, the antigen being detected was not defined, and it became clear that identification and purification of the circulating antigen(s) being detected in these assays would be a necessary prerequisite to developing specific monoclonal reagents that would enhance both their specificity and sensitivity.

Therefore, in the present study, we have utilized qualitative analytical procedures in conjunction with the quantitative radioimmunoassay used previously for first identifying the circulating antigen detected in these human filarial plasmas and then for determining its basic physicochemical characteristics.

MATERIALS AND METHODS

Patients and patient plasma. Several groups of patients were studied (Table 1). Patients with bancroftian filariasis were classified into three categories according to the signs and the symptoms of their disease (Ottesen, 1980). Asymptomatic microfilaremic (MF) individuals had microfilariae circulating in their blood either nocturnally (India) or subperiodically (Cook Islands). A second group of patients exhibited chronic lymphatic obstructive pathology of elephantiasis or hydrocoele (CP) with episodic lymphangitis and lymphadenitis. The third clinical category, tropical pulmonary eosinophilia (TPE), was characterized by acute pulmonary obstructive and restrictive findings with very high levels of blood eosinophils and anti-filarial antibodies. 'Endemic normals' (EC) in the subperiodic population were individuals clinically and parasitologically free of infection but living in the endemic region and manifesting vigorous antifilarial immune responsiveness (Ottesen *et al.*, 1982). North American controls were healthy individuals never exposed to lymphatic filarial infections. Non filarial helminthiasis controls were South East Asian refugees all of whom had multiple intestinal helminthic infections including hookworm, *Strongyloides stercoralis*, *Ascaris lumbricoides* and *Trichuris trichiura* but without evidence of filarial infection. Non-bancroftian filariasis controls were patients who had infection with *Onchocerca volvulus*, *Loa loa* or *Mansonella perstans* and who had microfilariae demonstrable in either the skin or blood.

Daytime heparinized plasma samples were collected from all individuals. Any microfilariae potentially present in these samples were removed by 0·45 μm membrane filtration (Millipore Corporation, Bedford, MA, USA) immediately after plasma collection, in order to prevent spurious degradation and release of microfilarial antigens. All samples were then stored in liquid nitrogen or at -70°C until use.

Table 1. Characteristics of patient and control populations

Clinical category	No.	Age (Range)	Sex (M/F)	Geographic location
Bancroftian filariasis				
Periodic				
Asymptomatic microfilaremia	8	21–60	8/0	India
Chronic lymphatic pathology	16	13–65	10/6	India
Tropical pulmonary eosinophilia (TPE)	8	18–52	4/4	India
Subperiodic				
Microfilaremia	5	39–71	5/0	Cook Islands
Endemic (exposed) normals	5	42–71	1/4	Cook Islands
Controls				
Non-endemic normal (North American)	5	17–25	2/3	USA
Non-filarial helminthiasis	10	4–50	4/6	South East Asia
Non-lymphatic filariasis				
O. volvulus	25	13–65	25/0	Ghana, Guatemala, Venezuela
L. loa	4	19–32	2/2	West Africa
M. Perstans	2	27, 29	0/2	West Africa

Buffers. (a) The working buffer was 0·05 M sodium phosphate buffer (pH 7·5) with 0·4 M NaCl, 0·05% (v/v) Tween 20, 0·2% BSA, and 0·05% $NaNa_3$. (b) The wash buffer was 0·05 M Tris-HCl with 0·15 M NaCl and 0·3% Tween 20, adjusted to pH 8. (c) The SDS sample buffer was 0·125 M Tris-HCl buffer at pH 6·8 containing 10% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0·002% bromophenol blue. (d) The blotting buffer was 0·025 M Tris-Glycine buffer with 20% methanol and 0·1% SDS, adjusted to pH 8·4. (e) Phosphate buffered saline (PBS) was 0·02 M sodium phosphate buffer with 0·15 M NaCl, adjusted to pH 7·4.

Filarial antigen and rabbit anti-filarial antisera. B. malayi adult antigen (BmA) was prepared as described previously (Hamilton *et al.*, 1984). Hyperimmune rabbit antiserum to BmA was partially purified by ammonium sulphate fractionation and coupled to CNBr-activated Sepharose CL4B (Pharmacia, Piscataway, NJ, USA) (3 mg/ml sorbent) for use as the solid phase antigen trap. Rabbit anti-BmA was affinity purified on a sepharose-BmA column and radiolabelled by the chloramine-T method as described elsewhere (Greenwood, Hunter & Glover, 1963; Hussain & Ottesen, 1983).

The solid phase antifilarial antibody was used to extract antigen from the plasma as previously described (Hamilton *et al.*, 1984). Fifty or 200 μl plasma was rotated overnight at room temperature in a total volume of 500 μl with 2·5% suspension of rabbit anti-BmA sorbent. Subsequently the sorbent was washed with working buffer. The last wash was carried out in working buffer containing 0·5 M NaCl, and the sorbent was aspirated dry. The pellet was vortexed and boiled in 50 μl of SDS sample buffer for 5 min. The sorbent was then microfuged and 25 μl of supernatent was applied to the gel.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The extracted antigens were separated on SDS-PAGE slab gel using a technique modified from that of Laemmeli (1970). A 7·5% to 12·5% gradient gel with a 4% stacking gel in a discontinuous Tris-HCl buffer system was run in 0·025 M Tris-glycine (pH 8·4) for 4 h at 12·5 mA and 25 mA constant current for the stacking and separating gels respectively.

Electroblotting. Electroblotting of antigens separated in the SDS-PAGE gell to nitrocellulose paper (NCP) was carried out according to the method of Towbin, Staehlin & Gordon (1979) with minor modifications. Electroblotting was performed in a Protean transblot cell (Bio-Rad Laboratories, Richmond, CA, USA) at a constant current of 0·1 to 0·15 mA per gel at 4–10°C for 14–18 h. The gels were equilibrated in blotting buffer without SDS for 30 min prior to blotting.

Detection of antigen on NCP. The NCP was removed after blotting and quenched for 3 h at room temperature in wash buffer containing 3% bovine serum albumin. It was subsequently washed four times in wash buffer; the last wash was carried out in buffer containing 0·05% Triton-X 100. The NCP was then cut into strips, and 8–10 of these strips were incubated with 5 μCi of ^{125}I-labelled rabbit anti-BmA in 20 ml wash buffer containing 5% heat inactivated fetal calf serum for 2 h at room temperature with gentle rocking. This incubation was followed by three washes in wash buffer containing 10 μM EDTA and one wash in buffer containing 0·5 M KI. The strips were dried and exposed to Kodak X-ray film (DEF-2) in Kodak X-Omatic cassettes with intensifying screens. The films were exposed for 1 day or 3 days at -70°C, developed and fixed.

Immunoradiometric assay (IRMA). Circulating antigen in the plasmas or sera was measured by a modified solid phase immunoradiometric assay (IRMA) (Hamilton *et al.*, 1984). In brief, 50 μl samples were rotated for 48 h with 0·5 ml of 0·2% (v/v) sepharose anti-BmA sorbent (presaturated with normal rabbit serum), washed four times in working buffer and incubated overnight by rotating with ^{125}I-labelled rabbit anti-BmA. The radiolabel was preabsorbed by rotating overnight with 25% anti-BmA sorbent to remove any non-specific activity. The radioactivity (ct/min) bound was compared with the counts bound by standards prepared by adding known quantities of BmA antigen to a North American control serum. The results are expressed as 'ng antigen equivalents' per ml of serum (Hamilton *et al.*, 1984).

Physicochemical characterization of the antigen.

(a) Heat sensitivity: A plasma sample positive for the antigen was diluted 1:8 in PBS and heated at 100°C for 5 min prior to absorption of the antigen onto the anti-BmA sorbent.

(b) Acid sensitivity: An antigen positive plasma was exposed to 0·1 M HCl for either 1 h or overnight. Samples were subsequently dialysed against working buffer before being reacted with the sorbent.

(c) Periodate sensitivity: The circulating antigen in the plasma was absorbed onto anti-BmA sorbent before being exposed for 1 h to 1, 10 and 100 mM sodium periodate (Aldrich Chemicals, Milwaukee, WI, USA) in PBS. The entire preparation was then boiled for 5 min in SDS sample buffer and applied to the gel.

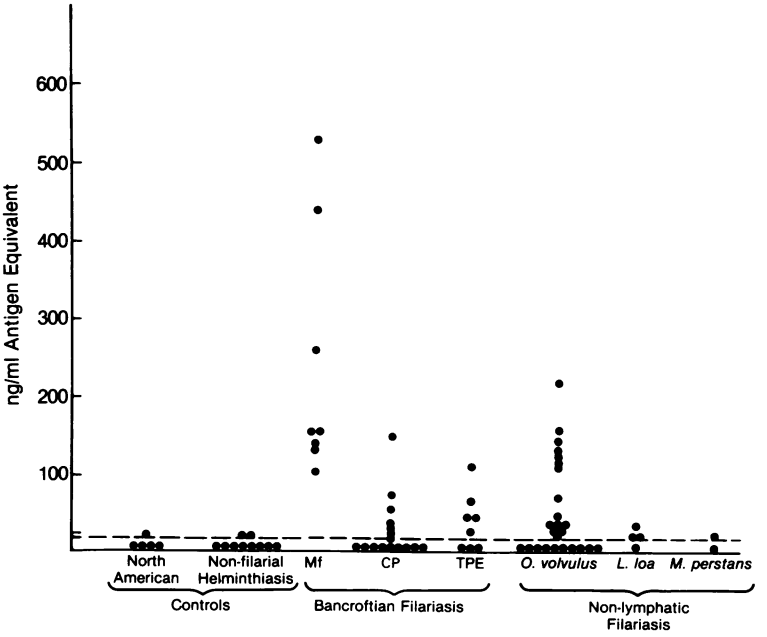

Fig. 1. Circulating antigen detected by IRMA in each patient or control specimen. Each point represents one individual and the dotted line at the bottom represents the mean+s.d. for North American normals and non-filarial helminth controls.

(d) Pronase sensitivity: The circulating antigen absorbed onto the sorbent was exposed to 0·1, 1·0, and 10 mg/ml of pronase (Protease type XIV from *Streptomyces griseus*, Sigma Chemical Co., St Louis, MO, USA) at 37°C for 4 h. The entire preparation was then boiled for 5 min with SDS sample buffer and applied to the gel.

RESULTS

Quantitative detection of circulating filarial antigen

The 'antigen equivalent' (Hamilton *et al.*, 1984) of the circulating antigen detected in the IRMA was calculated from the standard curve generated with standards prepared by adding known quantities of *B. malayi* antigen to a negative control serum. Fig. 1 shows the circulating antigen values in the plasmas from the 32 patients with periodic *W. bancrofti* infection and 46 controls.

Identification of the circulating antigen.

Plasma samples from patients with lymphatic filariasis and controls were analysed qualitatively by immunoblotting. As seen in Fig. 2, though prominent bands were evident in all autoradiographs in the molecular weight regions of ~44 and ~22 kD, these bands were also seen with both the control plasma and the buffer blank to which no human plasma was added. As they are 'nonspecific', their identification has not been pursued further. Much more important, however, was the prominent band seen in the ~200 kD molecular weight region in five of the 32 plasma from *W. bancrofti* infected patients when 50 μl plasma samples were used. With 200 μl specimens four additional positives were obtained (data not shown). Eight of the positive plasma samples came from microfilaremic patients and one from a patient with tropical eosinophilia; all were also positive for antigen by IRMA. In addition, four of the five microfilaremic subjects with subperiodic bancroftian filariasis were positive in the immunoblot assay with 50 μl plasma samples and one of five 'endemic normals' from the same endemic region was also positive (data not shown). Surprisingly, it was the same ~200 kD antigen that was identified in all 14 positive plasmas, and all of these 14 plasmas were also positive in the IRMA. No other specific bands were seen in any of these specimens.

Specificity of the antigen detected

The specificity of the antigen detected was established by analysing the plasma samples from five unexposed North American normals and 10 patients with non-filarial helminth infections. None of these controls showed the specific antigen band even when 200 μl plasma samples were used and the autoradiographs overexposed (Fig. 3). Among the 31 non-lymphatic filariasis plasmas tested three were positive for this antigen. All three positive plasmas were from onchocerciasis patients living in Africa.

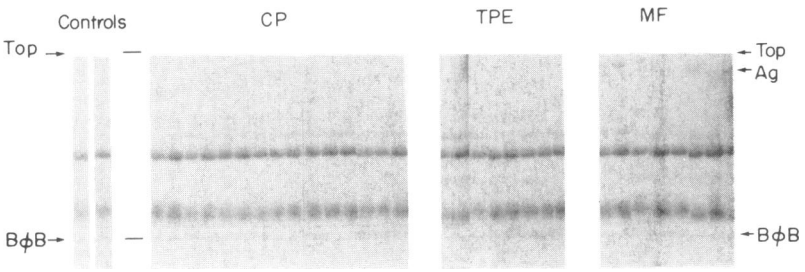

Fig. 2. The autoradiogram shows the qualitative analysis of 50 μl plasma samples from 32 patients with periodic *W. bancrofti* infection. The control lane at the extreme left shows the pattern of response for a buffer blank alone. The second control lane contained a normal North American plasma. A parasite specific band seen in the microfilaremic plasma is indicated by the arrow. Other bands are 'non-specific' (see text).

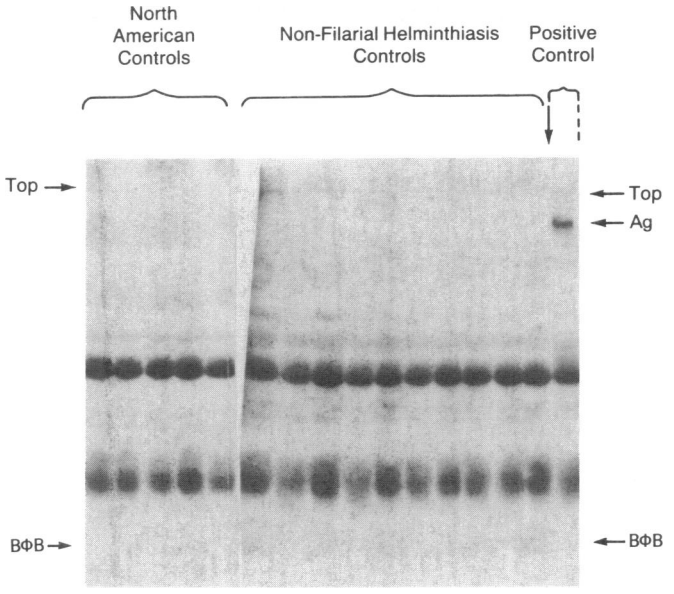

Fig. 3. The autoradiogram shows the qualitative analysis of 200 μl plasma samples from five North American control sera and 10 non-filarial helminthiasis control sera. At the far right is a serum with detectable antigen as indicated by the arrow. The autoradiogram was overexposed (6 days) in order to enhance the detection of antigen if it were present in the sera.

Table 2. Comparison between the sensitivities of the immunoradiometric assay (IRMA) and the qualitative (immunoblot) antigen detection system

Antigen equivalent Measured in IRMA (ng/ml)	Clinical category	200 kD antigen detected by immunoblotting in 50 μl plasma	in 200 μl plasma
< 50*	TPE, CP	–	–
56	CP	–	–
67	TPE	–	–
72	CP	–	–
110	TPE	–	+
105	Mf	–	+
132	Mf	–	+
140	Mf	–	+
150	CP	–	–
154	Mf	+	+
155	Mf	+	+
260	Mf	+	+
440	Mf	+	+
530	Mf	+	+

* Nineteen patients fell into this category.
CP Chronic Lymphatic Pathology.
TPE Tropical pulmonary eosinophilia.
Mf Microfilaremic.

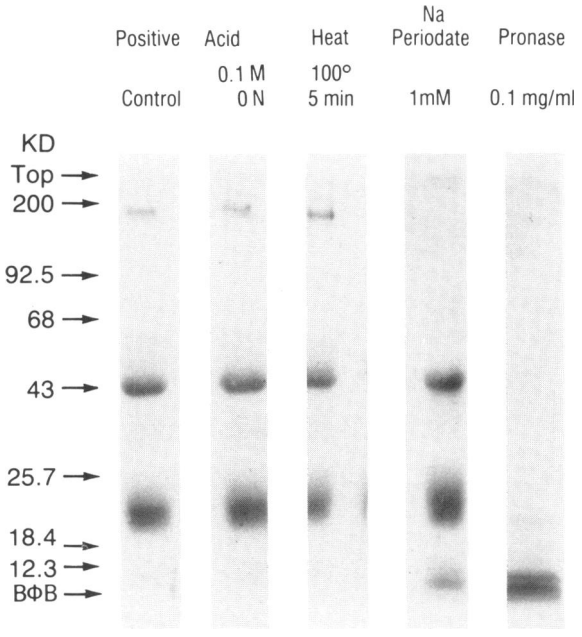

Fig. 4. The autoradiogram shows the qualitative analysis of the circulating antigen in a known positive sample after it was subjected to various treatments. See text for description.

Relative sensitivity of the IRMA and immunoblot assays

Both the quantitative (IRMA) and qualitative (immunoblot) assays were performed with 50 or 200 μl plasma from all 32 patients with periodic bancroftian filariasis. All plasmas with antigen equivalents greater than 150 ng were positive in the immunoblot assay with 50 μl plasma samples (Table 2) and four plasma samples with lesser antigen equivalent values were negative in the immunoblot assay with 50 μl of plasma but positive with 200 μl of plasma. These results suggest that the IRMA employed with identical reagents is at least three times more sensitive than the immunoblot assay carried out here. It is also important to note that plasmas negative in IRMA were never positive in the immunoblot analysis, even when 200 μl samples were used. These two observations suggest that the antigen detected by immunoblotting is an important component of the 'antigen' detected in the IRMA. Only one specimen was negative by immunoblotting despite an appreciable level of circulating antigen (150 ng/ml) detected by IRMA.

Characterization of the circulating antigen

Figure 4 shows the results of partial physicochemical characterization of this circulating antigen. All positive plasma exhibited the antigen specific band in the ~200 kD molecular weight region under both reducing and non-reducing conditions. The acid and heat stability of the antigen are indicated in lanes 2 and 3. The antigen was, however, destroyed when exposed to 0·1 mg/ml of pronase or 1 mM sodium periodate, observations compatible with its being a high molecular weight glycoprotein.

DISCUSSION

Among the various methods for immunodiagnosis of parasitic infections the detection of circulating antigens is probably the most desirable because, if sensitive enough, it should differentiate between current and past infection. This attribute would be of particular value in areas endemic for filariasis where all individuals are repeatedly exposed to filarial antigens during their

lifetimes through bites of mosquitoes carrying infective larvae so that all develop antibodies to parasite antigens (Ottesen *et al.*, 1982).

A number of attempts have been made to detect circulating antigen in filarial infections. Dissanayake *et al.* (1982) and Prasad *et al.* (1983) were able to detect circulating filarial antigen in sera from patients with bancroftian filariasis by using polyethylene glycol precipitation of immune complexes followed by an ELISA to detect the antigen. More recently, Dissanayake *et al.* (1984) and Hamilton *et al.* (1984) both used two-site immunoassays to detect circulating filarial antigens in their patients. Though most of these studies were successful in detecting the circulating antigens in microfilaremic subjects, none has been able to detect reliably the antigens in other clinical groups where the level of circulating antigen is presumably lower. If such assays are to become useful for diagnosing infection in all patients with filariasis, their sensitivity must be increased considerably. Unfortunately, there are neither appropriate reagents available for these assays nor much descriptive information about the nature and characteristics of the circulating antigen being detected.

In the present study therefore, we have used solid phase polyclonal antibody to capture filarial antigen in the circulation and then SDS-PAGE and 'Western blotting' for the qualitative analysis of the trapped antigen. With this technique we could identify a prominent circulating antigen in most of the plasma samples that were positive by immunoradiometric assay though the sensitivity of this qualitative assay appeared to be about three times lower than that of the quantitative assay. From this qualitative analysis, however, it was evident that a single antigen predominated in the circulation and that it was the same antigen in all positive plasma, 12 of which came from patients with microfilaremia, one from a patient with tropical eosinophilia and one from an 'endemic control' who likely had a cryptic infection (Ottesen, 1980; Ottesen *et al.*, 1982). The antigen appeared specific to filarial infections in that it was not seen in normal controls or patients with non filarial helminthic infections. However, it does appear to exist in the plasmas of patients with other filarial infections. The fact that the rabbit antiserum used as the principal reagent in the assay was raised against the closely related but heterologous *B. malayi* parasite makes it likely that the detected antigen will be found in at least other lymphatic filarial infections.

This single predominant circulating antigen was partially characterized as a step toward its isolation. It appears to be heat and acid stable without loss of immunoreactivity. Its high molecular weight ($\sim$200 kD) and sensitivity to both periodate reduction and pronase digestion suggest a glycoprotein nature. Current efforts are directed towards isolating this antigen for use in developing more specific reagents (i.e., monoclonal antibodies) that could be utilized to enhance the specificity and sensitivity of the quantitative assay to the point where IRMA or an enzyme-based immunoassay derived from it could be used for routine diagnosis of all active filarial infections. Such reagents would also be necessary to determine immunohistologically from which stage (adult worm or microfilaria) and anatomic site this antigen comes.

Apart from its use in immunodiagnosis this antigen might play an important role in other host-parasite interactions. As an $\sim$200 kD molecular weight antigen it is surprising that it is not rapidly cleared but persists in the circulation. Its being found predominantly in microfilaremic individuals who of all patients with filariasis, are the most profoundly immunosuppressed with respect to their responses to filarial antigens (Ottesen, Weller & Heck, 1977; Piessens *et al.*, 1980a, b) suggests that this antigen might be involved in modulating the immune response of the host. Studies of such questions, however, along with the development of improved immunodiagnostic techniques must first await purification of this antigen from the complex mixture of antigens derived from these filarial worms.

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