

A PRELIMINARY ANALYSIS OF *WUCHERERIA BANCROFTI* MICROFILARIAL ANTIGENS FOR POTENTIAL USE IN DIAGNOSIS

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Abstract. Several antigens from the microfilarial stage of *Wuchereria bancrofti* have been identified using immunoblots of microfilarial antigens and screening with immune sera and tropical pulmonary eosinophilia (TPE) sera. This analysis revealed an array of antigens with apparent molecular weights of 14kDa, 35kDa, 42kDa, 63kDa, 88kDa, 97kDa and 200kDa. Among these only the 14kDa and 42kDa antigens were consistently recognized by most of the immune sera. A 132kDa antigen was recognized only by TPE sera.

Analysis of rabbit immune sera revealed that the 42kDa antigen was shared by two developmental stages of *W. bancrofti*, namely L3 and mF. This antigen could become a potential vaccine candidate. The 14kDa antigen seems specific for the microfilarial stage and therefore could be a diagnostic marker for active infection. The 132kDa antigen could aid in the diagnosis of TPE.

INTRODUCTION

Lymphatic filariasis has been identified as the second leading cause of long-term and permanent disability (Ottesen *et al*, 1997). Although it causes little direct mortality, it results in profound debilitating morbidity, which has immense socio-economic impact especially in the developing countries. *Wuchereria bancrofti* and *Brugia malayi* are responsible for the majority of cases of lymphatic filariasis. An estimated 128 million people worldwide are diseased with lymphatic filarial species of which 115.12 million are caused by *W. bancrofti* (Michael and Bundy, 1997).

The intermediate vector for *W. bancrofti* is the mosquito species *Culex quinquefasciatus*. The microfilarial (mF) stage of the parasite is infective to the mosquito where it undergoes moulting to give rise to the second and the third stage larvae (L3). This stage is infective to humans where it matures into the adult parasite which resides in the lymphatics. The mF show nocturnal periodicity.

Although filariasis has been identified as one of only six infectious diseases that are currently considered to be "eradicable" or "potentially eradicable" (Ottesen *et al*, 1997), the control of this disease is curtailed by the lack of easy diagnostic and suitable prophylactic measures. Vast advances

have been made in the area of diagnosis of filariasis. These are based on immunodiagnosis and species-specific DNA techniques. Immunodiagnosis is based either on antibody detection (Kumari *et al*, 1994; Chenthamarakshan *et al*, 1996) or antigen detection (Chanteau *et al*, 1994; Ramzy *et al*, 1994; Dhas and Raj, 1995). Recently two antigen detection test kits have been described, one based on the monoclonal antibody Og4C3 and the other on the monoclonal antibody AD12 (Weil *et al*, 1997). In immunodiagnosis of filariasis antigen detection assays offer the best scope, while the antibody detection assays provide sensitivity but lack in specificity since they cannot distinguish active from past infection and gastro-intestinal parasitic infections. In DNA based techniques, many studies have described the use of PCR in the diagnosis of filariasis (Siridewa *et al*, 1996; McCarthy *et al*, 1996; Zhong *et al*, 1996; Furtado *et al*, 1997). Although these techniques are valuable in research, their use in field conditions is limited by the constraints of cost and the need for trained personnel for their implementation and interpretation. So, despite the development of many sophisticated diagnostic techniques, microscopy remains the conventional method in the rural areas of many developing countries. Therefore the need still remains to find diagnostic techniques which can be easily applied under field conditions.

Although filariasis has been identified as a potentially eradicable disease, there has been slow progress in its achievement. Mass chemotherapy remains the corner stone in the area of control and

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prevention (Ottesen, 1985). Many clinical trials with combination of diethylcarbamazine, ivermectin and albendazole have been carried out to arrive at a single-dose strategy (Ismail *et al*, 1996; 1998). A supplementary tool in control programs is vector control (Ottesen *et al*, 1997). Very little progress has been made in the area of vaccine research, mainly due to the paucity of parasite material and the lack of a suitable animal model. *W. bancrofti*, unlike *B. malayi*, cannot be maintained in laboratory animals (Maizels and Selkirk, 1988). Therefore much of the work has been carried out using cross-reactive antigens of *B. malayi* (Kaushal *et al*, 1984; Dissanayake *et al*, 1995; Wang *et al*, 1997).

In this study a preliminary analysis of *W. bancrofti* microfilarial antigens has been carried out with a view to identifying antigens that could be important in diagnosis and potential vaccine candidates.

MATERIALS AND METHODS

Isolation of microfilariae (mF)

Healthy asymptomatic microfilaremic individuals with high mF counts were selected for bleeding. After obtaining written consent, 250-500 ml of blood was withdrawn from each individual between 8.30 pm and 10.00 pm. The blood samples were kept at 4°C overnight and processed individually. mF were extracted using 1% SDS to lyse human red and white cells. The samples were first stirred at room temperature (RT) for 15 minutes. Four volumes of 1% sodium dodecyl sulphate (SDS) was then added and the stirring was continued for another 15 minutes at RT. Then it was centrifuged in a Beckman centrifuge using a JA-10 rotor at 9,5000 rpm for 25 minutes at 4°C. The pellet was resuspended in 1% SDS and centrifuged at the same speed. Again the pellet was resuspended in 10ml of 1% SDS and centrifuged in a JS7.5 rotor at 7,000 rpm for 20 minutes at 4°C. The supernatant was removed and the pellet was resuspended in 0.5 ml of phosphate buffered saline (PBS pH 7.4). A 5 µl aliquot was appropriately diluted and the mF count was taken. The mF were stored at -20°C until use.

Isolation of third stage larvae

Laboratory bred sterile *Culex quinquefasciatus* mosquitos were allowed to feed on infected human volunteers with high mF counts. Fourteen days

later the mosquitos were dissected individually and the L3 were collected in PBS (pH 7.4) and stored at -20°C until use.

Collection of human sera

Immune sera were collected from patients living in an endemic area of Sri Lanka. These patients attended the anti-filaria campaign clinic with symptoms and signs suggestive of filariasis. These included pain and or swelling of either the upper or lower limbs, arthralgia and in some, chronic lymphoedema. The sera were analyzed for the presence of antibodies against microfilarial surface antigens by immunofluorescence and were classified as fluorescent antibody test (FAT) positive, weakly positive and negative. Sera were also collected from two patients diagnosed as having TPE. Negative control sera were obtained from residents in Sweden, non-endemic for filariasis.

Preparation of antigens

1x 10⁵ mF were used for the preparation of antigens for polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Antigen extraction was carried out as described by Parab *et al* (1988). For the purpose of immunization, mF antigens were extracted from 1 million mF by ultrasonication in 0.1% TritonX100 at 7 micro tip limit. L3 antigens were prepared in three lots of fifty L3 each, by sonication in 1xPBS (pH 7.4).

Immunization of rabbit with L3 and mF antigens

A single New Zealand white rabbit was immunized intramuscularly with antigens from 50 L3, in Freund's complete adjuvant (FCA). Pre-immune serum was collected prior to immunization. Two booster doses of L3 antigens (50 L3 each) were given in Freund's incomplete adjuvant (FIA) at two to three weeks interval, and serum collected one to three weeks after each booster dose. The same rabbit was immunized about a month after the second L3 booster dose with two doses of 50 µg mF antigens first in FCA, and after three weeks, the second in FIA. One to three weeks after each dose, immune serum was collected.

Immunization of rabbits with specific antigens

Most of the immune sera consistently recognized two antigenic polypeptides on immunoblots. The bands corresponding to these polypeptides were cut out from the blots, ground in a mortar,

suspended in PBS, emulsified in FCA and used to immunize two individual New Zealand white rabbits. Pre-immune as well as two immune sera (after two booster doses) were collected. The sera were analyzed on immunoblots and indirect immunofluorescence test was carried out using whole mF.

SDS-PAGE and immunoblotting

Electrophoretic separation of filarial polypeptides by SDS-PAGE and transfer to nitrocellulose were done as described by Towbin *et al* (1979). The immunoblotting was performed essentially as described by Berzins *et al* (1983). The blots were blocked in PBS with 3% bovine serum albumin for two hours at RT, washed twice in PBS with 0.05% Tween 20 and incubated overnight at RT with sera diluted in PBS with 0.1% BSA and 0.05% Tween 20. After washing twice in PBS with 0.05% Tween 20 the strips were incubated in 1/500 dilution of alkaline phosphatase conjugated antibody for six hours at RT. After two more washes in PBS with 0.05% Tween 20, color development was carried out in substrate buffer containing 50 mM Tris-HCl buffer pH 8.6, 10 mM MgCl₂, *o*-naphthyl phosphate and Fast blue B salt (Berzins *et al*, 1983).

Indirect immunofluorescence test

Whole mF were used to analyze antibody reactivity of the sera. 1/100 dilution of biotinylated goat anti-human or goat anti-rabbit immunoglobulin was used as second antibody and fluorescence developed with 1/100 dilution of fluorescein avidin D (FITC).

RESULTS

In order to identify *W. bancrofti* antigens recognized by antibodies elicited by natural filarial infections, thirty human sera from a filaria endemic area were analyzed by immunoblotting using mF polypeptides as antigens. Most sera showed antibody reactivity with two polypeptides of apparent molecular weights of 14kDa and 42kDa. Out of the thirty serum samples five are represented in Fig 1 along with two TPE sera and one Swedish negative control. These polypeptides were also recognized by FAT negative sera, although less intensely than by FAT positive sera. In addition, the FAT positive sera frequently also recognized polypeptides of 35kDa, 63kDa, 88kDa,

97kDa and 200kDa (Fig 1). The Two TPE sera were FAT positive and also recognized the mF polypeptides of 14kDa, 35kDa, 42kDa and 63kDa as well as an additional 132kDa polypeptide (Fig 1).

Although filarial infections are associated with high levels of IgE and IgG4, only weak antibody reactivity with mF polypeptides was seen with these isotypes, mainly with the 14kDa and 35kDa polypeptides (Fig 2A and B). In contrast, the TPE sera showed strong antibody reactivity of both IgE and IgG4, mainly with polypeptides of 14kDa, 35kDa, 42kDa, 63kDa and 200kDa (Fig 2A and B).

In order to analyze the degree of cross-reactivity between antigens of the L3 and mF stages of *W. bancrofti*, a rabbit was immunized with a sonicate of L3. The sera obtained showed reactivity only with the 42kDa and 63kDa mF polypeptides, but were negative in immunofluorescence

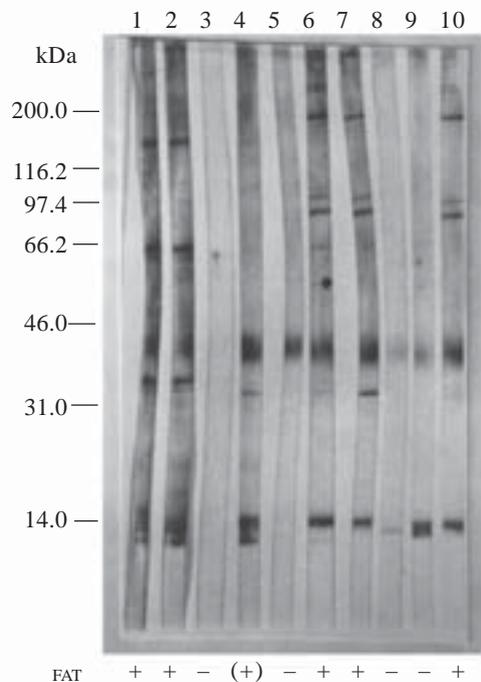


Fig 1—Immunoblotting analysis of the reactivity of human sera with mF antigens. Lanes 1 and 2 TPE sera, lane 3 Swedish negative control, lanes 4-10 sera from endemic patients. All sera were used in 1/250 dilution. The reactivity in FAT is indicated as + (positive), (+) (weakly positive) and - (negative).

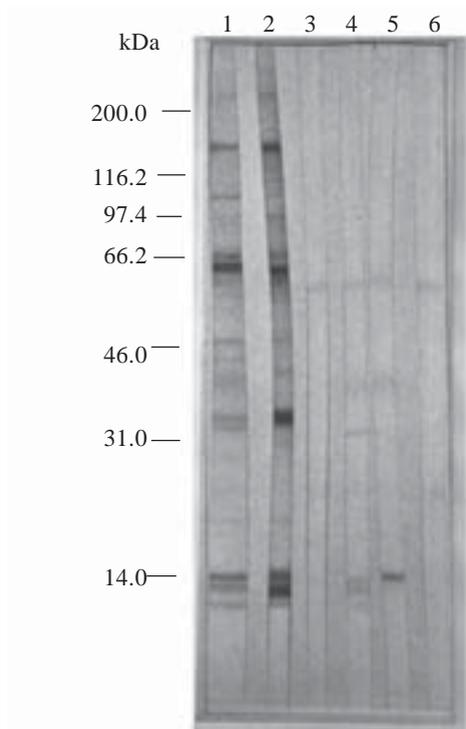


Fig 2A—Immunoblotting analysis of the reactivity of IgE antibodies in human sera with mF antigens. Endemic sera (lanes 3-6) and TPE sera (lanes 1 and 2). All sera were used in 1/100 dilution.

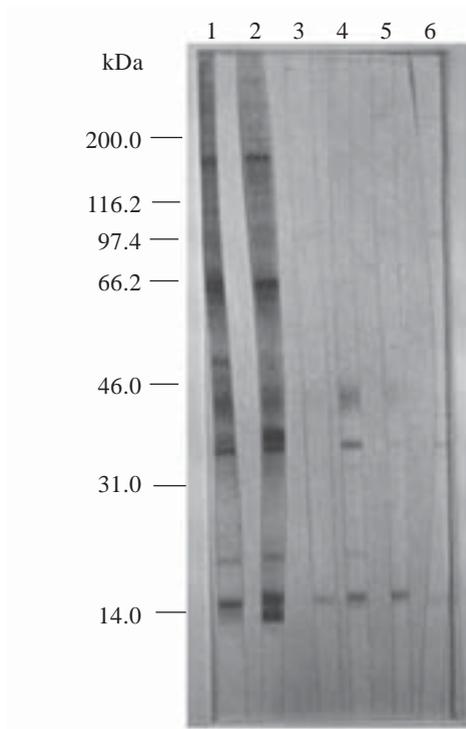


Fig 2B—Immunoblotting analysis of the reactivity of IgG4 antibodies in human sera with mF antigens. Endemic sera (lanes 3-6) and TPE sera (lanes 1 and 2). All sera were used in 1/100 dilution.

with mF (Fig 3). After subsequent immunization of the rabbit with mF extract, additional strong reactivity was seen with polypeptides of 14kDa, 35kDa, 88kDa, 97kDa and 200kDa (Fig 3), and the sera were positive in immunofluorescence.

In order to produce monospecific antisera to the two mF polypeptides most frequently recognized by human immune sera, bands with the 14kDa and 42kDa polypeptides were excised from immunoblots and used for immunization of rabbits. While no antibodies were detected in the antiserum against the 14kDa polypeptide, the antiserum against the 42kDa polypeptide showed reactivity with the corresponding antigen as detected by immunoblotting (Fig 4). However, the latter serum was negative in immunofluorescence with mF.

DISCUSSION

Identifying filarial antigens is a fundamental step towards improving immunological diagnostic

methods, and for providing basic insight which would aid in identifying potential vaccine candidates. Although closely related parasitic species share several antigens (Geiger *et al*, 1996), it cannot be disputed that the efficiency of any procedure can only be optimized by utilizing antigens from the same species that is responsible for the disease. Therefore in areas endemic for *W. bancrofti* filariasis it would be ideal to utilize antigens derived from the same species.

In order to analyze microfilarial antigens of *W. bancrofti*, sera were collected from patients living in an endemic area in Sri Lanka, who had symptoms and signs related to filariasis. Several antigens of apparent molecular weights of 14kDa, 35kDa, 42kDa, 63kDa, 88kDa, 97kDa and 200kDa were recognized by these sera when analyzed on immunoblots of *W. bancrofti* microfilarial antigens (Fig 1). Of these the most consistently recognized antigens were the 14kDa and 42kDa antigens. These were recognized even by sera that had been characterized as FAT negative. Thus fluorescent antibody test which is used routinely for screening of

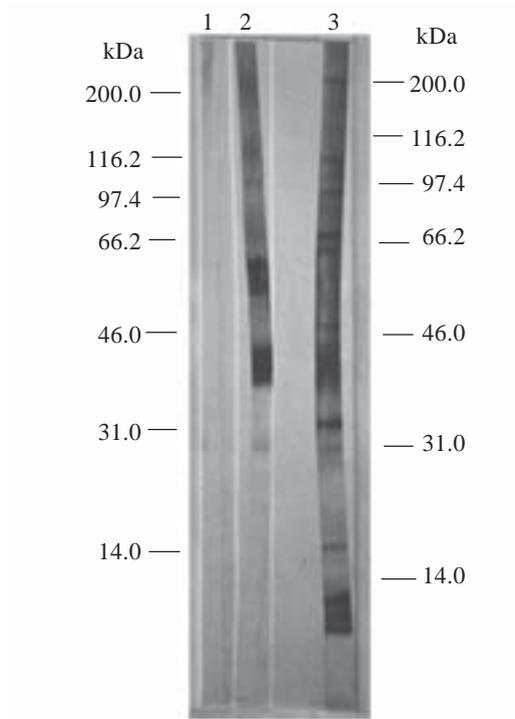


Fig 3—Immunoblotting analysis of the reactivity with mF antigens of antisera from a rabbit immunized with L3 extract and subsequently with mF extract. Pre-immune serum was included as control (lane 1), L3 extract (lane 2) and mF extract (lane 3). All sera were used in 1/100 dilution.

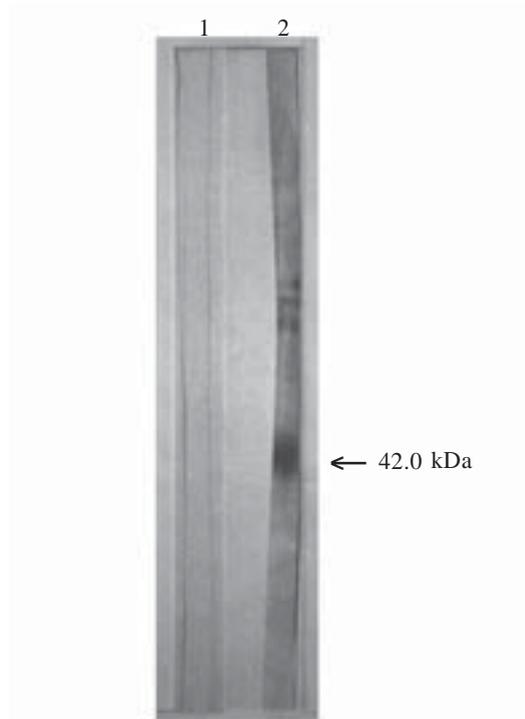


Fig 4—Immunoblotting analysis of the reactivity with mF antigens of a rabbit antiserum made against a 42kDa polypeptide of mF. The pre-immune serum of the rabbit as control (lane 1), and mF antigens (lane 2). Both sera were FAT negative.

filariasis is not a sensitive test for the diagnosis of filariasis. Although several antigens have been identified in this study, the method of isolation of mF using SDS could have resulted in the loss of some surface antigens.

The sera obtained from the rabbit after immunization with L3 recognized the 42kDa and 63kDa antigens but not the other antigens (Fig 3). Only the sera after immunization with mF recognized the other antigens including the 14kDa antigen (Fig.3). These findings suggest a strong possibility that the 42kDa antigen is shared by the two developmental stages namely L3 and mF. The 14kDa antigen seems specific for mF. Therefore antibodies to the 14kDa antigen could be an indicator of active infection and thereby a potential diagnostic marker, since lower molecular weight helminth antigens seem to show greater specificity than the higher molecular weight antigens (Kaushal *et al*, 1984).

The rabbit antisera against L3 when analyzed by indirect immunofluorescent antibody test on whole mF was negative, raising the possibility that the 42kDa antigen could be an internal antigen not found on the surface of mF.

It is interesting to note that some of the immune sera reacted only with the 42kDa antigen. This may suggest an early infection before the production of mF and/or successful elimination of L3 by the host's immune system. Sera from "endemic normals" could help to confirm this. It is now widely accepted that the L3/post-infective L3 is the most likely developmental stage that induces protective immune responses in humans (Day, 1991; Devaney *et al*, 1996; Kurniawan-Atmadja *et al*, 1998), while the later stages are involved in pathogenesis or tolerance. This has raised the strong possibility that the L3 stage seems to be the ideal target for vaccines (Maizels *et al*, 1991; Grieve *et al*, 1995). Thus the 42kDa antigen which is

recognized by most immune sera, could be considered a potential vaccine candidate.

Since monospecific antibodies could be raised in rabbits using the 42kDa antigen (Fig 4), the serum could be used to immunoscreen a cDNA library of *W. bancrofti* L3 to isolate and characterize the gene and the protein which it encodes.

Patients with TPE are known to have high antibody titers against filarial antigens (Ottesen, 1992) and therefore react strongly on immunoblots as is also shown in Fig 1, Fig 2A and B. Although many patients suffer from respiratory symptoms and signs simulating TPE, there is still no specific diagnostic test to confirm this disease; therefore treatment is instituted arbitrarily (Ong and Doyle, 1998). In this study, TPE sera recognized a 132kDa antigen which is not recognized by the rest of the immune sera. Studies with more TPE sera could confirm the diagnostic utility of this 132kDa antigen.

In this preliminary study, three antigens of either diagnostic or prophylactic utility in human lymphatic filariasis caused by *W. bancrofti* have been identified. These antigens show apparent molecular weights of 14kDa, 42kDa and 132kDa. The characterization of their genes and the proteins they encode would aid in the understanding of their usefulness which could be exploited successfully for diagnostic and therapeutic purposes.

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