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Identification of a potential marker for *Brugia malayi* infection by Western Blot analysis

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Abstract

Brugia malayi adult antigen was electrophoresed on SDS-PAGE gels and electrophoretically transferred onto PVDF membranes. The membrane strips were incubated with different categories of human sera, followed by successive incubations with blocking solution, monoclonal anti-human IgG4 antibody and peroxidase rabbit anti-mouse IgG antibody; with adequate washings done in between each incubation steps. Chemiluminescence detection was used to develop the blots. Two antigenic epitopes (molecular wieights of ~67-68 kDa and ~54-55 kDA) were found to be present in the Western blots of all microfilaraemic sera, all amicrofilaraemic sera with positive anti-filarial IgG4 antibodies, some treated patients and some elephantiasis patients. We hypothesized that last two groups are still harbouring live adult worms despite being treated or in the chronic stage respectively. The two epitopes did not simultaneously react with soil-transmitted helminth sera, normal endemic sera and sera of city dwellers. Therefore the simultaneous presence of both epitopes may potentially serve as a marker for *Brugia malayi* infection.

Introduction

Lymphatic filariasis caused by *Brugia malayi* and *Wuchereria bancrofti* is endemic in South East Asia (1). In the peninsular Malaysia filariasis is mainly caused by *B. malayi*. The laboratory diagnosis of brugian filariasis is still based on blood detection of microfilaria by thick blood smear and/or concentration methods. These methods lack sensitivity and thus many positive cases may be missed (2).

Newer methods of diagnosis of lymphatic filariasis have been developed. This include antigen detection tests for bancroftian filariasis (3) and polymerase chain reaction for both brugian and bancroftian filariasis (4, 5). Diagnosis based on nucleic acid amplification is very specific and sensitive but it probably cannot diagnose amicrofilaraemic cases and is very costly. Therefore a disgnostic assy for brugian filariasis that is cheaper and will also diagnose patients that are not having circulating microfilariae is still urgently needed.

Western blot analysis of *Brugia malayi* antigens have been reported by several investigators (6, 7, 8). However there was no demonstration of epitope(s) that is/are specific for diagnosis of *Brugia malayi* infection. Therefore the aim of this study was to identify potential antigenic epitope(s) that may be useful in the specific diagnosis of *B. malayi* infection.

Materials and Methods

Antigen preparation

Brugia malayi adult worms were harvested from gerbils infected three months before with the third larvae stage of the parasite. The adult worms were washed in several changes of phosphate buffered saline (PBS, PH 7.4). They were then cut, homogenized, sonicated and freeze-thawed in PBS containing protease inhibitor. The preparation was then centrifuged and the supernatant concentrated to half its volume in a vacuum concentrator. The antigen was then desalted and its protein content was determined by Bio-Rad assay to be ~ 2000 ug/ml.

Serum samples

A total of 200 serum samples were tested, they comprised of the following categories : 1. Serum samples from individuals with microfilaria (n = 20) 2. Serum samples from individuals with elephantiasis (n = 7). 3. Serum from previously microfilaraemic individuals i.e. they have been treated and were amicrofilareamic at time of sampling (n = 12) 4. Serum samples from individuals in endemic area whose anti-filarial IgG4 antibodies were (previously determined) was negative (n =40) 5. Serum samples from amicrofilaraemic individuals in endemic areas with high titres of anti-filarial IgG4 antibodies (n =35). Endemic areas sampled were Gua Musang and Setiu in Kelantan and Marang in Terengganu. 6. Serum samples from city-dwellers (Kota Bharu) and non-endemic area (Jeli) (n =60). 7. Serum samples

from patients with soil-transmitted helminth infections admitted at HUSM (n = 26).

SDS-PAGE and Electrophoretic Transfer

Phast Electrophoresis System (Pharmacia, Sweden) was employed for running of the SDS-PAGE gels and for electroblotting. 10-15 % gradient and 7.5% homogenous Phast gels were employed. 1 ul of a low molecular weight marker and 3 ul of the antigen/well (previously optimized volume) and was electrophoresed at 250V, 10.0 mA, 3.0W, 15 °C, 60 Vh.

After the electrophoresis, the gels were peeled off the plastic backing using the gel slicer provided and the protein transferred onto PVDF membranes using semi-dry electrophoretic transfer at 20V, 25 mA, 15 °C, 5 Vh, 30 minutes.

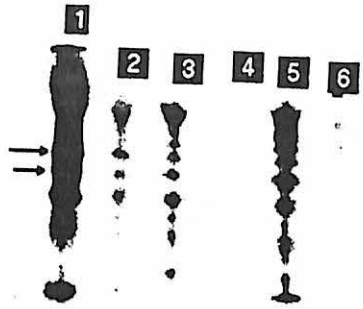
Western Blotting

Immediately after blotting of the antigen onto the PVDF membrane, the membrane was immersed in 10.5% Ponceau Red stain in order to visualize the bands while the membrane was being cut into strips. The lane containing the molecular weight marker was then washed in distilled water and immersed in Amido Black stain; the other strips were washed and incubated in 1% blocking solution (Boehringer Mannheim, Germany) for 30 minutes at room temperature (rt). The strips were then washed in TBS- 0.05% Tween 20 twice (10 mins/wash) followed by 2 times washing (10min/wash) in 0.5 % blocking

solution. Serum at 1: 800 dilution (in 0.5% blocking solution) was added to the strips and incubated for 2h, rt. This was followed by a washing step (as above) and the strips were then incubated with monoclonal anti-human IgG4 antibody at 1: 1000 for 30 minutes, rt. After another washing step, incubation of the strips was performed with anti-mouse IgG-HRP (horseradish-peroxidase) at 1: 2000 for 30 minutes, rt. Another washing step followed (5 x 15 minutes/wash with 0.5% TBS-Tween), then the blots were developed using a chemiluminescence blotting substrate (Boehringer Mannheim, Germany). The molecular weights of the antigenic bands were then determined using a digital image analyzer.

Results and Discussion

There were 9-10 major antigenic bands in the blots of all microfilaraemic individuals. Two of the antigenic bands with the approximate molecular weights of 67-68 kDa and 54-55 kDa always seemed to occur simultaneously in the following sera: all microfilaraemic sera, all sera from endemic area-individuals with high anti-filarial IgG4 antibody titre, some chronic elephantiasis cases (2/7) and some treated patients (6/12). These two bands did not occur simultaneously or did not occur at all in sera of city-dwellers, sera of soil-transmitted helminth patients, sera of endemic area-individuals with no anti-filarial IgG4 antibodies, some sera of chronic elephantiasis patients (5/7) and some sera of treated patients (6/12). Figure 1 shows an example of the results obtained.



1- Positive control (pooled microfilaraemic serum)
2- Serum from a microfilaraemic patient
3- Serum from a person in an endemic area with positive anti-filarial IgG4 antibody
4- Serum from a person infected with *Ascaris lumbricoides*
5- Serum from a treated microfilaraemic person
6- Serum from an elephantiasis patient

The presence of anti-filarial IgG4 antibodies has been strongly correlated to active infection in lymphatic filariasis (9, 10). Thus the observation that all individuals with high titres of the antibody demonstrated the marker is a contributory evidence that the occurrence of the two bands simultaneously may indicate infection with *Brugia malayi* . Chronic elephantaisis is a late stage lymphatic filaria infection, thus in most patients the adult worms are belived to dead or dying. Thus patients in this category who demostrated the bands simultaneously may harbour live or dying adult worms. The dying adult worms will still elicit antibody production against it, thus this may be the reason for the presence of the marker in 29% (2/7) of the elephantiasis patients.

Although treatment of microfilaraemic individuals with diethylcarbamazine is usually effective in killing the microfilaraie and adult worms, it is not uncommon to find treated people to be microfilaraemic some months post-treatment. This can be due to two reasons, firstly the drug causes some adverse reaction such as headache, nausea and low blood pressure. Therefore treatment compliance has been a problem especially in remote areas where the authorities cannot visit the patients on each treatment day. Secondly, the adult worms in deep lymphatics may not die with the normal one course of the drug. Another point to note is that upon treatment the microfilariae will quickly die, but the adult worms will die off slowly. Therefore this may explain why 50% (6/12) of the treated individuals demonstrated the infection marker.

Areas for further studies

There are several limitation of the study that we obseved :

1. In order to prove that the two bands can serve as an infection marker, a bigger population must be sampled.

2. The number of soil-transmitted helminth serum was small. Many more serum from this category should be tested since many cases of cross-reactivities in lymphatic filariasis serology were seen with serum from individuals with other helminthic infection.

3. Western blots using serum from *Wuchereria bancrofti* patients was not performed.

4. What is the evidence that shows the amicrofilaremic individuals with high anti-IgG4 antibody titres were really infected?

Thus, we must conduct an evaluation study using much larger sample number. We also will sample 50-100 soil-transmitted helminth individuals living in a non-endemic area for filariasis. We will try to obtain sera from other helminthic infection (e.g. Toxocara, Trichinella) and from individuals with *Wuchereria bancrofti* infection.

As for the last problem, we can treat the seemingly healthy individuals with high anti-filarial IgG4 antibody titres and perform Western blots on their sera before and after treatment. A drop in the intensity of the marker or a dissapearance of the marker will be a strong indication that the individuals were indeed actively infected despite being amicrofilaraemic.

In conclusion, this potential marker for *Brugia malayi* infection seemed to be promising and thus must be evaluated further.

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