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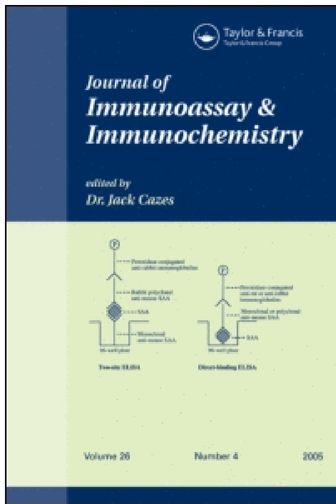

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DIAGNOSTIC USE OF POLYCLONAL ANTIBODIES RAISED IN MOUSE ASCITIC FLUID IN BANCROFTIAN FILARIASIS

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ABSTRACT

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Polyclonal antibodies were produced against *Brugia malayi* adult antigens (BmA (PBS) SAg and BmA (SDS) SAg) in mouse ascitic fluid by immunising Balb/c mice intraperitoneally with high ratio of adjuvant to immunogen. The diagnostic use of these antibodies in detecting circulating filarial antigen in bancroftian filariasis was studied by sandwich enzyme-linked immunosorbent assay (sandwich ELISA) using stick assay system. Both antibodies raised against PBS and SDS soluble antigens were found to be equally sensitive and relatively specific in detection of circulating filarial antigen. When anti BmA (PBS) SAg antibody was used in sandwich ELISA, 90% of microfilaraemic sera, 30-40% of acute and sub acute filarial sera, 20% of chronic filarial sera, 7% of endemic normal sera and none of 15 non-endemic normal sera were positive for filarial antigen. Using anti BmA (SDS) SAg antibody, 93% of microfilarial sera, 40% of acute and sub acute filarial sera, 20% of chronic filarial sera and none of 15 endemic and non-endemic normal sera showed the presence of filarial antigen. The filarial antigen detection using anti BmA S Ag antibodies produced in mouse ascitic fluid in sandwich ELISA may be useful in detection of active stage (microfilaraemia) of infection.

(KEY WORDS : Filariasis, ELISA, Polyclonal antibodies, ascitic fluid, filarial antigens, diagnosis).

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INTRODUCTION

Filariasis is a major health problem in tropical countries. The precise diagnosis of human lymphatic filariasis is based on the detection of microfilariae in the blood taken at night, a test that often fails in low parasite density and in detection of prepatent and occult infections. At present, the main focus is on immunodiagnosis for detection of either parasite antigen or antibody in the infected host (1). Diagnostic methods based on detection of parasite antigens are more useful in detecting active infection. Detection of circulating filarial antigen in the sera, urine and hydrocoele fluid samples of filarial patients has been reported by several workers. Hamilton *et al.*have used antibodies specific for *Brugia malayi* adult worms to detect cross reactive filarial antigens in *Wuchereria bancrofti* infection by immuno radiometric assay (2). Mostly polyclonal antibodies are raised in rabbits, sheep or goats. Due to the limited amount of *B.malayi* filarial antigens available in the laboratory from infected animals, it is not possible to immunize a large number of these animals. Antibodies produced in the mouse ascitic fluid may be used as an alterna-

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tive for immunological assays (3). It would be easy and relatively inexpensive to immunize large groups of mice intraperitoneally with a high ratio of adjuvant to immunogen and pristane (Sigma, USA) which induces the formation of ascitic fluid (4). This communication describes the production of polyclonal antibodies to *B.malayi* adult antigens in mouse ascitic fluids and their use in detection of circulating antigen in sera of filarial patients by enzyme linked immuno sorbent assay.

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MATERIALS AND METHODS

Sera

Human sera belonging to different groups viz. normal (endemic and non endemic) and filarial (microfilaraemia and clinical filariasis) were screened in this study. Filarial blood samples were collected from Sevagram and surrounding villages, an endemic region for nocturnally periodic *Wuchereria bancrofti*. The presence of microfilariae in microfilaraemia cases was confirmed by night blood (wet smear) examination. Clinical disease refers to acute filariasis (Lymphangitis and lymphadenitis), sub acute filariasis (Hydrocoels and soft oedema of limbs persisting over 2-5 years) and chronic diseases (elephantiasis of limbs/genitalia with evidence of gross fibrosis). Endemic normal

blood samples were from healthy individuals living in an endemic region having no history of filariasis. Non-endemic normal blood samples were collected from students coming to this Institute from places like Chandigarh and Kashmir, India where there is no filariasis. Sera were separated from blood samples and stored at -20°C with sodium azide as preservative.

Sera from patients who were positive for Onchocerciasis, Strongyloides, Toxocariasis, Echinococcus were provided by Dr. N. Weiss from Swiss Tropical Institute (Socinstrasse, Switzerland); sera from patients with *Brugia malayi* infection and from clinical filarial patients of *W.bancrofti* infection were provided by Dr. S. K. Kar from Regional Medical Research Centre (Bhubaneswar, India).

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Antigens

Somatic extracts from adult worms of *B.malayi* were prepared as described by Kaushal *et al*. and Maizels *et al*. (5,6). Briefly, freeze dried adult female worms of *B.malayi* recovered from the peritoneal cavity of jirds, *Merionus unguiculatus* were pulverized by manual homogenization. The homogenate was extracted with phosphate buffered saline (PBS,0.05M,pH 7.2), overnight at 4°C. Proteins soluble in PBS were recovered by centrifugation at 13000 RPM for 30 min

at 4°C. The supernatent was separated, dialyzed and labelled as PBS soluble antigens (BmA (PBS) SAg). The pellet was subjected to the harsher extraction conditions of 5% sodium dodecyl sulfate (SDS), 5% 2-Mercaptoethanol and 8M urea in 0.01M, sodium phosphate buffer (SPB, pH 7.2). The SDS soluble fraction was recovered by centrifugation (13000 RPM) at 4°C for 30 min and the supernatent was separated and labelled as SDS soluble antigens (BmA (SDS) SAg). The soluble extract was then dialyzed against 0.01M SPB, pH 7.2 and the protein was estimated by Lowry's method (7).

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Anti B.malayi antibodies

Polyclonal antibodies against *B.malayi* adult antigens viz.,BmA (PBS) SAg and BmA (SDS) SAg were produced in mouse as described by Overkamp *et al*. (3). Briefly, female Balb/c mice were immunized by repeated intraperitoneal injections of an emulsion of BmA SAg in complete Freund's adjuvant (200 ug Ag per mice in 5 doses) together with a single intraperitoneal injection of pristane which induced ascites formation in most mice within 5 weeks. Ascitic fluid was obtained by abdominal tapping of immunized mice. Antifilarial antibodies were separated from ascitic fluid by 33% ammonium sulphate

saturation. The precipitate was resuspended in 0.01M SPB (pH 7.2), dialysed against excess SPB and the protein was estimated by Lowry's method (7).

Sandwich ELISA

Conjugation of anti BmA (PBS) SAgAb and anti BmA (SDS) S AgAb with penicillinase (Sigma Chemical Co., USA) was achieved by the method of Avrameas (8). The substrate consisted of soluble starch (150 mg) in 27.5 ml of 0.25M SPB (pH 7.2) containing 10.6 mg of penicillin V and 100 ul of 0.08M iodine in 3.2M potassium iodide solution. The substrate was prepared fresh before use.

Sandwich ELISA was carried out using a stick assay system (9). The optimal dilutions of anti BmAS-AgAb and anti BmAS AgAb-penicillinase conjugate were determined by chequer board titration. Since 6 of 15 non-endemic normal sera gave a positive reaction at serum dilution of 1:150 when screened for antigen, we considered 1:300 titre as positive reaction for filarial antigen and the sera were screened at 1:300 dilution. ELISA was carried out in small plastic vials using cellulose acetate membrane squares(5x5 mm) fixed to the plastic strips (5x70 mm) as the solid support for antibody binding. Optimally diluted

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anti BmASAgAb (100 ng/stick) in 0.05M SPB (pH 7.2) were coated to the sticks and air dried. The unbound sites were saturated with 3% gelatin in SPB (0.05M, pH 7.2). After washing with PBS (0.01M, pH 7.2) containing 0.05% (v/v) Tween 20 (PBS/T) for 5 times at 5 min intervals, the sticks were incubated with 0.5 ml of optimally diluted test sera (1:300) or serially diluted (2 fold) test sera in PBS/T with 0.5% bovine serum albumin (PBS/T-BSA) at 37°C for 2 hrs. After washing as above, the sticks were incubated with 0.5 ml of anti BmA SAgAb-penicillinase conjugate (1:2000) diluted in PBS/T-BSA at 37°C for 2 hrs. Following the final washing, the immune reaction was observed by incubating the sticks with starch-iodine-penicillin substrate. The complete decolourization or decolourization with a slight tinge of substrate colour denoted a positive reaction, while negative reaction was confirmed by the persistence of blue colour.

RESULTS

A total of 120 sera belonging to different groups were screened by the sandwich ELISA using anti BmA SAgAb and the results are summarised in Fig.1 and 2. Among the bancroftian filarial sera, 27 out of 30 microfilaraemic sera (90%), 3 out of 10

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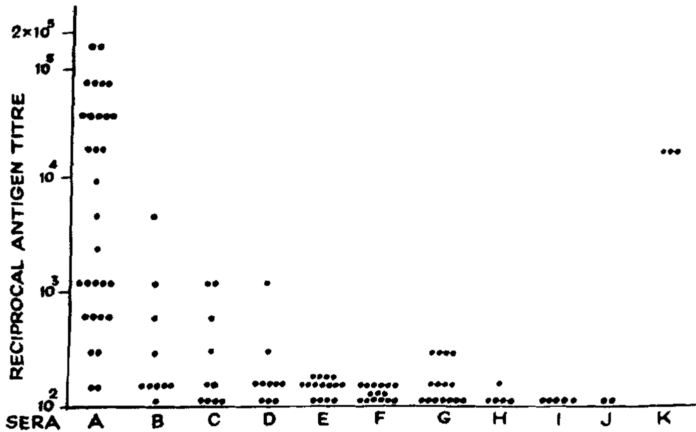

FIGURE 1 : Scattergram of the filarial antigen levels against anti BmA (PBS) SAg-antibody in sera samples of (A) microfilaraemia, (B) acute filariasis (C) subacute filariasis (D) chronic filariasis, (E) endemic normals, (F) non-endemic normals, (G) Onchocerciasis, (H) Strongyloides (I) *Toxocara canis*, (J) Echinococcus and (K) *Brugia malayi*. The sera dilution at 1:300 was taken as the threshold level for positivity.

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actute filarial sera (30%), 4 out of 10 sub acute filarial sera (40%), 2 out of 10 chronic filarial sera (20%) and only 1 out of 15 endemic normal sera (7%) showed the presence of filarial antigen against anti BmA (PBS) SAgAb. All the 3 brugian filarial sera (100%) and 3 out of 15 onchocercal sera (20%) were positive for filarial antigen while all the 15 non endemic normal sera, 5 strongyloides sera, 5 toxocariasis sera and 2 echinococcus sera were

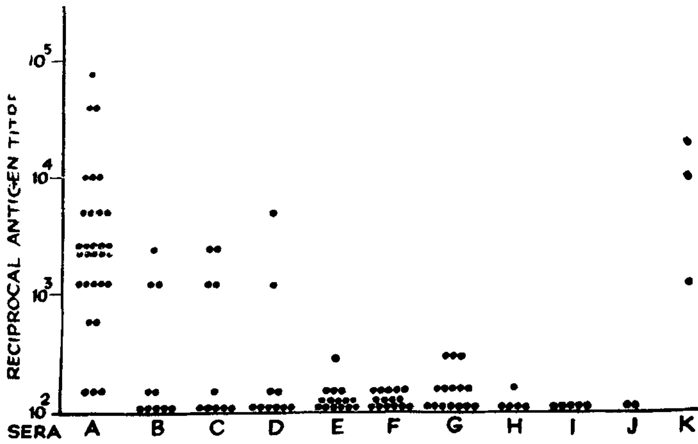

FIGURE 2 : Scattergram of the filarial antigen levels against anti BmA (SDS) SAg-antibody in sera samples of (A) microfilaraemia, (B) acute filariasis, (C) subacute filariasis, (D) chronic filariasis, (E) endemic normals, (F) non-endemic normals, (G) Onchocerciasis, (H) Strongyloides, (I) *Toxocara canis*, (J) Echinococcus and (K) *Brugia malayi*. The sera dilution at 1:300 was taken as the threshold level for positivity.

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negative for filarial antigen against anti BmA (PBS) SAgAb. The microfilaraemic sera showed a higher geometric mean of the reciprocal of antigen titre (GMRT 2512) than clinical filarial sera (GMRT 217) and the difference was statistically significant ($p < 0.001$) by the chi square test. While this test showed specificity of 93%, the sensitivity and accuracyvalues were higher for microfilaraemics (90% & 92% respectively) than for clinical filariasis (30% & 71.3% respectively).

When anti BmA (SDS) SAgAb was used, 28 out of 30 microfilaraemic sera (93%), 4 out of each 10 acute and 10 sub acute filarial sera (40%), 2 out of 10 chronic filarial sera (20%), all the 3 brugian filarial sera (100%) and 4 out of 15 onchocercal sera (27%) were positive for filarial antigen. All the 15 endemic normal sera, 15 non-endemic sera, 5 strongyloides sera, 5 toxocariasis sera and 2 echinococcus sera were negative for filarial antigen. The geometric mean of the reciprocal of antigen titre of microfilaraemic sera (GMRT 5639) was higher than that of clinical filarial sera (GMRT 217) and the difference was significant ($p < 0.001$) by the chi square test. While the test was relatively specific (93%) the sensitivity and accuracy values were higher for microfilaraemics (93%) than for clinical filariasis (93% and 72% respectively).

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DISCUSSION

The diagnosis of filariasis based on the detection of antifilarial antibody have proved to be of minimal use as an index of active infection (Microfilaraemia). In contrast to specific antibody detection, circulating filarial antigens are better markers for confirmation of current filarial

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infection (10). Circulating filarial antigen was first demonstrated in bancroftian filariasis by passive cutaneous anaphylaxis (11). More recently, a number of studies have been reported detecting circulating filarial antigens in bancroftian filariasis (2,9,12-19). However, these tests are not used in field studies possibly due to the sophistication of the techniques and limitations of the availability of assay reagent. We have raised a polyclonal antibody in mouse ascitic fluid and used in stick ELISA for the detection of circulating filarial antigen in bancroftian filariasis. Utilization of anti BmASAg antibody in sandwich ELISA increased the sensitivity of antigen detection to 93% in microfilariae carriers. In addition, antigen was detected in about 33% of clinical filarial cases including acute, sub acute and chronic filariasis. The high reciprocal filarial antigen titre in the microfilariae carriers suggested a possible association between the occurrence of this antigen in serum and active infection. A significant correlation was observed between serum antigen levels and the number of adult worms in canine dirofilariasis (20). The moderate levels of antigen detected in the acute filariasis

such as lymphangitis and lymphadenitis and much lower levels in the late chronic cases such as elephantiasis where adult worms are unlikely to be living after prolonged treatment, also provided supporting evidence for an association between the occurrence of this antigen in serum and active infection.

The presence of detectable antigen in the sera of amicrofilaraemic, asymptomatic individuals from endemic areas may be due to subclinical infections in these individuals. Autopsy data suggested that asymptomatic infections, and in some cases, unisexual infections can occur (21). Longitudinal follow-up of these patients is necessary to determine whether they are in the early phase of infection. All the 3 B.malayi infected sera showed the presence of filarial antigen, where as 4 out of 15 onchocercal sera showed the presence of filarial antigen, indicating the limited cross-reactivity of this antibody.

The lower levels of circulating antigen seen in most of the clinical filarial patients were most probably due to the fact that circulating antigen was complexed with specific antibody forming immune complexes. Significantly higher level of filaria

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specific circulating immune complexes have been detected in clinical filarial patients compared to microfilaraemic cases (22,23). Zheng *et al.*(24) reported that approximately one half of the sera from patients with chronic lymphatic filariasis contain parasite antigens. These groups of patients is heterogenous in its immune response to filarial antigens and suggest that active infections are present in half of these individuals (25). Further no reaction was observed with strongyloides, toxocariasis and echinococcus sera, thus increasing the specificity of this assay system. The advantages of producing antibody in mouse ascitic fluid compared to conventional immunization methods makes the process of producing the assay reagents easier. Stick ELISA system system has been found to be simple and economic. Thus the detection of circulating filarial antigen using anti *Bm*ASAgAb in stick ELISA may be useful for the detection of active infection in filarial control programmes.

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