Antibody responses of *Wuchereria bancrofti* patients to recombinant *Brugia pahangi* superoxide dismutase

S Rathaur, S Sharma & RN Singh
Department of Biochemistry, Faculty of Science, Banaras Hindu University, Varanasi 221 005, India,
e-mail - sushma@banaras.ernet.in
Kimberley Henkle
BNI, Bernhard-Nocht-str,74, 20359, Hamburg, Germany
and
Murray E Selkirk
Department of Biochemistry, Imperial College of Science & Technology, London, UK.

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Lymphatic filarial parasite *Brugia malayi* contains significant amount of Cu/Zn superoxide dismutase (SOD) activity in the extract of different life stages and in the excretory-secretory product of adults. In the present study recombinant SOD from *B. pahangi* has been used to see the antibody response in *Wuchereria bancrofti* infected patients. The recombinant SOD from *B. pahangi* reacted specifically with *W. bancrofti* infected sera in ELISA and immunoblotting. The reactivity of IgM subclass was more as compared to IgG subclass both in the asymptomatic microfilaraemic and symptomatic amicrofilaraemic when tested by ELISA. Serum from other helminthic infection was very low and found to be insignificant. The antibody response to rec SOD was directly proportional to the number of microfilariae in infected patients. The circulating filarial SOD was detected in filarial patients using polyclonal antibodies raised against recombinant Cu/Zn SOD in rabbits. The apparent molecular masses as determined by immunoblotting were 29 and 22 kDa. The specificity of recombinant SOD could be explored for its use in immunodiagnosis of lymphatic filariasis.

Lymphatic filariasis is one of the most pervasive disease in the developing world. In India alone an estimated 874 million living are in endemic areas and 45 million are infected with disease. Although the disease does not result in immediate mortality, the associated morbidity is believed to cause significant disability. Lymphatic parasite of man are long lived organisms which persist in mammalian host despite vigorous and sustained immune response. The ability to neutralise or subvert host immunity is thus crucial for their survival. One important factor against invading organism may be the production of toxic oxidants by the host phagocytes. Antibody dependent cellular cytotoxicity (ADCC) has been invoked as a major effector mechanism against filarial nematodes and free radicals generated via the “oxidative burst” are thought to contribute to the killing process. The antioxidant enzymes like superoxide dismutase (SOD) and glutathione peroxidase (GSHPx) of the parasite may play important role to combat the reactive oxygen species. The enzyme SOD has been detected in *Brugia malayi*, a lymphatic filarial parasite of man and in *Onchocerca volvulus*. Two distinct types of Cu/Zn SODs, extracellular (EC) and cytoplasmic forms from filarial worm *Brugia pahangi* have been cloned and expressed. *B. pahangi* enzymes are very similar to *O. volvulus* cytoplasmic Cu/Zn SOD. Both forms are dimeric and their derived amino acid sequences are highly homologous to each other. However, the sequence of EC form contained an additional 43 residues at the N-terminus, the first 16 of which were markedly hydrophobic. In the present study, *Brugia pahangi* rec SOD reacted with sera from Wuchereria bancrofti infected filarial patients when tested by ELISA and immunoblotting. The circulating SOD was identified in microfilaraemic patients using polyclonal antibodies raised in rabbit against the recombinant enzyme.

**Materials and Methods**

*B. pahangi* recombinant Cu/Zn SOD and its polyclonal rabbit antiserum were prepared as described by Tang et al. Alkaline phosphatase and horseradish peroxidase conjugated antibodies and antihuman immunoglobulins; orthophenylenediamine,
4- chloronaphthol, 5-bromo indoyl phosphate and nitroblue tetrazolium were procured from M/s Sigma Chemical Company, U.S.A. All other chemicals used were of analytical grade.

**Collection of sera**—Sera from filaria infected humans were collected by field study in two areas of Varanasi, Sunderpur, an urban slum area and Chiraigaon, an agricultural area. These two areas are endemic for the nocturnal periodic form of *W. bancrofti*. Out of the total population of 6000 in Sunderpur and 8000 in Chiraigaon, randomly 500 and 1250 people were examined for microfilaraemia and chronic infections. Microfilaraemia was detected between 22.00 - 00.00§. The blood from patients having 250-1000 mf/ml was collected. Acute and chronic manifestations of filariasis were also recorded after physical verifications of all persons. Sera were separated by keeping blood at 37°C for 2 hr and centrifuged at 1000 g for 10 min in a refrigerated centrifuge and stored at -70°C with protease inhibitors till further use.

**Preparation of immunoglobulin free sera**—Immunoglobulin free sera were prepared by precipitating with 33% ammonium sulphate. The supernatant (free from Igs) was dialysed before use.

The filarial cases were divided into asymptomatic microfilaraemic (subjects having microfilariae in their blood but are devoid of any superficial symptoms) and symptomatic microfilaraemic (patients having superficial symptoms but they do not have microfilariae in their blood). The asymptomatic microfilaraemics were further categorized into two groups on the basis of the number of microfilariae in circulation: group I patients were having 250-500 mf/ml and group II patients were having 500-1000 mf/ml of blood. The controls included apparently endemic normals. During selection of cases it was ensured that the filarial patients were not suffering from any other infection.

**Enzyme linked immunosorbant assay**—Enzyme linked immunosorbant assay (ELISA) plate was coated with 2 µg/ml of recombinant enzyme or 5 µg/ml of different category of sera (free of immunoglobulins) in carbonate buffer (0.66 M, pH 9.6) and left overnight at 4°C; 100 µl of 1:50 diluted human sera or polyclonal antibodies raised in rabbits against rec SOD (1:100) was added into the wells and left at 37°C for 3 hr. After washing thrice with PBS tween (0.1%) 100 µl of horseradish peroxidase conjugated goat antihuman IgM or IgG (1:4000) or polyvalant anti rabbit alkaline phosphatase conjugate (1:4000) were added and incubated for further 3 hr. After washing with PBS tween the presence of antibodies was detected with orthophenyl diamine containing H₂O₂ in peroxidase conjugate or using BICP and NBT for alkaline phosphatase. The absorbance was read at 492 nm for peroxidase conjugate and at 405 nm for alkaline phosphatase using a Biorad ELISA reader. The positivity was determined with nonendemic samples (> mean OD + 3 SD).

**Western blotting**—Sera from endemic normal subjects and microfilaraemic patients were run on 12.5% SDS PAGE prior to blotting by standard protocol. Rabbit anti Cu/Zn SOD polyclonal antibody was used at a dilution of 1:100 in PBS containing 5% skimmed milk powder and 0.1% Tween 20, and left overnight at 4°C. Horseradish peroxidase conjugated anti rabbit IgG was used at the dilution of 1:1000 for 3 hr at room temperature in PBS alone. Binding was visualised by 4-chloronaphthol at a final concentration of 0.5 mg/ml.

**Results**

**Population study**—Out of 1250 persons examined, 9% were harbouring microfilariae in their circulation (asymptomatic microfilaraemics) without any symptoms of the disease. Elephantiasis patients (patients with chronic irreversible oedema of extremities were only 3.1% and rest were asymptomatic microfilaraemics). Out of these 40 cases of microfilaraemics, 40 cases of elephantiasis and 40 endemic normals were chosen for the detection of circulating antigen or antibody response.

Sera from other helminthic infections were procured from the Department of Microbiology, Institute of Medical Sciences, B. H. U., Varanasi.

**Antibody response to B. pahangi rec SOD in ELISA**— Reactivity of B. pahangi rec SOD with IgG and IgM subclass antibodies from different category of human filarial sera has been shown in Fig.1. Antibody levels in asymptomatic microfilaraemics (AS) and chronic patients (CP) do not differ much but are significantly higher (P<0.01) than those of endemic normals.

All of chronic patients as well as microfilaraemia positive showed 100% seropositivity for both IgG and IgM subclass antibodies, however, IgM showed higher reactivity than IgG when compared with endemic normals in elephantiasis patients. Reactivity
with sera obtained from other helminthic infected patients was very low and was found to be insignificant (P>0.05).

Figure 2 shows the correlation of antibody response to *B. pahangi* rec SOD with the number of microfilariae in filarial patients. A significantly higher response was observed in Group II patients as compared to Group I suggesting a positive correlation.

**Detection of circulating parasitic SOD**—Figure 3 shows the bar diagram for detecting the circulating antioxidant enzymes using a rabbit polyclonal *B. pahangi* rec SOD. The parasitic SOD was detected in asymptomatic and elephantiasic groups. Sera from other helminthic viz. hookworm (10 no.) and ascariasis (10 no.) infections did not show the presence of parasitic antioxidant enzymes in the infected sera.

**Immunoblotting**—Figure 4 shows the immunoblotting of infected sera with recombinant *B. pahangi* SOD antibodies raised in rabbit. At 1:50 dilution, rec SOD antibodies reacted with two bands of molecular mass 29 and 22 kDa in asymptomatic microfilaraemic sera.

**Discussion**

Antioxidant enzymes have been detected enzymatically in many helminth parasites, both in somatic extract and tissue culture fluid⁶. Filarial
parasites B. malayi, B. pahangi and O. volvulus contain two forms of the enzyme, the cytoplasmic and extracellular (EC) form. The EC form of the enzyme from Brugia and Onchocerca is secreted in vivo\(^7,8\). In this communication antibody response to rec SOD from B. pahangi in W. bancrofti infected patients as well as circulating parasitic enzymes in the infected patients is reported.

Generally the asymptomatic microfilaraemic group is immunologically hyporesponsive to the parasite as they have restricted low serum level of antifilarial antibodies and poor ability to mount B and T cell response\(^10\). In contrast, patients with pathology associated with infection have relatively strong cellular and humoral response\(^11\). We have detected antibody response to rec SOD from B. pahangi in W. bancroftii infected patients. However in the present study, both the chronic filarial patients as well as asymptomatic microfilaraemic carriers showed positive response to IgG and IgM subclass antibodies, compared to endemic normals in ELISA. The antibody response to IgM subclass was more significant as compared to IgG. Earlier Bal and Das\(^12\) have also shown the positive antibody response to SOD from cattle parasite Setaria digitata in bancroftian filariasis patients. Filarial infections are associated with corresponding elevation in polyclonal IgE level and eosinophilia although the antigen specific antibody profiles vary according to the clinical status of the affected individual\(^13\). For instance, infections with Acanthocheilonema viteae was reported to be associated with an IgG and IgM antibody response against ES 62 antigen\(^14\). Prasad et al\(^15\) have reported IgM antibody response to various antigenic fractions isolated from immune complexes separated from human filarial sera. They found that IgM antibody response to all antigenic fractions were higher in microfilaraemic group compared to symptomatic microfilaraemic and thus proposed that IgM in the microfilariae carriers has been replaced by IgG isotype in the latent stage of infection.

In the present study a comparable IgG and IgM subclass response who observed against rec SOD antigen in both the asymptomatic microfilaraemics and symptomatic microfilaraemic patients. The continuous release of the SOD antigen from the parasite which is exposed to the host's immune system may be responsible for the observed antibody response.

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Fig. 3—Detection of circulating parasite SOD using rabbit polyclonal B. pahangi rec. SOD antibodies (1:100). [a = endemic normal, b = asymptomatic microfilariae, c = symptomatic microfilaraemic. The no. of sera in each group was 40].

Fig. 4—Immunoreactivity of filaria infected sera with (a) normal rabbit serum and (b) Rec B. pahangi SOD antibodies raised in rabbits.
The antibody response to *B. pahangi* rec SOD is directly proportional to the number of microfilariae in asymptomatic microfilaraemic patients showing its relation to parasitemia. Microfilaraemic patients have altered B-cell responses as indicated by their relatively low serum levels of antibodies. The lack of well defined parasite antigen has been a major obstacle to the study of parasite specific immune responses. Studies on total antibody response and isotypic antibody response against characterized antigen have just of well defined parasite antigen has been a major obstacle to the study of parasite specific immune responses. The antibody response to antigen, appears promising for use in epidemiological surveys but its sensitivity is unknown in persons with ultralow microfilarial density.

An interesting observation is that the nonfilarial helminthic infected sera have not shown reactivity with the recombinant antioxidant enzyme suggesting that there is no cross reactivity between the filarial antioxidant enzymes and nonfilarial intestinal worms like hookworm or Ascaris.

The polyclonal antibody to a partial rec Cu/Zn SOD of *B. pahangi*, that recognize both the cytoplasmic enzyme with an estimated mass of 19 kDa and extracellular enzyme with an estimated mass of 29 kDa\(^7\), has been used for the detection of circulating SOD antigen in *W. bancrofti* infection. In western blotting, these antibodies reacted to the 29 and 22 kDa proteins of the asymptomatic microfilaraemic human sera. Further, the *B. pahangi* rec SOD antibodies also reacted to the 29 kDa EC form of Cu/Zn SOD in the lavage of Mongolian jirda infected intraperitoneally with *B. malayi* indicating that this form of SOD is secreted in vivo\(^7\).

In *W. bancrofti* infection, the 29 kDa protein may be the extracellular form of Cu/Zn SOD and the 22 kDa protein may be either the cytosolic form of the enzyme or a break down product of the 29 kDa protein. The digestion of *B. pahangi* EC SOD with N-glycanase resulted in a protein of 22 kDa\(^7\). The 29 and 22 kDa proteins observed in *W. bancrofti* infected human sera are of parasite origin as the filarial EC SOD differs from human EC enzyme in that it is dimeric and may result from the truncated N-terminus; the N-terminus has been proposed to mediate association into a tetrameric form for the mammalian EC SODs\(^9\). This is the first report indicating the presence of parasitic SOD in *W. bancrofti* infection suggesting the active secretion of the enzyme under in vivo conditions.

The continuous turn over of the soluble EC SOD from *W. bancrofti* in the host circulation may perform one of the two functions; bulk scavenging of superoxide anion or an anti-inflammatory activity. Further investigations are underway for the determination of the diagnostic value and vaccine potential of the parasitic SOD in lymphatic filariasis.

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**References**