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Brugia malayi Thioredoxin Peroxidase as a Potential Vaccine Candidate Antigen for Lymphatic Filariasis

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Abstract Attempts were made to evaluate the protective efficacy of *Brugia malayi* thioredoxin peroxidase (BmTPX) in a mouse model. Mice immunized with a protein vaccine containing rBmTPX developed higher titres (1:5,000/1:10,000) of anti-BmTPX antibodies, compared with the mice immunized with the alum control. There was a higher level of cellular proliferative response in mice immunized with BmTPX compared with the alum control ($p<0.05$), which was associated with a Th2-type of response. In order to compare the prophylactic efficacy of BmTPX in natural infection, we evaluated the human immune responses to these antigens in endemic normals (EN) and infected individuals (microfilaraemic and chronic pathology). Results showed that EN subjects carry BmTPX-specific IgG1 and IgG3 circulating antibodies against natural exposure to filariasis. Peripheral blood mononuclear cells from EN subjects responded strongly to rBmTPX by proliferating, as well as by secreting interferon (IFN)-γ (Th1) and IL-5 (Th2), a mixed type of response to rBmTPX. In the case of infected individuals, there was no IFN-γ or IL-5 response. Thus, there was a clear dichotomy in the cytokine production by infected versus EN individuals. Our findings suggest that BmTPX may be a suitable antigen candidate for lymphatic filariasis, but a further study is still required.

Keywords Filariasis · Vaccines · *Brugia* · Thioredoxin peroxidase · Antigens

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Introduction

Human lymphatic filariasis is a mosquito-borne tropical disease, caused by infection with the nematode parasites *Wuchereria bancrofti* and *Brugia malayi*. The disease is endemic in 80 countries, and more than 1.2 billion people worldwide are estimated to be at risk. Approximately 120 million people in tropical and subtropical areas of the world are infected [1, 2].

Currently, the most commonly used measures, chemotherapy and vector control, have not adequately controlled filariasis, pointing to the imperative need for a multi-pronged approach that includes simpler diagnostic methods, surveillance, vaccine development and drugs based on molecular pathogenesis and pharmocogenomics. Diethylcarbamazine, ivermectin and albendazole, the drugs commonly used to treat the disease [3], have the inherent disadvantages of the requirement of prolonged treatment for years and the development of drug resistance. Moreover, these drugs are principally microfilaricidal with the associated danger of dead or dying adult worms worsening lymphatic pathology when the macrofilaricidal effect is achieved. Therefore, alternative strategies such as vaccination could aid in eradicating filariasis from endemic areas, as is the case with many such persistent infectious diseases.

Vaccination as a preventive measure has been a crucial component for the control of many infectious diseases of bacterial and viral origin (e.g. smallpox, measles, diphtheria, etc). However, successful vaccines should be able to induce strong immune responses which are long lasting and, in most cases, providing protection against different strains of the same pathogen. Application of this new vaccination technology with regard to parasitic infection provides new hope for significant advances in anti-parasitic vaccine research. An important consideration in developing vaccines against parasites is the complexity of parasitic diseases. Parasites have complex life cycles, and host immunity to stage-specific antigens may not overlap with other later stages or vector-borne stages. Antigenic variation and other immune evasion mechanisms also complicate the development of vaccines against parasites. However, with recombinant technology, it is now possible to take rational parasite-specific strategies to vaccine design and overcome the obstacles presented by parasitic disease.

As part of the filarial genome project at our centre, the expressed sequence tags of the cDNA library of *B. malayi* were randomly screened, and several clones were deposited in the Gene Bank. Among these is one clone named *B. malayi* thioredoxin peroxidase (BmTPX), which was kindly provided by Alan L. Scott (Johns Hopkins University, Baltimore, USA). TPX is a 29-kDa thiol-specific antioxidant detoxification enzyme which has been shown to be a critical component in the parasite's defence against injury caused by oxygen radicals derived from the endogenous and exogenous environment (Inca Ghosh 1998). Previously, TPX was also identified from L_3 stages of *B. malayi* as an endemic normal (EN) reacting protein [4]. Filarial nematodes indeed produce enzymes with antioxidant capabilities. Earlier studies have shown that one such antioxidant enzyme, glutathione-S-transferase, has shown protection against microfilariae and infective larvae of *B. malayi* in mastomys. Studies using TPX of *Dirofilaria immitis* suggest that in addition to its antioxidant property, *D. immitis* may be a protective antigen. TPX in *B. malayi* has been shown to be transcribed or detected in adult and microfilarial stages, but its antigenicity, immunoprophylactic and immunological responses in humans are yet to be studied.

In the present study, we evaluated the immune responses of BmTPX in an animal model and in filarial patients. For this, we expressed and purified the BmTPX protein, and the cellular and humoral responses were studied in Balb/c mice; further, the cellular and humoral

responses of BmTPX were studied in a cohort of subjects residing in a village endemic for lymphatic filariasis.

Materials and Methods

Study Population

Standardized histories were obtained, and physical examinations were done during routine epidemiological surveys on subjects living in and around Vellore, India, an area endemic for *W. bancrofti* infections. Patients identified positive were then recruited through the National Filarial Control Units under the Directorate of Public Health (Vellore, India) after obtaining informed consent with protocols approved by Institutional Review Boards of Anna University, Chennai. Parasitological examination of all individuals was done by detection of microfilariae in night blood smears. Heparinized blood was collected with prior consent from all individuals in the study. The study population was divided into three categories [endemic normals (EN) and infected individuals: chronic pathology (CP) and microfilaraemic (MF)] based on the detection of circulating parasites and antigens or by evaluating the clinical symptoms of the disease. Circulating microfilariae in the blood samples were identified as described previously [4], and the circulating filarial antigens were detected using a commercially available Og4C3 kit and a WbSXP-based enzyme-linked immunosorbent assay (ELISA). Individuals with no circulating antigen or microfilariae were considered as EN, whereas subjects with circulating microfilariae and/or circulating antigens as detected by Og4C3/WbSXP ELISA were considered as MF. Subjects showing lymphoedema and other visual clinical symptoms of filariasis were classified into the CP group. Sera samples from non-endemic normal (NEN) individuals were a kind gift from Dr. Thomas B. Nutman, NIAID, USA.

Recombinant Protein Expression and Purification

For expression, salt-inducible *Escherichia coli* GJ1158 cells (Bangalore Genei, India) were transformed with the recombinant plasmid and allowed to grow. When the cultures reached an optical density (OD) of 0.6 at 600 nm, 0.3-M NaCl was added to the cultures to induce gene expression, and the cultures were incubated for 3 h. Total cell proteins were analysed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the presence of histidine-tagged recombinant protein was confirmed by immunoblotting with anti-histidine tag antibodies (anti-HisG; Invitrogen, San Diego, USA). Subsequently, the histidine-tagged recombinant protein was purified from pelleted bacteria using chelating sepharose fast flow (Amersham Biosciences, Uppsala, Sweden) as per the manufacturer's recommendations.

Immunization of Mice

The responsible ethical committees at Anna University, Chennai approved the experiment protocols in the animal model. Male BALB/c mice, 6 to 8 weeks old, were obtained from Kings Institute, Chennai, India. Animals were maintained under standard laboratory conditions with food and water. Animals were grouped into two groups, with four animals each. Group A received alum alone; group B received rBmTPX. Each group was vaccinated at week 0 and boosted 4 weeks later by intraperotoneal injection of 10-μg recombinant protein.

Serological Studies

Mice antibodies to rBmTPX were detected by immunoblot as previously described [5]. Similarly, antibodies to purified recombinant protein rBmTPX, prepared by metal affinity chromatography, were measured in mice sera by ELISA as previously described [5]. Antibody titres were defined as the highest serum dilution that produced an OD greater than the mean OD plus 3 standard deviations observed with a pool of normal mouse sera.

For isotype-specific ELISA, after 1-h incubation with mouse sera diluted (1:1,000), the plates were incubated with goat anti-mouse IgG1, IgG2a, IgG2b or IgM for 1 h at 37 °C. After washing, the plates were incubated with rabbit anti-goat IgG labelled with alkaline phosphatase for 1 h at 37 °C. The plates were washed and developed with substrate (pNPP) for 30 min at room temperature. OD was measured against a phosphate-buffered saline (PBS) blank at 405 nm. Antibody titres were defined as the highest serum dilution that produced an OD greater than the mean plus 3 standard deviations above the value obtained with the preimmune sera.

Splenic T-Cell Proliferative in Vaccinated Mice

The spleens of vaccinated alum control group and rBmTPX-vaccinated mice were removed aseptically after the micropore chamber experiment and washed in Petri plates containing PBS. Splenocyte cells were separated and washed twice with fresh culture Dulbecco's modified Eagle's medium (DMEM). Lysis buffer (0.83% ammonium chloride) was added to the pellet to remove the erythrocytes, and the cells were counted. The splenocyte cell suspension was then distributed into a 96-well tissue culture plate (Nunc, Denmark) so that each well contained about 2×10^5 cells/100 μl of cell suspension. The cells in triplicate wells were stimulated by the addition of 10 μg/well of BmTPX antigen or 10 μg/well of concanavalin A (ConA) (mitogen). Unstimulated cell cultures in triplicate wells served as controls. The final volume of the culture fluid in each well was adjusted to 200 μl with DMEM (containing 10% fetal calf serum) medium. The plates were incubated for 72 h at 37 °C in a CO_2 incubator (Forma Scientific Inc., Marietta, USA) with 5% CO_2. After 72 h, cell proliferation was measured by the MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (CellTiter 96® aqueous non-radioactive cell proliferation assay; Promega, USA). The proliferation was expressed as the stimulation index (SI) by the following formula: SI=geometric mean (GM) of absorbance in experimental stimulated/absorbance control.

Cytokine Response in Vaccinated Mice

Separate cultures were set up for cytokine assay. Briefly, spleen cells were washed and plated onto 24-well flat-bottomed tissue culture plates (Costar) at 4×10^6 cells/ml in a volume of 1 ml of DMEM supplemented with 10% fetal calf serum (Gibco-BRL) and 80-μg/ml gentamicin. Immunized and control spleen cells were then stimulated by the addition of 10 μg/well of BmTPX antigen. Supernatants were harvested at 48 h and on day 5 after cultures and stored at −70 °C before use. Capture ELISAs (Pierce Biotechnology, Rockford, IL) were performed to detect interferon (IFN)-γ (for Th1 responses), IL-5 and IL-10 (for Th2 responses) in culture supernatants as described previously [6]. All values were derived from the interpolation of standard curves run simultaneously on every plate. The sensitivities of the assays for IFN-γ, IL-5 and IL-10 were 39 pg/ml, respectively.

Humoral Immune Response to *Brugia malayi* Thioredoxin Peroxidase Protein in an Endemic Population

Levels of BmTPX-specific antibodies in sera from human subjects were studied by ELISA as previously described [7]. Briefly, polyvinyl microtitre plates (Nunc, Germany) were coated with rBmTPX protein at a concentration of 1 μg/ml at 4 °C and blocked with 3% bovine serum albumin in 0.01-M PBS at 37 °C for 2 h. After blocking the plates with blocking buffer, diluted (1:100) serum samples in 0.01-M PBS were added, and the plates were incubated at 37 °C for 2 h. After further washes, the plates were incubated with peroxidase-conjugated affinity-purified goat anti-human IgG (GibcoBRL) for 1 h at 37 °C, washed and developed with a substrate containing H_2O_2 and 3,3′,5,5′-tetramethylbenzidine (Sigma Chemical, USA) after 10 min at room temperature. The OD was measured at 490 nm.

ELISA to Determine Isotype-Specific Antibodies

ELISA was carried out as described [7] with few modifications using biotin-conjugated mouse monoclonal anti-human IgG1, IgG2, IgG3 and IgG4 and avidin horseradish peroxidase (HRPO) conjugate (Sigma Chemical, USA). The wells of a polystyrene microtitre plate (Nunc, Germany) were coated with rBmTPX (at a concentration of 1 μg/50 μl) in 0.06-M carbonate buffer (pH 9.6) and incubated at 4 °C overnight and blocked with 100 μl of 2% bovine serum albumin at 37 °C for 1 h; 50 μl of diluted sera (1:100) was added and incubated at 37 °C for 1 h. Wells were washed and further incubated with biotinylated monoclonal mouse anti-human IgG isotype antibodies (IgG1 at 1:1,000; IgG2 at 1:15,000; IgG3 at 1:5,000; and IgG4 at 1:15,000) at 37 °C for 1 h; 50 μl of avidin peroxidase conjugate (1:20,000) (Sigma Chemical, USA) was added and incubated for 30 min. After final washing, each well was incubated with 50 μl of substrate consisting of *O*-phenylene diamine dihydrochloride (OPD) (Sigma Chemical, USA) and H_2O_2 in citrate phosphate buffer (pH 5.0). The reaction was stopped after 15 min by adding 5-M HCl. OD_{490} values were read in an ELISA reader.

Human Peripheral Blood Mononuclear Cell Response to *Brugia malayi* Thioredoxin Peroxidase

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by gradient centrifugation over lymphocyte medium (Lymphoprep; Nycomed Pharma AS, Oslo, Norway). The cells were washed, and the viability was determined by the tryptan blue dye exclusion method. The cells were then cultured in round-bottomed microtitre plates at a concentration of 0.2×106 cells/well in DMEM(Sigma, St. Louis, USA) supplemented with gentamicin (80 μg/ml) (Ranbaxy Laboratories, India), 25-mM HEPES (USB, Amersham Pharmacia, UK), 2-mM glutamine (USB, Amersham Pharmacia, UK) and 10% human AB serum or fetal calf serum and stimulated with a soluble crude extract of *Brugia* malayi Antigen (10 μg/ml), ConA (10 μg/ml) or medium alone as the negative control. A dose–response study using rBmTPX was carried out with PBMCs from normal healthy individuals to determine the optimum proliferative doses from 1 to 20 μg/ml. Cultures were performed in triplicate wells in a humidified atmosphere of 5% CO_2 at 37 °C for 72 h. Cell proliferation was determined by an MTT assay using the formula: SI=absorbance recombinant stimulated/absorbance unstimulated.

Cytokine Assays

Separate cultures were set up for cytokine assay. Briefly, PBMCs were washed and plated onto 24-well flat-bottomed tissue culture plates (Costar) at 4×10^6 cells/ml in a volume of 1 ml of DMEM supplemented with 10% fetal calf serum (Gibco-BRL) and 80-μg/ml gentamicin. PBMCs were then stimulated with or without rBmTPX at 10 μg/ml. Supernatants were harvested at 48 or 96 h after stimulation and stored at −70 °C before use. Capture ELISAs (Pierce Biotechnology, Rockford, IL) were performed to detect IFN-γ (for Th1 responses) and IL-5 (for Th2 responses) in culture supernatants as described previously (Mahanty et al. 1996). All values were derived from the interpolation of standard curves run simultaneously on every plate. The sensitivities of the assays for IFN-γ and IL-5 were 39 pg/ml, respectively.

Statistical Analysis

Statistical analysis was performed using XL STAT software v.7.5.2 (Kovach Computing Services, Anglesey, UK). Statistical significance between comparable groups was evaluated using appropriate non-parametric tests with the level of significance.

Results

Expression of *Brugia malayi* Thioredoxin Peroxidase

The rBmTPX cloned in pRSET-B was expressed as histidine-tagged fusion protein. The molecular mass of the recombinant fusion protein (with the histidine tag) was approximately 29 kDa (Fig. 1). SDS–PAGE analysis showed that expressed rBmTPX consisted of over 10% of the total *E. coli* proteins. Subsequently, the recombinant protein was purified using metal affinity column chromatography (Fig. 1a), and size was confirmed by immunoblotting using anti-histidine antibody (Fig. 1b).

Humoral Immune Response in Vaccinated Mice

Antibodies present were determined by ELISA in mice immunized with rBmTPX (Fig. 2a). Antibodies were present 2 weeks after the first immunization and stronger after the second immunization. An antibody response to rBmTPX was not detected in the control group. Similarly, 29-kDa rBmTPX antigen was bound to BmTPX antibodies and was detected until 1:5,000 dilutions (Fig. 2b).

In order to determine the nature of the immune response elicited in mice following immunization with rBmTPX and its potential relevance to the induced protection, mouse anti-rTPX IgG1, IgG2a, IgG2b and IgG3 isotype profiles were measured. As shown in Fig. 3, the antibody response in protected mice following vaccination with rBmTPX consisted of very high levels of IgG3 and IgG1 and very low levels of IgG2a and IgG2b.

Splenocyte Proliferation in Vaccinated Mice

Splenocytes from the mice immunized with rBmTPX (GM SI=2.09, $p<0.05$) produced an enhanced proliferative response to rBmTPX. Cells from control mice immunized with alum did not produce a significant proliferation response to BmTPX. However, stimulation with ConA induced significant proliferation in both the control and vaccine-immunized animals.

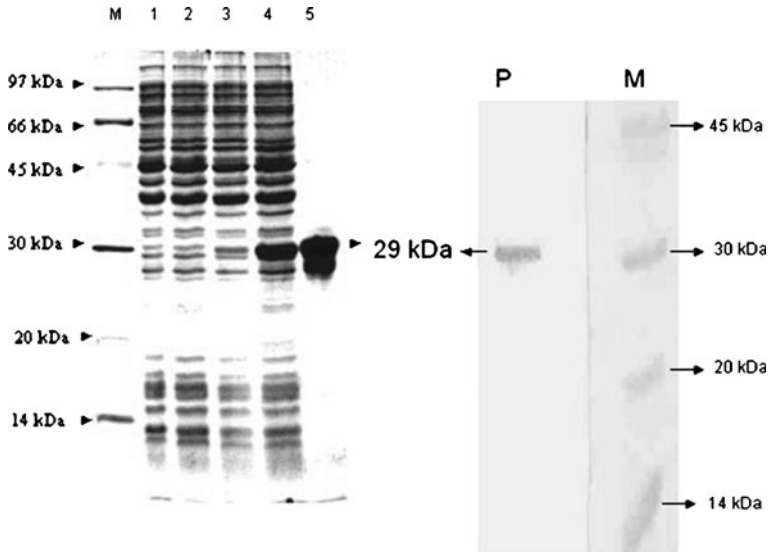

Fig. 1 Expression of *Brugia malayi* thioredoxin peroxidase (BmTPX) in *Escherichia coli* GJ1158 host. **a** Total protein extracts from BmTPX and control pRSET-B were solubilized in 1×SSB, separated on a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel and stained with Coomassie brilliant blue dye. A quantity of 50-μg protein was loaded in each of the respective lanes. *Lane M* molecular weight marker, *lane 1* vector uninduced, *lane 2* vector induced, *lane 3* 0.3-M NaCl BmTPX uninduced, *lane 4* BmTPX induced at 3 h, *lane 5* purified protein eluted at 200-mM immidazole. **b** Total protein extracts after inducing the recombinant clone BmTPX with NaCl were separated on 12% SDS–PAGE, transferred to nitrocellulose membrane and probed with mouse monoclonal anti-histidine antibody (1:2,000), followed by incubation with goat anti-mouse antibody conjugated with alkaline phosphatase (1:30,000) and developed with NBT and BCIP. *Lane P* anti-histidine antibody, *lane M* marker

Cytokine Response in Vaccinated Mice

To assess the spleen cytokine repertoire, spleen cells immunized with rBmTPX or alum alone were cultured with the BmTPX antigen, and IFN-γ (Th1), IL-5 and IL-10 (Th2) production was assessed by ELISA. rBmTPX was able to induce only significant levels of IL-5 (32 pg/ml), moderate levels of IL-10 (25 pg/ml) and lower levels of IFN-γ (8 pg/ml). Cells from the control group did not produce a significant proliferative response to rBmTPX.

Humoral Immune Response to Recombinant BmTPX Protein in an Endemic Population

ELISA results presented in Fig. 4a show that significant ($P<0.01$) amounts of anti-rBmTPX IgG antibodies were present in the sera of EN, MF and CP individuals compared to sera from NEN individuals. Further analyses suggest that EN individuals carry significantly ($P<0.05$) higher titres of anti-rBmTPX IgG antibodies in their sera compared to individuals in the MF or CP group.

Isotype Profiles to rBmTPX in Human Sera

Since BmTPX IgG antibodies were significantly increased in all the group of subjects except NEN, further analyses were performed to determine the isotype of BmTPX IgG antibodies. These analyses showed that majority of the BmTPX IgG antibodies in the sera of EN

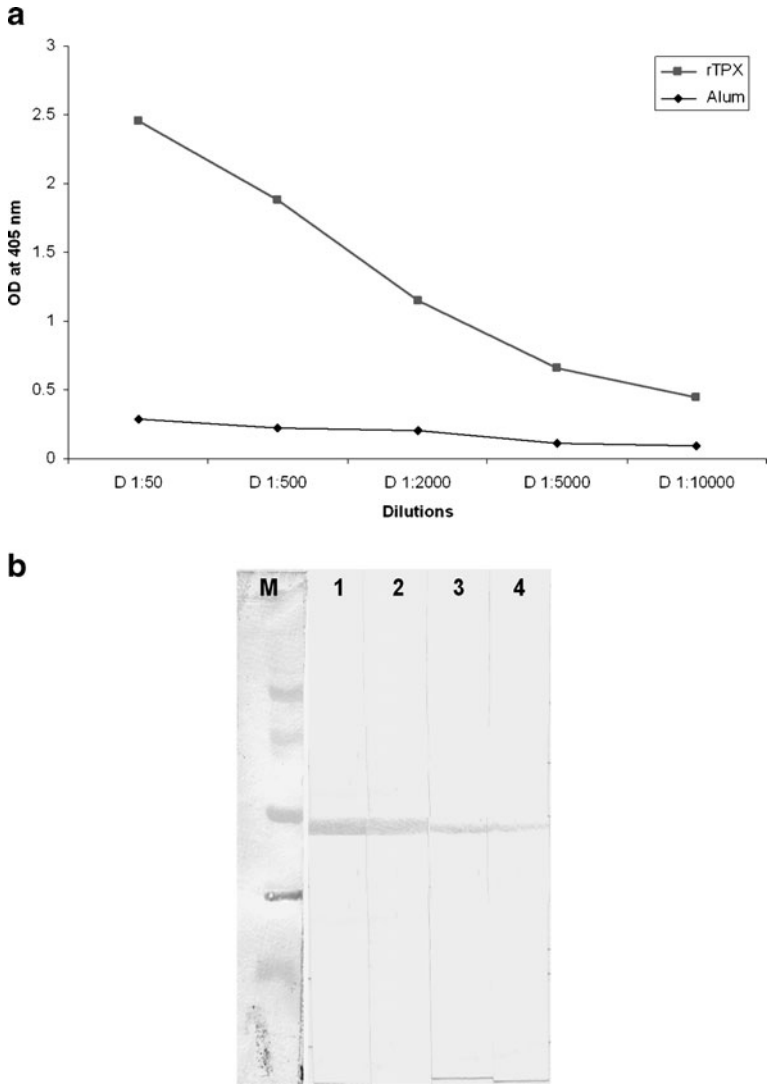

Fig. 2 Levels of antigen-specific antibodies (total IgG) in sera from immunized mice. **a** The points represent the mean value of titres from different mice in each group. Mice were immunized with recombinant protein (10 μg). Four doses at 2-week intervals were administered. The trend of antibody levels (total IgG) induced in mice administered with *Brugia malayi* thioredoxin peroxidase (BmTPX) group compared with control groups. IgG isotypes were measured by enzyme-linked immunosorbent assay (ELISA) at 405 nm. Results are expressed as the mean and standard deviation of four mice in each group, with control optical density (OD) values subtracted. **b** Total protein extracts after inducing the recombinant clone BmTPX with NaCl were separated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose membrane and probed with anti-rBmTPX antibody followed by incubation with goat-anti-mouse antibody conjugated with alkaline phosphatase (1:30,000) and developed with NBT and BCIP. *Lane M* marker, *lane 1* anti-rBmTPX antibody (1:500), *lane 2* anti-rBmTPX antibody (1:1,000), *lane 3* anti-rBmTPX antibody (1:2,000), *lane 4* anti-rBmTPX antibody (1:5,000)

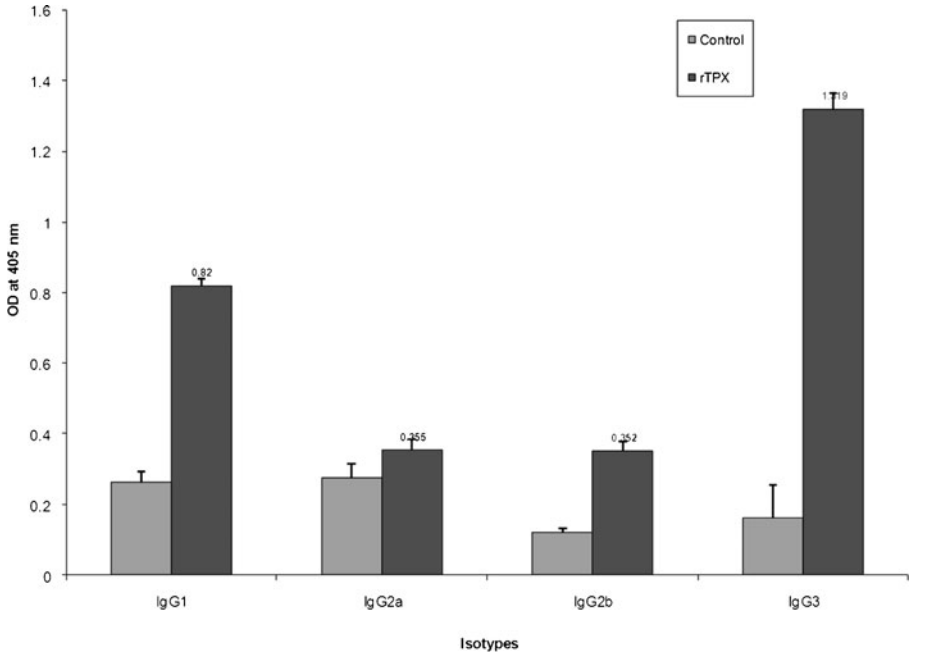

Fig. 3 Isotype profiles of antibodies: subclass-specific anti-*Brugia malayi* thioredoxin peroxidase (BmTPX) antibodies in mice immunized with BmTPX. The *bars* represent the mean optical density at 405 nm of four mice. The levels of BmTPX-specific IgG1 and IgG3 were significantly higher in the case of mice immunized with BmTPX compared with the control ($p<0.05$)

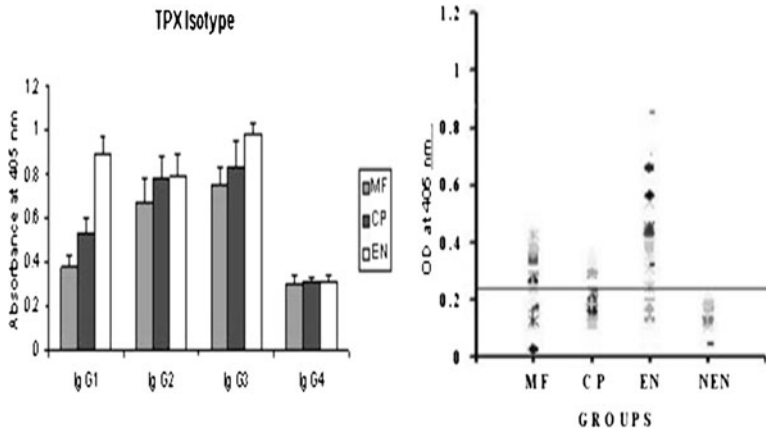

Fig. 4 Levels of *Brugia malayi* thioredoxin peroxidase (BmTPX)-specific IgG antibodies in the sera of various clinical groups of lymphatic filariasis. Wells were coated with rBmTPX, and sera collected from endemic normal (*EN*), microfilaraemic (*MF*) and chronic pathology (*CP*) patients were added at 1:100 dilution. Levels of total IgG reactivity were determined by an enzyme-linked immunosorbent assay (ELISA). **a** Sera from ten subjects were tested in each group. Each *dot* in the scatter plot indicates individual sera. Total IgG reactivity with BmTPX was significantly higher in EN ($p<0.05$) when compared with CP or MF groups. **b** Isotype analysis of the BmTPX-specific IgG antibodies in the various clinical groups was determined by isotype-specific ELISA for IgG1, IgG2, IgG3 and IgG4. Levels of IgG1, IgG2 and IgG3 antibodies were significantly higher ($p<0.05$) in EN individuals. However, comparatively, levels were lower than in EN and CP individuals. Interestingly, IgG4 levels were consistently low in all individuals

individuals were of IgG1, IgG2 and IgG3 isotypes (Fig. 4b), BmTPX-IgG4 isotype of antibodies were barely detectable in all three groups (EN, MF or CP) of subjects.

Human T-Cell Response to rBmTPX

It has been demonstrated that cellular responses to mitogens do not differ among the various clinical groups of lymphatic filariasis; however, hyporesponsiveness to adult parasite soluble crude extract antigen (BmA) is a characteristic feature of patent filarial infection. To examine whether purified rBmTPX behaves similarly to BmA, PBMCs were obtained from EN, MF and CP individuals and were stimulated with 10 μg/ml of rBmTPX or 10 μg/ml of BmA or ConA. BmA-induced proliferative responses were diminished in MF (GM SI=0.67, $p<0.05$) patients compared to either the EN (GM SI=4.62, $p<0.05$) or CP (GM SI=3.07, $p<0.05$) individuals. In contrast, rBmTPX induced higher proliferative responses in PBMCs collected from EN (GM SI=4.0, $p<0.05$) subjects compared to those from MF (GM SI=1.8, $p<0.05$) and CP (GM SI=1.9, $P<0.05$) individuals (Fig. 5.). ConA induced significant proliferation of PBMCs collected from all groups.

Cytokine Levels

Levels of IFN-gamma (marker for Th1 immune response) and IL-5 (marker for Th2 immune responses) were determined in the PBMC culture supernatants stimulated with rBmTPX. Levels of IFN-gamma (Fig. 6) were significantly higher ($p<0.05$) (337.6 pg/ml) in the culture supernatants of PBMCs from EN, compared to similarly stimulated PBMC from MF (81.4 pg/ml) and CP individuals (93.8 pg/ml) ($P<0.05$). Interestingly, IL-5 levels (Fig. 6) were significantly ($p<0.05$) elevated in the culture supernatants of rBmTPX-stimulated PBMC from EN individuals (119.5 pg/ml) compared to MF (78.8 pg/ml) and CP

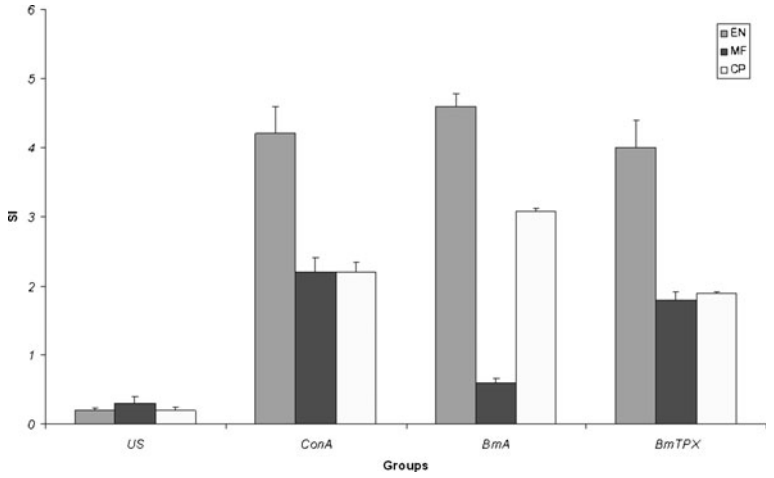

Fig. 5 Lympho-proliferative responses to *Brugia malayi* thioredoxin peroxidase (BmTPX). Peripheral blood mononuclear cells (PBMCs) from endemic normal (*EN*), microfilaraemic (*MF*) and chronic pathology (*CP*) individuals were cultured at 0.2×10^{6} cells/ml and stimulated either recombinant *BmTPX*, *BmA* or concanavalin A (*ConA*). Cells were incubated for 72 h in a 5% CO_2 environment. Lymphocyte proliferation was measured by a CellTiter 96® aqueous non-radioactive cell proliferation assay kit. Results are expressed as stimulation index (*SI*). The *horizontal bar* denotes the geometric mean of all ten samples from each group

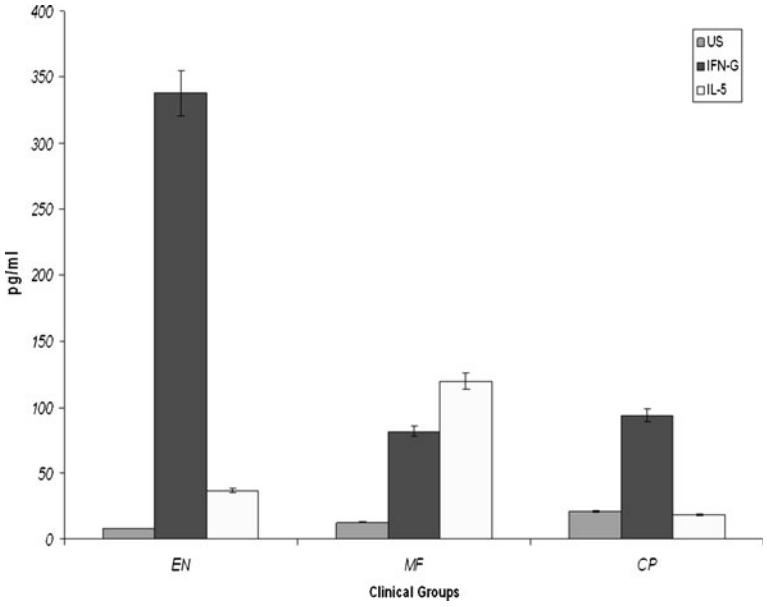

Fig. 6 Interferon (IFN)-γ and IL-5 responses to recombinant *Brugia malayi* thioredoxin peroxidase (BmTPX) in human peripheral blood mononuclear cells (PBMCs). PBMCs collected from endemic normal (*EN*), microfilaraemic (*MF*) and chronic pathology (*CP*) individuals were cultured at 4×10^6 cells/ml and stimulated with rBmTPX (10 μg/ml) for 72 h at 37 °C. After incubation, culture supernatants were collected, and levels of IFN-γ and IL-5 were measured using an enzyme-linked immunosorbent assay (ELISA). Cells cultured in media alone were used as controls. Results show that IFN-γ levels were significantly increased ($p<0.05$) in EN individuals compared with CP or MF, whereas IL-5 levels were significantly elevated in MF individuals compared with EN and CP groups. *Bars* represent the geometric mean of ten samples

(18.7 pg/ml) individuals. Cytokine levels in the culture supernatants of unstimulated control cultures were below detection limits.

Discussion

Parasite-derived antioxidant proteins have been implicated in playing an important role in protection against the oxygen radicals that are generated during aerobic metabolism and in defence against host immune cell attack. Filarial nematodes indeed produce enzymes with antioxidant capabilities. Numerous antioxidant enzymes such as glutathione peroxidase and glutathione-S-transferase have been identified. One such class of molecule that has been identified as a thiol-specific antioxidant from *B. malayi* is TPX. BmTPX, apart from its pivotal antioxidant property [5, 8], may be an immunodominant antigen inducing antibodies in putative immune individuals [4]. TPX is essential for the detoxification of oxygen free radicals produced by it and the host. Hence, targeting this enzyme will be lethal to the parasite.

The possible role for BmTPX in inducing a protective immune response is strengthened by the identification of TPX from the cDNA library of *D. immitis* using serum from dogs vaccinated by chemotherapeutically abbreviated *D. immitis* larval infections [8]. BmTPX has been shown to be transcribed in all the stages of the parasite and to be localized in the cells of the hypodermis and lateral chord, suggesting a role for TPX in counteracting oxygen

free radicals derived from endogenous and exogenous sources [5]. TPX has also been shown to be an excretory–secretory product of *B. malayi* [5] and eggs of schistosomes [9]. Ov TPX has been shown to be expressed in higher intensities during differentiation of the infective L_3 stage compared with the other stages of the parasite. Another role for TPX in granuloma formation in schistosoma infections has been shown by inducing significant production of IFN-γ, IL-2 and IL-5, and essentially no IL-4 in $CD4^+$ cells from mice [9]. Hence, all of these features make TPX an ideal target for prophylaxis against *B. malayi* in animal models.

The humoral arm of the immune response has been observed to play a crucial role in the development of protective immunity in lymphatic filariasis. In the present study, the humoral immune response induced by vaccination with recombinant protein induced a stronger antibody response.

The profile of antibody isotype distribution is considered as a reliable indicator of the type of response. Hence, in the present study, the isotype profile was assessed from vaccinated sera which showed increased levels of IgG1 and IgG3, biased towards a Th2 response. These findings are consistent with those earlier reported for rBmALT-2 [4, 10] and recombinant paramyosin [11]

As well as the isotype profile, we were also interested in evaluating the cytokine profile. For this, the splenocytes of mice vaccinated with rBmTPX were re-stimulated with either mitogen or with BmTPX. The result showed specific T-cell proliferation and significant higher levels of IL-5 and moderate levels of IL-10 production upon stimulation by the recombinant antigen, compared with alum alone ($p<0.01$), which enumerated towards a Th2 type of response.

There is increasing evidence that IL-5 may be involved in immunity to filarial nematodes. Humans with active infection have depressed levels of IL-5 production despite enhanced IL-4 levels. In the mouse *Litomosoides sigmodontis* model, it has been shown that IL-5-deficient animals are unable to resist the challenge of L_3 infection unlike their wild-type or IL-4-deficient counterparts. In addition, MF of the filarial nematode *Onchocerca volvulus* are able to survive in IL-$5^{-/-}$ animals but not in IL-4 mice. Several studies insist that IL-5 knockouts prove a role for eosinophils in defence against nematodes, as antibody responses were unaltered in these animals. What is increasingly apparent is that there is a dysregulation of type 2 responses seen in filariasis, where IL-5 levels appear to be independent of the presence/absence of IL-4 (or IL-13). This dysregulation may play a critical role in filarial immunity and may prevent parasite destruction by an IL-5-dependent mechanism.

The fate of an infection in a host, either susceptibility or development of resistance to the infection, is thought to be determined by the quality and the character of the immune response generated by the host. In lymphatic filariasis, the development of immune response to the incoming infective L_3 stage larvae determines the outcome of the challenge. Studies measuring the antibody response to parasite antigen in an endemic population have provided conflicting evidence regarding the role of humoral immune response in protective immunity.

Analysis of the humoral immune responses to rBmTPX in patients exposed (EN) or infected (MF and CP) with *W. bancrofti* showed that EN individuals carry significant levels of anti-BmTPX antibodies when compared to CP, MF or NEN individuals. However, the isotype profile of IgG antibodies showed that anti-BmTPX antibodies were predominantly of the IgG1 and IgG3 isotypes followed by IgG2 in the sera of EN individuals, whereas infected individuals (MF and CP) carry predominantly IgG3 antibodies followed by IgG2 and IgG1 isotypes. These findings were consistent with what we reported earlier [4]. In a recent study with the serpin secreted by *Brugia malayi* microfilariae, Bm SPN-2 displayed

potent humoral immune response to both IgG1 and IgG4 in human patients [12]. A study with Bm-SL3 revealed a significant increase in IgG1 and IgG2 antibodies in endemic normal sera compared with other groups [13]

Antigen-specific hyporesponsiveness is the hallmark of patent filarial infections, but it leaves intact the ability to respond to mitogen [14]. Although stimulation with mitogens is an acceptable means of assessing general lymphocyte functions, it is not always the best in vitro correlate of cellular immune responsiveness. Therefore, the present study was designed to study the rBmTPX-specific responses to evaluate an antigen-specific function in vivo. Similar to antibody responses, rBmTPX induced high levels of PBMC proliferation in endemic individuals when compared to MF or CP. Earlier studies have reported increased lymphocyte proliferation in endemic group in response to *Seteria digitata* antigens than those of microfilaraemics or clinical filarial cases. Similarly, microfilarial and larval soluble antigen showing specific sero reactivity with endemic normals has also shown increased proliferation in endemic normals [15]. A recent study with analysis of lymphocyte proliferative response against *Brugia* mf S-7 showed the endemic normals having significantly high proliferative response compared to that in microfilaraemics and chronic filarial cases.

In the present work, IFN-γ and IL-5 were the cytokines chosen as indicators of Th1 and Th2 type of responses, respectively. When peripheral T-cell population was challenged in vitro, BmTPX induced more consistent IFN-γ response and IL-5 in the endemic subjects compared to filarial infected individuals (MF and CP) as significant levels of IgG1, IgG2a and IgG3 isotypes were found as an isotype that is known to be cell-mediated immunity by mixed Th1/Th2 responses in humans. Several evidences have confirmed that Th2 responses are responsible for resistance to intestinal helminths; however, it is difficult to extrapolate such results to tissue-dwelling nematodes like filariae. Several recent studies in different models of filarial diseases have shown that Th1 type responses are also involved in the immune protection to filarial infection. These data were supported by the fact that the early human response to the first exposure to live L_3 of *Brugia malayi* has shown to be Th1 dominated; numbers of $CD4^+$ and $CD8^+$ T cells expressing IFN-γ, TNF-α and GM-CSF were increased on exposure to L_3 in vitro but not T cells expressing Th2 cytokines. A recent study with Bm-SL3 showed an enhanced level of IFN-γ (Th1) in the PBMCs of the endemic normal. Ravichandran et al (1997) [16] have shown that Th1 cytokines predominate in putative immune individuals. Patients with elephantiasis, who have successfully cleared the parasite but have developed lymphoedema in the process, also have a relative prominence of Th1 cytokines, similar to endemic normals. Considering endemic normal persons to be putatively immune to filarial infection, the present observation indicates that Th-1 response is protective.

Hence, in the present study, our data show that BmTPX is the efficacious vaccine candidate against lymphatic filariasis and provides strong support for further investigation and development of thioredoxin peroxidase as a recombinant vaccine against lymphatic filariasis. We hope that these studies will help us to characterize protective responses of jirds to *B. malayi* infection and develop effective vaccination strategies using recombinant thioredoxin peroxidase in the future.

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