Evaluation of Immunoprophylactic Efficacy of *Brugia malayi* Transglutaminase (BmTGA) in Single and Multiple Antigen Vaccination with BmALT-2 and BmTPX for Human Lymphatic Filariasis

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Abstract. An attempt was made to study the immunoprophylactic efficacy of recombinant *Brugia malayi* transglutaminase (BmTGA) as protein vaccine along with two other recombinant proteins, *Brugia malayi* abundant larval transcript-2 (BmALT-2) and *Brugia malayi* thioredoxin peroxidase (BmTPX), in single and multiple antigen form for human lymphatic filariasis. Parasite challenge studies in jirds exhibited protection of 30%, 69%, and 43% against BmTGA, BmALT-2, and BmTPX, respectively, in single antigen vaccination mode. The protective efficacy of BmTGA was enhanced significantly (74%) by immunizing the jirds in multiple antigen vaccination mode along with BmTPX, whereas immunizing with the combination of BmTGA and BmALT2 conferred only 47% protection. The same protection profiles were obtained by *in vitro* antibody–dependent cellular cytotoxicity, using live microfilariae and L3 stage larvae. The immune response was Th2 biased, irrespective of single or multiple vaccinations. The combination of BmTGA and BmTPX seems to be a promising vaccine candidate against lymphatic filariasis.

INTRODUCTION

Human lymphatic filariasis is a disease caused by the nematode parasites *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*. At present, 120 million people in 80 countries are infected by this disease, and several millions of people are under risk of infection. The current treatment of this disease relies on chemotherapeutic agents such as diethylcarbamazine (DEC), ivermectin, and albendazole. However, these drugs have only microfilaricidal effect, and the adult worms continue to persist in the infected individuals even after prolonged treatment with these drugs. Furthermore, they also pose problems because of undesirable side effects and development of drug resistance. Hence, there is an urgent need to identify vaccine candidates that can be used for the elimination of lymphatic filariasis by immunoprophylaxis. It has been reported that different stages of the parasite life cycle are antigenically distinct, and a state of concomitant immunity is therefore possible that prevents superinfection, thus emphasizing both the possibility and the need for an effective vaccine against filarial parasites that will avoid any risk of increasing disease severity. The use of attenuated L3 and naïve proteins as vaccine candidates have been demonstrated with various levels of protection in animal models.

In the process of evaluating a candidate antigen, its significance in the life cycle or biology of the parasite needs to be taken into consideration. Among the crucial parasite proteins, transglutaminases (TGAs) are a family of enzymes that catalyze the formation of polyamine linkages between or within the proteins of the exoskeleton. The resulting bonds are covalent, stable, and resistant to chemical, enzymatic, and histologic degradation. Such linkages are present in the exoskeleton or cuticle of the parasites. During the development from one larval stage into another, the cuticle is renewed; this process is termed molting. When the new cuticle is formed, TGA plays a very important role in the formation of the ε-(γ-glutamyl) lysine cross-links, resulting in the stabilization of the exoskeleton. Hence, an attempt was made to study the immunoprophylactic efficacy of recombinant TGA from the parasite *B. malayi* for filariasis.

Furthermore, the complexity posed by the multicellular helminth parasites demands the development of a multiple vaccine comprising more than one recombinant antigen in a cocktail mix to expose the maximum number of antigenic epitopes for binding to the various receptors that might confer enough protection against subsequent challenge. Multiple vaccination studies using more than one antigen showed promising results in parasitic diseases such as malaria, leishmaniasis, and *Schistosomiasis*. It has been reported that filarial parasites express antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione-S-transferase (GST), and thioredoxin peroxidase (TPX) to counteract the free radical damage produced by the host. Indeed, studies using TPX of *Dirofilaria immitis* suggest that, in addition to its antioxidant property, *D. immitis* may be a protective antigen. Similarly, *B. malayi* abundant larval transcript (BmALT-2) has been extensively studied in our laboratory, and results from these studies indicated that recombinant BmALT-2 protein vaccine gave up to 74% protection in mouse and jird models. Hence, BmALT-2 and BmTPX antigens have been used along with BmTGA in a cocktail mix to study the immune responses generated by multiple antigen vaccination in a jird model.

MATERIALS AND METHODS

Recombinant clones used in this study. The *BmTGA* gene was subcloned from pTrcHisB vector (GenBank accession no. AY273895) into pRSETB vector. The details of the cloning are reported elsewhere. The clone pRSETB-TGA was expressed and purified by immobilized metal affinity chromatography (IMAC), and the immunoreactivity of the recombinant protein was checked with anti-histidine and anti-TGA antibody. The enzymatic activity was checked before immunization.
Enzymatic assay for BmTGA. The enzymatic activity of recombinant BmTGA was determined using the $N,N'$-dimethylcasein solid-phase microtiter plate assay. The substrate 5-biotinamido pentylamine was covalently incorporated into $N,N'$-dimethylcasein by rBmTGA in a calcium-dependent reaction. Briefly, ELISA plates were coated with 200 µL of $N,N'$-dimethylcasein (10–20 mg/mL), sealed with paraffin paper, and stored at 4°C overnight. After the unbound $N,N'$-dimethylcasein was discarded, the wells were blocked with nonfat dry milk (0.5% in 0.1 mol/L Tris-HCl, pH 8.5) for 30 minutes and washed two times with 350 µL of 0.1 mol/L Tris-HCl, pH 8.5. Reagents were added to each well as follows: 5 mmol/L CaCl$_2$, 10 mmol/L dithiothreitol, 0.5 mmol/L 5-biotinamido pentylamine, 500 ng of rBmTGA, and 0.1 mol/L Tris-HCl, pH 8.5, to obtain a total volume of 200 µL/well. After the microtiter plate was incubated for 2 hours at 37°C, the liquid was discarded, and the reaction was stopped by washing twice with 350 µL of EDTA (200 mmol/mL) followed by two washes with 350 µL of 0.1 mol/L Tris-HCl, pH 8.5.

The streptavidin-alkaline phosphatase (0.25 mg/mL) was diluted 1:150 with nonfat dry milk (0.5% in 0.1 mol/L Tris-HCl, pH 8.5) before adding 250 µL of the solution per well for a 1-hour incubation at room temperature. The plate was washed once with 350 µL of 0.01% Triton-X100 followed by four 350-µL washes with 0.1 mol/L Tris-HCl, pH 8.5. Then, 200 µL of 0.1 mol/L Tris-HCl, pH 8.5, and 50 µL of phosphate substrate, p-nitro phenylphosphate (1 mg/mL) were added to each well. A kinetic measurement of absorbance at 405 nm was determined at 30-second intervals for a period of 30 minutes using a Vmax Kinetic Microplate Reader (Labmate, Chennai, India). TGA activity is expressed as units of optical density (mOD/min). All reactions were performed in triplicate. Guinea pig liver TGA (Sigma, St. Louis, MO) was used as a positive control.

Apart from TGA, two other clones were used in this study for multiple antigen vaccination in jirds. The recombinant plasmid containing gene for BmALT-2 (pRSETB-ALT2) was a kind gift from Dr. Thomas B. Nutman (GenBank accession no. BMU47100). These two clones were also expressed and purified, and the recombinant proteins were used for immunization studies.

Animals. Groups of male outbred mongolian jirds (Meriones unguiculatus) weighing 35–40 g were bred conventionally and maintained at the animal house facility at Mahatma Gandhi Institute of Medical Sciences (MGIMS), Sevagram, which is registered with CPCSEA (Committee for the Purpose and Control of Supervision on Experimental Animals) approval. The animals were maintained under standard laboratory conditions, and strict aseptic conditions were followed for surgical procedures. Housing, handling, and treatment of animals were performed in accordance with animal ethical committee guidelines.

Brugia malayi infection. Brugia malayi was established and maintained in jirds and mastomys. B. malayi–infected mastomys obtained from CDRI, Lucknow, were used for infecting other animals. For maintaining the cycle of infection, 4-day-old mosquitoes were used to feed on infected mastomys having 80–100 microfilariae (mf) in their circulation. Colonies of the liver pool black eye strain mosquitoes, Aedes aegypti (SS strain), obtained from Hindustan Ciba Geigy Research Center, Mumbai, India, were maintained at the MGIMS animal house facility. After 12–14 days, the mosquitoes were dissected and checked for L3 stage larvae. For mass dissection, the mosquitoes were collected using a mosquito suction gun (Hausherr’s Machine Works, Toms River, NJ), stunned by shaking, placed in a petri dish with 2–3 mL of insect saline (0.6% NaCl), and were gently crushed to release the L3 larvae. The contents were transferred to a Bearmann apparatus and kept at 35–40°C for 45–60 min. The third stage larvae collected at the bottom were removed, counted with the help of a dissecting microscope, and used for infecting fresh animals. Male Mastomys (normal), 6–8 weeks old, were infected by subcutaneous injection, and male jirds (normal) of the same age were infected by intraperitoneal injection of 100 L3, respectively, so that the microfilariae were available after 3 months or more after infection, which are collected by peritoneal lavage (in jirds).

Immunization of jirds. Three groups, each consisting of five jirds (4–6 weeks old), were immunized with individual proteins of TGA, ALT, and TPX, respectively. Another two groups of jirds were immunized with mixtures of ALT + TGA and TPX + TGA, respectively. About 25 µg of recombinant protein adsorbed in alum was immunized intraperitoneally. Each jird received a total of five doses (inoculums) of immunogen along with an equal volume of alum (Sigma-Aldrich, Bangalore, India) as adjuvant at 15-day interval. Before immunization, the animals were bled from the retro-orbital plexus to obtain preimmune serum. Control groups were immunized every time with alum alone.

Antigen-specific antibodies and isotypes in animal models. The antibody titer was determined by ELISA method. The sera were collected on a day before subsequent immunization and checked for the antigen-specific antibodies. The 96-well high-binding polystyrene, nonsterile, flat bottom plates were coated with purified recombinant antigen. The coating concentration of the different antigens—ALT, TPX, and TGA—were optimized in an earlier study. For ALT, 100 ng/well was coated, whereas TPX and TGA proteins were coated at 500 ng/well in 100 µL of coating buffer (NaHCO$_3$/Na$_2$CO$_3$ 0.067 mol/L, pH 9.6) and incubated at 4°C overnight. For the combination groups, ALT + TGA, TPX + TGA, the plates were coated with an optimized concentration of both recombinant antigens.

The dilutions of sera used were 1:250, 1:500, 1:1,000, 1:2,000, 1:4,000, 1:8,000, 1:15,000, and 1:30,000. Similarly, 100 µL of preimmune jird serum was also added to separate wells at the same dilutions. The plates were washed with PBS/T; the substrate ParaNitroPhenyPhosphate (PNPP) (Sigma) was added to the wells, and the color developed was quantitatively measured as the absorbance at 405 nm using a microplate ELISA reader (Labmate).

For determining the antibody titer, a cut-off value was fixed as the mean plus 3 SD of the OD value obtained from the preimmune serum. The highest dilution of the antisera that shows an OD value above the cut-off value was taken as the antibody titer. The isotype antibody detection for IgG1, IgG2a, IgG2b, and IgG3 was done with Pierce Isotyping kit per the manufacturer’s instructions (cat. no. 37502; Pierce, Rockford, IL). The optimal dilution of the sera used for isotyping was 1:500.

Splenocyte proliferation assay. Splenocyte proliferation was carried out as described previously. After counting the
cell number and determining viability using trypan blue dye exclusion, ~0.2 × 10^6 cells were cultured per well in 200 µL of complete Roswell Park Memorial Institute (RPMI) 1640 medium. The splenocytes were proliferated with three different conditions—unstimulated (medium [RPMI + 10% fetal calf serum]), ConA (1 µg) stimulated, and protein stimulated (10 µg in case of single antigen or 5 µg + 5 µg in case of dual antigen), and incubated for 72 hours at 37°C in a CO_2 incubator. The concentration of the antigens for stimulation was optimized earlier. Cell proliferation was measured by nonradioactive MTS one-solution cell proliferation assay (cat. no. G3580; Promega, Madison, WI). The absorbance was recorded at 490 nm. The percentage of splenocyte proliferation was calculated as the ratio of the experimental absorbance to that of control absorbance multiplied by 100.

Cytokine analysis by ELISA method. The media from the jird splenocyte culture were collected and stored at −80°C for analyzing the level of cytokines such as interleukin (IL)-4, IL-5, and interferon (INF)-γ by ELISA method. Cytokine kits obtained from Pierce (cat. no. IL4-EMIL4, IL5-EMIL5, IFNγ-EM1001) was used as per the manufacturer’s protocol for measuring the concentration of soluble cytokines at the protein level.

Parasite challenge studies. The immunized groups of jirds were infected intraperitoneally with 100 B. malayi L3 larvae after the final vaccination. After 120 days, the numbers of adult worms established were counted by sacrificing the jirds. The thorax and abdomen were opened to collect adult filarial worms from the abdominal cavity, testes, heart, lungs, and other organs. The number of adult worms recovered from each animal was recorded, and the percentage reduction in worm establishment was calculated as the average number of worms from the control group minus the average number of worms recovered from the vaccinated group divided by the average number of worms from the control group multiplied by 100.¹³

In vitro antibody–dependent cellular cytotoxicity assay with L3 and mf. Peritoneal exudates cells (PECs) were collected from normal jirds, and samples with > 95% viability were used. The cytotoxicity assay was carried out as described by previous methods.¹² Briefly 100 mf or 10 L3 larvae of B. malayi in 50 µL of RPMI 1640 were incubated with 50 µL of normal jird PECs (5 × 10^6 cells/100 mf/10 L3) and 50 µL of normal jird serum or sera from each of the immunized group of jirds in a 96-well culture plate. The 96-well plate was incubated for 48 hours at 37°C in a 5% CO_2 atmosphere. The parasites were examined under a microscope after 48 hours to check for viability. The parasites were considered dead if not motile and adhered by PECs. The percentage cytotoxicity was expressed as the ratio of the number of dead parasites to that of the total number recovered within the experimental period multiplied by 100.

Statistical analysis. All statistical analysis were done using GraphPad Prism software version 5. A probability value (P) ≤ 0.05 was considered statistically significant.

RESULTS

Antibody response and isotype distribution to single and multiple protein vaccination. There was significant antibody production against the different antigens in the immunized groups compared with controls. The dose-response curves for antibody titer are given in Figure 1A. The titers of the antigens-specific antibodies were as follows: ALT, 1:20,000; TPX, 1:15,000; TGA, 1:15,000; ALT + TGA, 1:25,000; TPX + TGA, 1:25,000. The isotype antibody analysis showed higher IgG1 and IgG3 isotypes compared with IgG2a and IgG2b (IgG1/IgG2a > 1) in all groups (Figure 1B).

Splenocyte proliferation assay. The results of proliferation in the cultures of splenocytes of vaccinated jirds indicated that there was significant proliferation in response to stimulation with various recombinant antigens compared with control groups immunized with alum. The data were analyzed statistically and found to be significant (P < 0.05). Stimulation with Concanavalin A (ConA) induced significant proliferation in both control and vaccinated jird splenocytes. The values are summarized in Table 1.

Cytokine analysis by ELISA method. The results of the cytokine analysis carried out in the supernatants of the splenocyte cultures of the immunized jirds are shown in Table 2. All groups other than the control group showed a predominance of IL-4, followed by IL-5, whereas IFN-γ levels were very low. The values obtained were statistically significant (P < 0.05).

Protection studies in jirds by in vivo adult worm recovery. The results of the protection studies are shown in Figure 2. In single antigen vaccination, TGA conferred only 30% protection, whereas ALT and TPX conferred 69% and 43%, respectively. In multiple antigen vaccination, ALT + TGA showed up to 47% protection, but it was lower than single antigen vaccination with ALT alone (69%). However, the combination of TPX and TGA (TPX + TGA) vaccination provided the maximum level of protection (up to 73%) compared with the other vaccinated groups.

Figure 1. A, Immunization and bleeding schedule for parasite challenge study in jirds. B, Isotype distribution of antibodies in jirds immunized with various recombinant protein vaccines. All groups showed predominance of IgG1 and IgG3, thus indicating a Th2-biased immune response.
In vitro antibody–dependent cellular cytotoxicity assay with L3 and MF. The results of antibody–dependent cellular cytotoxicity assay (ADCC; Figure 3) showed that TGA confers only 16% and 18% cytotoxicity with mf and L3, respectively (P > 0.05), whereas ALT showed 72% (mf) and 70% (L3) (P < 0.05). TPX showed 19% (mf) and 23% (L3) (P > 0.05), ALT + TGA showed 60% (mf) and 62% (L3) (P < 0.05), and TPX + TGA showed 79% (mf) and 76% (L3) protection (P < 0.05). The control group showed 12% (mf) and 16% (L3) cytotoxicity.

DISCUSSION

Lymphatic filariasis continues to be a worsening problem in many places, especially in Africa and the Indian subcontinent,23 causing elephantiasis, lymphoedema, and genital pathology in men, women, and children. Because of the various drawbacks of chemotherapeutic methods of treatment, there is an immediate need for development of vaccines by the identification and evaluation of candidate antigens from the different stages in the life cycle of the parasite by more reliable recombinant DNA technology.24 Enzymes involved in the molting and development of the filarial worms, such as TGAs,8 are ideal candidates for testing vaccine efficacy. The role of TGAs in parasite larval molting and survival has been well documented in filarial25–27 and malarial parasites.28 Hence, we attempted to study the immunoprophylactic efficacy of BmTGA. The BmTGA gene was subcloned from the pTrcHisB vector into pRSETB, where it was expressed, purified, and characterized further. These details have been reported elsewhere16 (GenBank accession no. EU429936). This study was undertaken to evaluate the immune response of the clone pRSETB-TGA as a single antigen vaccine in the form of recombinant protein and also as a multiple antigen protein vaccine in combination with other known vaccine candidates such as ALT-2 and TPX.

Multiple antigen vaccination in jirds. Because parasites are complex multicellular organisms, sometimes a single recombinant antigen might not be sufficient to elicit appropriate immune response to confer good protection. With their large gene complement, it seems likely that filariae have evolved a spectrum of immune evasion products that underpin their ability to live for many years within the human host. These gene products provide a molecular repertoire that helminth parasites have evolved to manipulate and evade the mammalian immune response.29 Hence, more than one recombinant antigen in a cocktail mix might confer enough protection, and to overcome the complexity of these parasites, a multivalent approach might be needed. There are many instances where combinations of two or more antigens have conferred more protection than a single antigen alone and have produced immune responses comparable to those observed after immunization with a single molecule vaccine.30,31,32 Hence, we decided to undertake a multiple antigen vaccination in our study and to evaluate the protection conferred by BmTGA along with other known recombinant antigens, such as BmALT-213–15 and BmTPX11–13,33 as a multiple antigen vaccine in a suitable animal model.

Gerbils or jirds (M. uunguiculatus) are well-established animal models for B. malayi infections,34 in which the parasite passes from L3 to adult, and parasite challenge studies can be effectively carried out. Hence, the level of protection can be determined in jirds by a more reliable parasite challenge method. In view of the above features, multiple antigen vaccination studies were carried out with BmTGA, BmALT-2, and BmTPX proteins in a jird model.

Humoral and cellular immune responses. There was significant antibody production against the different antigens in the immunized groups compared with control groups. The profile of antibody isotype distribution in jirds showed the predominance of IgG1 and IgG3 isotypes compared with IgG2a and IgG2b. Similarly, there was significant proliferation of the splenocytes of immunized jirds in response to stimulation with various recombinant antigens compared with control groups. The analysis of the cytokines in the immunized jirds showed predominance of IL-4 in all groups, followed by significant levels of IL-5, and with very low levels of IFN-γ (Table 2). Thus, the isotype antibody and the cytokine profile indicates a Th2-biased immune response for single and multiple protein vaccine. As previously mentioned, immunity to nematode worms is critically dependent on a type 2 cytokine response.
Protection studies. Protection studies in jirds showed that BmTGA alone conferred only 30% protection as obtained earlier in a mice model with 24% protection by the micro-pore chamber method (unpublished data). The 69% protection conferred by BmALT-2 in our study is in accordance with that of 74% in jirds obtained previously. Further, multiple antigen vaccination of BmTGA along with BmALT-2 increased protective efficacy of BmTGA up to 47% compared with BmTGA single antigen vaccination (30%) while reducing the efficacy of the BmALT-2 single vaccine (69%). At present, we cannot rule out the possibility that a direct interaction between BmALT-2 and BmTGA adversely affected the immunogenicity of BmALT-2. A similar situation was encountered for a multiple vaccination study in malaria where two recombinant merozoite surface proteins (MSPs) of Plasmodium yoelli, PyMSP-1 42 and PyMSP-8, were tested for their protective efficacy, and it was found that the immunogenicity of PyMSP-1 42 was further reduced when mice were immunized with a mixture of PyMSP-1 42 and PyMSP-8. The antigenic epitopes on BmALT-2 might have been masked by BmTGA, leading to the downregulation of the efficacy of BmALT-2 in the combination vaccine.

The combination of the BmTPX and BmTGA vaccination provided a significant level of protection (up to 73%) compared with the other vaccinated groups. Although, as single antigens, BmTGA (30%) and BmTPX (43%) failed to provide much protection, the combination of both greatly enhanced protection. One possible explanation for the unexpected increase in the level of protection in the combination is that this mixture may behave like a multiple-subunit vaccine. In this context, it is a known fact that parasite TGAs are a family of enzymes that share catalytic function with thior-odoxin and protein disulphide isomerases (PDI), apart from their usual protein cross-linking function. Thioredoxin per-oxidases are also a class of antioxidant enzymes belonging to the thioredoxin family of proteins. Hence, TPX and TGA are members of the same thioredoxin family. When these two are brought together in combination, the number of available protective epitopes cumulatively increase, in a non-antagonistic manner, and it is probably this availability of a maximum number of antigenic epitopes that brings effective protection.

The immunological mechanism used by the host to kill a large multicellular helminth is classically thought to involve ADCC and is not attributed to complement proteins. This mechanism of killing may depend on a type 2 response, in which large amounts of antibody of different isotypes, including IgE, are produced. In vitro, there is a large body of evidence showing that all mammalian stages of filarial parasites can be killed by ADCC. Hence, in this study, it was also attempted to find whether ADCC has a role in conferring protection. The results of ADCC for multiple antigen vaccination, against mf and L3, are in accordance with that obtained in the protection studies after parasite challenge, with the BmTPX and BmTGA protein combination showing significantly higher cytotoxicity than either single antigen group.

Another important aspect is that all the antigen-immunized jirds showed similar profiles of immune responses in terms of splenocyte proliferation and cytokine secretion pattern (IL4, IL5, and IFN-γ), but only differ in terms of protection in vivo and in vitro ADCC, where killing was much higher in dual vaccinations than single vaccinations. One probable reason might be that the mechanism of ADCC, which is characteristic of the immune responses in helminth parasites, differs in the different immunized groups, which in turn affects adult worm recovery as seen in protection studies. Hence, the profiles of adult worm recovery and in vitro ADCC are comparable, whereas other immune responses (splenocyte proliferation, cytokine secretion) do not show much variation between the single and dual-antigen immunized groups of jirds.

Thus, a putative vaccine candidate BmTGA is characterized, and its immunoprophylactic potential was studied in a jird model. In multiple antigen form, BmTGA + BmTPX seems to be a promising multiple antigen vaccine candidate for human lymphatic filariasis.

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