Immune responses generated by intramuscular DNA immunization of *Brugia malayi* transglutaminase (BmTGA) in mice

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SUMMARY

An attempt was made to evaluate the immunoprophylactic efficacy of *Brugia malayi* transglutaminase (BmTGA) as a DNA vaccine, for human lymphatic filariasis. BmTGA was cloned and characterized in the DNA vaccine vector pVAX1. Further, the tissue distribution study of the DNA construct, pVAX-TGA was carried out in mice and the DNA vaccine was shown to be efficiently distributed to all the organs, was accessible to the immune system, and at the same time was metabolized quickly and did not pose problems of toxicity. Intramuscular immunization in mice showed significant antibody production and splenocyte proliferation upon antigenic stimulation. The immune responses were biased towards the Th1 arm, as evaluated in terms of isotype antibody distribution and cytokine profile. Thus, analysis of the humoral and cellular immune responses indicated that BmTGA is a potent immunogen. However, protection studies as determined by the micropore chamber method using live microfilarial larvae, showed that the DNA vaccine could confer only partial protection in the mouse model. We conclude that despite the induction of sufficient humoral and cellular immune responses, BmTGA as a DNA vaccine could not confer much protection against subsequent challenge and other aspects of the immune responses need to be further investigated.

Key words: filariasis, DNA vaccine, transglutaminase, cytokine, micropore chamber.

INTRODUCTION

Human lymphatic filariasis is one of the nematode parasite-borne diseases and is one of the leading causes of disability, infecting some 120 million individuals worldwide, with a further 1·3 billion people at risk (WHO, 2006) and 40 million seriously incapacitated or disfigured by the disease (Ottesen, 2000). The disease is caused by the nematode parasites *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*. The widely used drugs of choice for the treatment of filariasis are diethyl carbamazine (DEC), ivermectin and albendazole. However, the effectiveness of these drugs in killing the adult stages of these parasites has been more difficult to establish and emerging parasite resistance has been reported (Ottesen, 1985; Lustigman and McCarter, 2007). Hence there is an urgent need for immunologists to try to produce vaccines which will help to consolidate other methods of control. There has been a great deal of epidemiological and experimental evidence to suggest that a filarial vaccine is feasible, and antigen discovery has progressed relatively rapidly within just the past few years and a variety of fascinating vaccine candidates have been cloned (Grieve *et al*. 1995). Many workers have reported the use of live/attenuated L3/stage-specific recombinant proteins as vaccine candidates and have demonstrated various levels of protection in animal models.

Since it is very difficult to generate enough parasite material for vaccination studies, we have to rely on recombinant DNA technology for the large-scale production of these vaccines. Potentially, protective parasite antigens can now be produced in recombinant form in a variety of vectors and this represents a key breakthrough on the road to commercial vaccine production (Knox *et al*. 2001). The most exciting developments in the field of vaccine research in recent years has been DNA vaccines, with which immune responses are induced subsequent to the *in vivo* expression of antigen from directly introduced plasmid DNA. Strong immune responses have been demonstrated in a number of animal models against many viral, bacterial and parasitic pathogens, and several human clinical trials have been undertaken. Genetic immunization may be both time and labour-saving in such instances when the isolation of enough

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pure protein for vaccination is time-consuming, and may offer a unique method for vaccination (Tang *et al*. 1992). These features encourage the development of a DNA vaccine for human lymphatic filariasis.

Towards targeting candidate antigens for vaccine development, it is necessary to have a clear idea about the different biochemical pathways operating in the parasite and which enzymes are critical for the survival of the parasite, so that blocking a particular enzyme or pathway would lead to instantaneous death of the parasite. Among these, transglutaminases (TGA) (EC 2.3.2.13) are a family of enzymes that catalyse the post-translational modification of proteins to form either ε-(γ-glutamyl)lysine or (γ-glutamyl) polyamine linkages between or within the proteins. The resulting bonds are covalent, stable and resistant to chemical, enzymatic and histological degradation (Folk, 1983). Such linkages are present in the exoskeleton or cuticle of the parasites. When the new cuticle is formed during the moulting process, TGA plays a very important role in the formation of the ε-(γ-glutamyl)lysine cross-links, resulting in the stabilization of the exoskeleton. There is ample evidence through earlier studies on TGA indicating the significance of this enzyme in the filarial parasites (Rao *et al*. 1991; Lustigman *et al*. 1995; Chandrashekar *et al*. 1998). Thus, TGA-catalysed reactions appear to be critical for the growth, development and maturation of nematodes because of their role in cuticle biosynthesis (Mehta *et al*. 1996). Their presence in all the parasitic stages and in higher levels in the adult female stages makes them ideal candidates for testing the vaccine efficacy.

The preliminary characterization of recombinant transglutaminase from the filarial parasite *B. malayi* (rBmTGA) has already been reported and the immune responses have been evaluated in jirds (Uma *et al*. 2006, 2009). In this study, we have aimed to analyse the immunoprophylactic potential of BmTGA as a DNA vaccine and evaluate the level of protection. This report is focused on the preparation and characterization of the DNA vaccine construct of BmTGA and analysis of the humoral, cellular and protective immune responses generated thereof in a mouse model.

MATERIALS AND METHODS

Cloning of BmTGA in the DNA-vaccine vector pVAX1

For cloning in pVAX1 vector (Invitrogen), BmTGA (GenBank Accession no. EU429936) was amplified using gene-specific primers incorporating the restriction sites for *Pst1* (forward primer) and *Xba1* (reverse primer). The cycling protocol for amplification was an initial activation and denaturation step of 5 min at 95 °C, followed by 35 cycles each consisting of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C and a final extension was given at 72 °C for 10 min. All amplifications were carried out using MJ Minicycler (Perkin Elmer, USA). For cloning, the PCR products were purified and then digested with restriction enzymes *Pst1* and *Xba1*. The restricted PCR products were then ligated into the multiple cloning site of the DNA vaccine vector, pVAX1 following digestion with *Pst1* and *Xba1*. The ligation mixture was then transformed into *E. coli* host DH5α. For screening the transformants, lysate PCR was employed. The positive clones were further confirmed by restriction digestion and checked for the release of the insert. The DNA vaccine vector containing BmTGA was named as pVAX-TGA. For the large-scale production of the DNA vaccine construct pVAX-TGA, the plasmid was purified using endotoxin-free plasmid gigaprep kit (Qiagen, Hilden, Germany) as per manufacturer's instructions.

Transient transfection of CHO cell line by pVAX-TGA

The CHO cell line cryopreserved and maintained by the Tissue Culture Laboratory, Centre for Biotechnology, Anna University, India, was used for the transient transfection of DNA vaccine construct of BmTGA to check for its expression. CHO cells were transiently transfected using lipofectamine reagent (GibcoBRL/Life Technologies, Gaithersberg, MD, USA) as described by the manufacturer. The cells were incubated at 37 °C in a CO_2 incubator. The transfected cells were harvested after 72 h. The cells were also transfected with a positive control plasmid pEGFPN3 (Clonetech, USA), which contains a CMV promoter and expresses green fluorescent protein. RNA was extracted from cells by using TRIZOL reagent (Invitrogen) and converted into cDNA using MMLV Reverse Transcriptase (NEB) by standard protocols. The cDNA was checked for the presence of message level of pVAX-TGA gene in the transfected CHO cells by amplifying with pVAX-TGA-specific forward and reverse primers. The level of β-actin m-RNA (a housekeeping gene) was simultaneously assayed as an internal control.

Parasites and animal studies

Six to eight-week-old inbred BALB/c mice were purchased from Veterinary College, Madhavaram, Chennai. Each group consisted of 5 mice. Animals were moved to the laboratory on the day of the experiment and maintained under standard conditions with food and water at the animal house facilities of the Centre for Biotechnology, Anna University. For micropore chamber experiments employing live

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parasites, the mice were transported to the animal house facility at the 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.

For *B. malayi* infection, the mosquito colony and parasites were maintained in jirds at the animal house facility at MGIMS, Sevagram. Colonies of the liver pool black eye strain mosquitoes, *Aedes aegypti* (SS Strain) obtained from Hindustan Ciba Geigy Research Centre, Mumbai were maintained at a temperature of 25–28 °C and relative humidity of 70–80%. For maintaining the cycle of infection, 4-day-old mosquitoes were allowed to feed on *B. malayi*-infected mastomys. After 12–14 days, the mosquitoes were dissected and checked for third-stage larvae (L3). For mass dissection the mosquitoes were collected using a mosquito suction gun, 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 then transferred to a Bearmann's apparatus and kept at 35–40 °C for 45–60 min. The L3 collected at the bottom were removed, counted with the help of a dissecting microscope (Suzuki and Seregeg, 1979) and used for infecting fresh animals, so as to render the microfilariae (mf) available.

The mf were obtained by lavage of the peritoneal cavities of jirds having an intraperitoneal L3 filarial infection of 3 months or more duration. The mf were washed with RPMI 1640 medium (GIBCO Laboratories, USA) (containing 20 mg/ml gentamycin, 100 mg/ml penicillin, 100 mg/ml streptomycin) plated on sterile plastic Petri dishes and incubated at 37 °C for 1 h to remove the jird peritoneal exudate cells. The mf were collected from Petri dishes, washed with RPMI-1640 medium and suspended in the same for loading in the micropore chamber (Sahare *et al*. 2008).

Tissue distribution study of pVAX-TGA

A sample of 100 μg of the DNA vaccine pVAX-TGA was injected intramuscularly and each animal was sacrificed by cervical dislocation at different time-points, namely 24 h, 7 days and 14 days after DNA vaccine administration. Different organs and cell types like muscle, spleen, kidney, liver, heart and colon were isolated from each mouse and approximately 100 mg of tissues were taken for DNA isolation. The DNA from different tissues at different time-points was subjected to PCR amplification with pVAX-TGA gene specific primers for studying the distribution and *in vivo* expression of the DNA vaccine construct of BmTGA. The levels of expression at different time-points in the various tissues were evaluated based on the band intensity of the PCR amplified product.

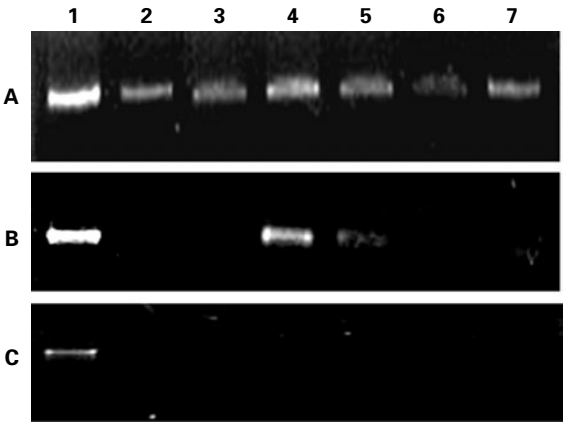

Fig. 1. Tissue distribution study of pVAX-TGA. Lane 1, injected site muscle; Lane 2, lung; Lane 3, heart, Lane 4, spleen; Lane 5, kidney; Lane 6, liver; Lane 7, colon. (A) After 24 h. Extensive distribution of DNA vaccine in all tissues with the majority of the DNA vaccine existing in the injected site muscle. (B) After 7 days. Plasmid DNA was detectable in injected site muscle, spleen and in very small amounts in the kidney, showing the quick metabolism of DNA vaccine in important organs, thereby posing less toxicity. At the same time, the long-term existence of the plasmid DNA in spleen implies the production of an effective immune response in mice. (C) After 14 days. Plasmid DNA was detectable only in injected site muscle as it is metabolized completely in other organs.

Immunization of mice

A sample of 100 μg of plasmid DNA vaccine was injected intramuscularly for the vaccination studies in mice. Control groups were injected with pVAX1 vector alone. The dose of the DNA vaccine for immunization in mice was optimized in an earlier study. Five mice were used per group. Prior to immunization the animals were bled from the retro-orbital plexus to obtain pre-immune serum. Thereafter, 5 doses, at weekly intervals were administered. Each time, the hyperimmune serum was collected on a day prior to subsequent immunization. A final booster dose was also given.

After administration of the final booster dose, the blood was checked for high antibody titre by ELISA. Thereafter, a micropore chamber containing live mf was implanted in the peritoneal cavity of mice on the 42nd day, and recovered after 48 h.

Antigen-specific antibodies and isotypes in animal model

The antibody titres in the immunized mice sera were analysed by ELISA as described previously (Thirugnanam, 2007). Dilutions of mice sera from 1 : 50, 100, 250, 500, 1000, 2000 and 4000 were used. The same dilutions of pre-immune mice sera were simultaneously used. For determining the antibody

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titre, a cutoff value was fixed as the mean plus 3SD of the OD value obtained from the pre-immune serum. The highest dilution of the antiserum that showed an OD value above the cutoff value was taken as the antibody titre. The isotype antibody detection for IgG1, IgG2a, IgG2b and IgG3 was done using a Pierce Isotyping kit as per the manufacturer's instructions (Pierce, USA, Cat. no. 37502). The optimal dilution of the sera used for isotyping was 1 : 100.

Splenocyte proliferation assay

Splenocyte proliferation was carried out as described previously (Thirugnanam, 2007). After counting cell numbers and determining the viability using trypan blue dye exclusion approximately $0{\cdot}2 \times 10^5$ cells were cultured per well in 200 μl of complete RPMI medium. The splenocytes were stimulated under 3 different conditions – unstimulated (medium (RPMI + 10% FCS)), ConA (1 μg) stimulated and protein stimulated (with recombinant BmTGA purified in *E. coli* (10 μg)), and incubated for 72 h at 37 °C in a CO_2 incubator. The concentration of the antigens for stimulation was optimized earlier. Cell proliferation was measured by non-radioactive MTS one-solution cell proliferation assay (Promega, cat. no. G3580). The absorbance was recorded at 490 nm. The proliferation was calculated as the ratio of the experimental absorbance to that of control absorbance multiplied by 100.

Cytokine analysis by ELISA

The medium from the mice splenocyte culture was collected and stored at −80 °C for analysis of the level of cytokines such as IL-4, IL-5 and IFNγ by the ELISA method. Cytokine kits (Pierce, Rockford, USA (cat. nos IL4-EMIL4, IL5-EMIL5, IFNγ-EM1001) were used as per the manufacturer's protocol for measuring the concentration of soluble cytokines at the protein level.

Statistical analysis

All statistical analysis was done using graphpad prism software version 5. Statistical significance of cytokine analysis was evaluated by non-parametric Mann-Whitney test. For protection studies, non-parametric ANOVA test was used. A probability (P) value $<=0{\cdot}05$ was considered statistically significant.

Protection study by micropore chamber method

A protection study was done using the micropore chamber method as described by Abraham *et al.* (1993) and Chenthamarakhshan *et al.* (1995). After the final booster immunization, the mice were challenged by intraperitoneal implantation of a micropore chamber containing 100 *B. malayi* mf larvae in RPMI-1640 medium, as described previously (Gnanasekar *et al.* 2004). After 48 h, the micropore chamber was harvested and the contents were removed onto a glass slide and examined microscopically for cytotoxicity. Numbers of live and dead mf were enumerated by observing their movement under a Nikon microscope. The tissue-culture plate containing the micropore chamber was kept at 37 °C for 30 min and counted again to see the live and dead mf. The percentage protection was calculated as the ratio of the number of dead mf to that of the total number of mf recovered during the experimental period multiplied by 100.

RESULTS

Characterization of BmTGA in DNA vaccine vector pVAX1

Cloning of BmTGA into pVAX1 vector. BmTGA was cloned into pVAX1 at the sites *Pst1* and *Xba1* and the recombinant construct containing BmTGA was named pVAX-TGA, and was sequenced completely. The presence of the insert was confirmed by restriction digestion of the plasmid with *Pst1* and *Xba1* (data not shown).

Transient transfection of CHO cell line by DNA vaccine construct of BmTGA

The transient expression of the DNA vaccine construct was confirmed in CHO cell lines at the message level of pVAX-TGA gene by RT-PCR from the cDNA of the transfected cells. The cells were also transfected with a positive control plasmid pEGFPN3 which contains a CMV promoter and expresses green fluorescent protein. The level of β-actin mRNA (a house-keeping gene) was simultaneously assayed as an internal control (data not shown).

Tissue distribution study of pVAX-TGA

The results of the tissue distribution study as determined by PCR amplification of pVAX-TGA in the different tissues, suggested that the DNA vaccine has quick absorption and extensive distribution within 24 h of injection, and that the majority of the plasmid DNA exists in the injected local muscles of all treated mice. By 7 days after injection, plasmid DNA was detectable in injected muscle, spleen and in small amounts in the kidney. The long-term existence of the plasmid DNA in spleen implies the production of an effective immune response in mice. Plasmid DNA was detected only in injected site muscle after 14 days of injection. It is quickly metabolized within 1 week in important organs,

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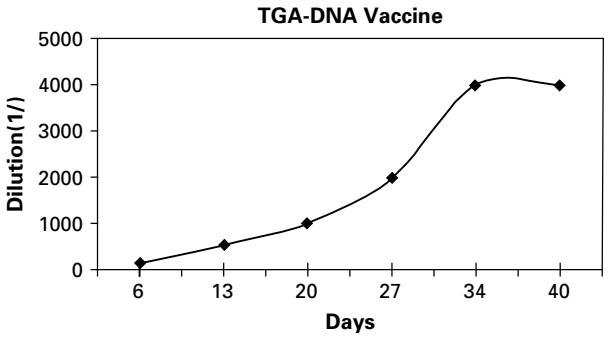

Fig. 2. Levels of antigen-specific antibodies (total IgG) in sera from DNA-immunized mice. The points represent the mean value from 5 different mice, of the antibody titre at different intervals after immunization.

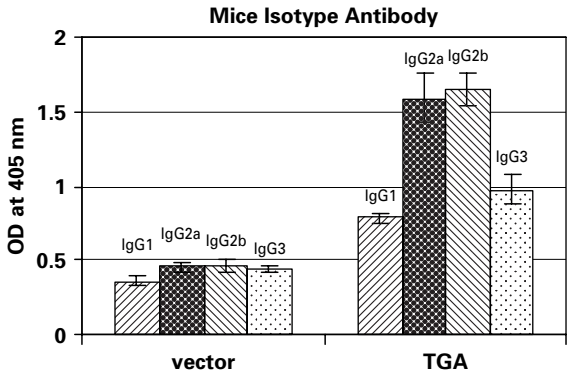

Fig. 3. Isotype distribution of antibodies in mice immunized with pVAX-TGA. Isotype antibody analysis showed that there were higher levels of IgG2a and IgG2b and a very low level of IgG1 (IgG1/IgG2a = 0·492). Thus immunization with DNA vaccination biased towards the Th1 arm of the immune response.

which indicates the low possibility of toxicity to these organs.

Fig. 1 shows the PCR amplification of DNA extracted from different tissues at the different time-points of 24 h, 7days and 14 days respectively, using pVAX-TGA primers.

Immune responses of BmTGA DNA vaccine in mice

Antibody response and isotype distribution to pVAX-TGA vaccination. There was significant antibody production against BmTGA in the immunized mice as shown in Fig. 2. The dose-response curve and the titre obtained at different intervals of the immunization schedule are given in Fig. 2. The control group immunized with pVAX1 vector showed most of the reactivity below the cutoff value (0·105) (data not shown). The titre of the antiserum on the day of the final bleed was 1 : 4000. The isotype antibody analysis revealed that there were higher levels of IgG2a and IgG2b and a lower level of IgG1 (IgG1/IgG2a = 0·492). Thus it appeared that immunization with DNA vaccine was biased towards the Th1 arm of the immune response (Fig. 3).

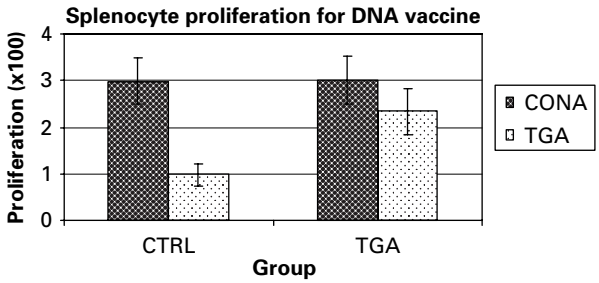

Fig. 4. Splenocyte proliferation as determined by MTS assay after 72 h of stimulation with BmTGA/ConA. The stimulation index values are given as the geometric mean (GM) ± S.D. of 5 mice. Significant proliferation in response to rBmTGA stimulation was observed in the vaccinated (2·331 ± 0·503) groups compared to control groups (0·975 ± 0·233) ($P < 0·05$), while stimulation with ConA induced significant proliferation in both vaccinated (3·018 ± 0·514) and control (2·984 ± 0·506) groups.

Splenocyte proliferation. The results of the splenocyte proliferation studies, as shown in Fig. 4, revealed that there was significant proliferation in response to stimulation with rBmTGA, in the splenocytes of mice immunized with pVAX-TGA (2·331 ± 0·503) as compared to control groups immunized with pVAX1 vector (0·975 ± 0·233). Stimulation with ConA induced significant proliferation in both control (2·984 ± 0·506) and vaccine-immunized (3·018 ± 0·514) mice splenocytes. The proliferation of the immunized groups in response to rBmTGA was found to be statistically significant as compared to control groups ($P < 0·05$).

Cytokine analysis by ELISA

The supernatants from the stimulated splenocyte cultures of the immunized mice were assayed for the amounts of various cytokines, such as IL-4, IL-5 and IFNγ by ELISA, and the results are shown in Fig. 5. Cytokine analysis revealed that upon stimulation of the splenocytes with recombinant BmTGA, the IFN-γ level was higher (13 ± 0·508 pg/ml) than IL-4 (4 ± 0·651 pg/ml) and IL-5 (4 ± 0·585 pg/ml) in the pVAX-TGA group. The amounts of cytokines in the pVAX1 control group are IFN-γ – 2 ± 0·475 pg/ml, IL-4 – 1 ± 0·411 pg/ml and IL-5 – 0·82 ± 0·354 pg/ml. The data obtained were analysed and found to be statistically significant ($P < 0·05$). Thus the cytokine analysis also shows that DNA immunization is biased towards the Th1 type of immune response.

Protection studies by the micropore chamber method

The results of *in situ* micropore chamber studies showed that the DNA vaccine construct pVAX-TGA induced 21% (21 ± 2·33) protection compared to the control group immunized with pVAX1 vector

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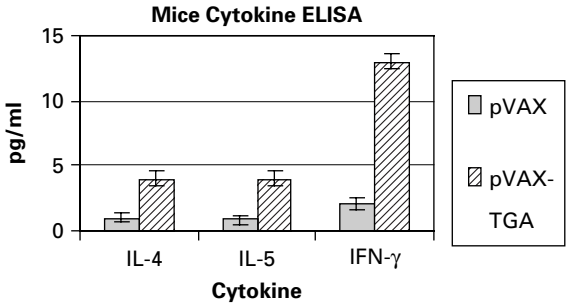

Fig. 5. Cytokine secretion profile of proliferated splenocytes of vaccinated mice. Cytokine analysis by capture ELISA shows that IFN-γ ($13 \pm 0{\cdot}508$ pg/ml) was higher than IL-4 ($4 \pm 0{\cdot}651$ pg/ml) and IL-5 ($4 \pm 0{\cdot}585$ pg/ml) in the pVAX-TGA-immunized group while control groups(pVAX1) showed significantly lower amounts of all the cytokines (IFN-γ: $2 \pm 0{\cdot}475$ pg/ml, IL-4: $1 \pm 0{\cdot}411$ pg/ml and IL-5: $0{\cdot}82 \pm 0{\cdot}354$ pg/ml) ($P < 0{\cdot}05$).

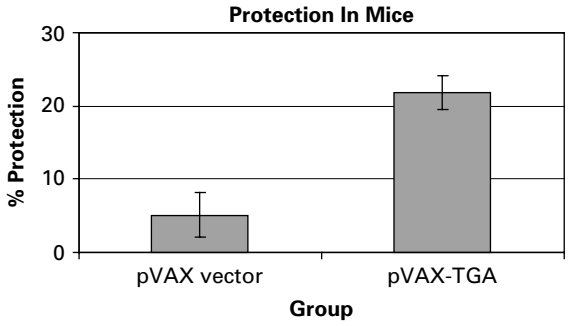

Fig. 6. Protection studies in mice using the *in situ* micropore chamber method. pVAX-TGA conferred 21% ($21 \pm 2{\cdot}33$) protection while the control group (pVAX1) showed 5% ($5 \pm 3{\cdot}062$) protection.

which showed only 5% ($5 \pm 3{\cdot}062$) protection. The results are shown in Fig. 6. The data obtained were statistically analysed and the difference between the control and test groups was significant ($P < 0{\cdot}05$).

DISCUSSION

Towards developing a vaccine for human lymphatic filariasis, the main aim of this study was to evaluate the immune responses and protection conferred by DNA immunization. Among the various candidate vaccines, enzymes involved in the moulting and development of the filarial worms, such as transglutaminases (TGA) have been well documented. The enzyme activity was shown to be essential for *in utero* growth and development of microfilariae. Adult worms of *B. malayi* have a large amount of epsilon-(gamma-glutamyl) lysine isopeptide bonds, a product of physiologically active transglutaminase, and contained several proteins that could serve as suitable substrates for the enzyme. During the process of their development, TGA is involved in covalent incorporation of host proteins into the embryos and microfilariae of *B. malayi*. Inhibition of the enzyme activity by an enzyme-specific pseudosubstrate, monodansylcadaverine (MDC), led to a time- and dose-dependent inhibition of microfilariae production and release by gravid female worms (Mehta *et al*. 1992, 1996). The possible involvement of transglutaminase-catalysed reactions in survival of adult worms, microfilariae (mf), and infective larvae of the filarial parasite *B. malayi* was studied *in vitro* by using the specific pseudosubstrate MDC and the active-site inhibitors cystamine or iodoacetamide. These inhibitors significantly inhibited parasite mobility in a dose-dependent manner. This inhibition was associated with irreversible biochemical lesions followed by filarial death. These studies suggest that transglutaminase-catalysed reactions may play an important role in the growth, development, and survival of filarial parasites (Rao, 1991). These features make them ideal candidates for testing the vaccine efficacy.

Recombinant *B. malayi* transglutaminase (BmTGA) protein has been previously characterized and the immune responses have been studied in jirds (Uma *et al*. 2006, 2009). Hence, an attempt was made to prepare the DNA vaccine construct of BmTGA and evaluate the immune responses and level of protection in a mouse model. BmTGA was cloned into the DNA vaccine vector pVAX1. The recombinant construct pVAX-TGA was purified in large-scale using a giga-prep kit (Qiagen) and the transient expression was checked in CHO cell lines. The presence of the message level of BmTGA suggests that the DNA vaccine pVAX-TGA is successfully expressed in mammalian cell lines.

In the context of DNA vaccination, understanding the fate of plasmids after immunization is important for the successful development of a DNA vaccine, enabling the use of lower doses and thereby reducing the risks in the clinical application of DNA vaccines. In order to understand the dissemination of plasmid DNA into different tissues, a tissue distribution study was carried out the mouse model after intramuscular injection of the DNA vaccine construct of pVAX-TGA. DNA was isolated from different tissues at different time-points and PCR amplified with pVAX-TGA gene-specific primers. This experiment confirmed the distribution of DNA vaccine and the results from this experiment suggested that the majority of the plasmid exists in the injected local muscles for all treated mice. The PCR assay also suggested that the plasmid DNA was quickly absorbed and extensively distributed in different organs of the immunized mice, such as heart, lung, and liver within 24 h after injection and all plasmid DNA was quickly metabolized within 1 week, indicating the low possibility of toxicity to these organs. The long-term existence of the plasmid DNA in spleen implies the production of an effective immune response in mice. As the construct pVAX-TGA does

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not contain eukaryotic origin of replication it cannot replicate in eukaryotic tissues, which once again implies low toxicity.

The susceptibility to *B. malayi* infection has been reported to vary in different laboratory hosts ranging from fully susceptible jirds to semi-susceptible mice. Semi-permissive hosts such as Balb/c mice have been proposed as suitable models to assess immunity against developing larvae of *B. malayi* (Ash and Riley, 1970). Immunological parameters like antibody isotyping, splenocyte proliferation studies, cytokine analysis at the message level by RT-PCR and at the protein level by ELISA are feasible in the mouse model by the use of commercially designed reagents and kits. Thus a certain level of immunological characterization of the antigens can be achieved in this model. Hence, with the above considerations in mind, an attempt was made to evaluate the immune responses of BmTGA as a DNA vaccine in the mouse model and the level of protection conferred in mice was assessed by the micropore chamber method.

There was significant antibody production against pVAX-TGA in the immunized groups as compared to control groups immunized with vector (pVAX1) alone. The isotype profile showed an increased level of IgG2a with lower levels of IgG1, thus indicating a bias towards a Th1 response (IgG1/IgG2a $<1{\cdot}0$). The results of splenocyte proliferation studies showed that there was significant proliferation of the splenocytes of immunized mice, in response to stimulation with rBmTGA, as compared to control mice immunized with pVAX1 vector alone. The cytokine analysis revealed that the IFN-γ level was higher than IL-4 and IL-5. Thus the cytokine analysis also shows that DNA immunization induced a Th1 type of immune response.

Several studies have implicated immunostimulatory CpG sequences as the causative factor in the development of Th1 immune responses to DNA immunization. The route of administration of plasmid DNA vaccines also influences the strength and nature of immune responses in mice and non-human primates. Responses to intramuscular injection were predominantly IgG2a (Th1-like) at all times (McCluskie *et al*. 1999), in contrast to the preferential induction of Th2-associated responses by gene gun immunization, probably by the migration of epidermal dendritic cells to the draining lymph nodes as a result of direct transfection, thereby presenting the antigen to the immune system (Robinson and Torres, 1997).

The level of protection conferred by BmTGA in mice was assessed by the *in situ* micropore chamber method. The micropore chamber technique in which a closer physiological environment can be provided for parasite growth and survival has been used by many investigators to analyse the protection levels. The chambers loaded with filarial larvae and implanted in the peritoneal cavity provoked infiltration of cell populations into the chamber and, after 48 h, the larvae were killed by cytotoxic effect. The results of *in situ* micropore chamber studies showed that the DNA vaccine construct pVAX-TGA induced 21% cytotoxicity as compared to the control group immunized with pVAX1 vector which showed only 5% cytotoxicity ($P<0{\cdot}05$).

From the above studies, it was observed that BmTGA could confer only partial (21%) protection as a DNA vaccine, which indicates that there is a need to further explore and examine the value of this important molecule in the control of filariasis. Earlier studies have shown that when compared to recombinant protein vaccination, the protection level conferred by DNA vaccination was generally low. There are many such instances in which DNA vaccines have failed to confer much protection in animal models, compared to vaccination with recombinant proteins or live attenuated larvae. Intramuscular immunization in mice with plasmid DNA that encodes *B. malayi* paramyosin (BM5) produced strong antibody and cell-mediated responses to paramyosin, but failed to induce the level of protection conferred by the recombinant protein (Liu *et al*. 2008).

Earlier experiments in our laboratory with the DNA vaccine construct of *B. malayi* Abundant Larval Transcript-2 (1020-BmALT-2) showed only partial protection in mice (30%) as a single antigen vaccination (Ramachandran *et al*. 2004). Recent studies with BmALT2, in our laboratory, have shown that protein vaccination conferred approximately 75% protection compared to DNA vaccination that conferred only 57% protection (Thirugnanam *et al*. 2007). Previously, we have shown that rBmTGA protein conferred 30% protection in jirds against challenge with L3 larvae and that rBmTGA in combination with recombinant *B. malayi* thioredoxin peroxidase (rBmTPX) protein (BmTGA + BmTPX) conferred 73% protection in jird parasite challenge studies (Uma *et al*. 2009). BmTGA DNA vaccination can induce both humoral as well as cellular immune responses to filarial antigens in mice, but confers only partial protection upon subsequent larval challenge, suggesting that apart from the usual humoral and cellular immune responses, some other co-stimulatory molecules and transcription factors may be involved in protection and need to be further investigated. One possible approach would be the inclusion of certain murine cytokine DNA constructs to enhance the immune responses that contribute to protection. Anyhow, it is realized that much more work is needed before practical DNA vaccines for filariasis become reality. In addition, more work is needed to optimize vaccination protocols, and many other candidate antigens need to be tested and the refinement of DNA vaccines needs to be carried out to effectively prime the protective immune responses for human lymphatic filariasis.

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