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Immunoprophylactic potential of filarial glutathione-s-transferase in lymphatic filariaisis

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ABSTRACT

Objective: To elucidates the immunoprophylactic potential of glutathion-s-transferase (GST) from cattle filarial parasite *Setaria digitata (S. digitata)* against lymphatic filariasis. **Methods:** GST was purified through affinity chromatography (SdGST) and chacterized by SDS-PAGE and Nano-LC MS/MS analysis. Antibody isotypes to SdGST were measured by ELISA. Antibody dependant cellular cytotoxicity (ADCC) was performed *in vitro* using sera from immunized animals and immune individuals. T-cell proliferation and cytokine response to SdGST in different groups of filariasis were measured. Immunoprophylactic potential of SdGST was evaluate in animal model. **Results:** SdGST exhibited 30-fold enhancement of enzyme activity over crude parasitic extract. It was found to be 26 kDa by SDS-PAGE. Nano LC-MS/MS analysis followed by blast search showed 100% homology with *Dirofilaria immitis (D. immitis)* and only 43% with *Homo sapiens (H. sapiens)*. Immunoblotting analysis showed putatively immune individuals carry significant level of antibodies to SdGST as compared with microfilaraemics. Immunized sera and sera endemic normal could neutralize the enzymatic activity of SdGST and inducing *in vitro* cytotoxicity of microfilariae. Peripheral blood mononuclear cells (PBMC) from endemic normals upon stimulation with SdGST showed a mixed type of Th1/Th2 response. SdGST immunization clear microfilariae from circulation in *S. digitata* implanted mastomys. **Conclusions:** The heterologous GST could be potentially developed as a vaccine candidate against lymphatic filarial parasite.

1. Introduction

Lymphatic filariasis is an infectious disease caused by *Wuchereria bancrofti (W. bancrofti)*, *Brugia malayi (B. malayi)* and *Brugia timori (B. timori)*. It affects 120 million people in 73 countries and more than a billion are at risk of infection[1]. It is considered as a major obstacle to economic development in endemic countries and identified as the second leading cause of permanent and long-term disability in developing countries including India[2]. The main problem regarding the chemotherapy of filariasis is that no safe and effective drug is available yet to combat the adult human filarial worms. In spite of all the advances in chemotherapy and vector control, still there is need of an effective vaccine candidate against filariasis. The filarial parasites have many developmental stages in their life cycle, which poses a major setback in developing an effective vaccine. A number of vaccine studies were found to be effective in inducing protection against filarial infection to a certain extent[3,4]. However, studies on the use of purified native antigen in immunoprophylaxis are scanty which might be more useful in achieving the desired result. Filarial parasite survives for years inside hostile environment of the mammalian host without any damage. This is mostly possible due to very strong antioxidant system to evade the host immunity and helped them in there prolong existence. If host antibodies can block the function of parasite antioxidant secretions, the secreted antigens may be critical in the formulation of future vaccines against parasitic infections. Glutathione-s-transferase (GST) may be one of the major phase-II detoxification enzymes in helminthes and its ability to effectively neutralize cytotoxic products arising from reactive oxygen species attack on cell membrane provide evidence that GST have potential to protect the parasite against the host immune responses[5]. GST has been well documented as a promising vaccine candidate against a number helminthes disease like fascioliasis and schistosomiasis[6,7]. Mice immunize with *Fasciola gigantica* GST26 could confer protection against challenge infection[8]. Some studies have also been shown the role of GST in human and experimental filariasis[9-11]. Recombinant WbGST showed its potential as a vaccine candidate by giving 61% protection against *B. malayi* challenge infection in jird model[12]. In onchocerciasis

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recombinant GST has been used as a vaccine candidate but with limited success. All these studies suggest that parasitic GST may be primary target for vaccine development in helminthes as the worm GST and Human GST are structurally different[13]. Further native antigen and recombinant antigen differ in their structure and immunological property. For these reasons, the present study has been focused on the use of native purified GST from *Setaria digitata (S. digitata)* (SdGST). We characterized the native SdGST; evaluate its humoral, cellular immune response and immunoprophylactic potential in experimental and human filariasis.

2. Materials and methods

2.1. Parasite collection and purification of SdGST

Adult *S. digitata* was collected from the peritoneal cavity of cattle of local slaughterhouse. The parasites were homogenized at 4 ℃ in 22 mmol/L potassium phosphate buffer pH 7.0 containing 1 mmol/L EDTA and then centrifuged at 12 000 g for 20 min. The resulting supernatant was used for purification of SdGST. SdGST was purified by affinity chromatography using Glutathione-Agarose column (Sigma, USA) as described previously[9]. Briefly, the column was equilibrated with several column volumes of the equilibration buffer PBS (10 mmol/L phosphate buffer pH 7.4, 150 mmol/L NaCl) before loading the clarified supernatant of adult worm extract. The bound protein was eluted with 10 mmol/L glutathione in 50 mmol/L Tris-HCl pH 9.6. The eluting fractions were neutralized to pH 7.0 by adding 2 mol/L Tris pH 6.0, dialyzed against PBS and stored in −70 ℃. The GST enzymatic activities of the purified fraction were measured spectrophotometrically at 340 nm with reduced glutathione and 1-chloro-2,4-dinitrobenzene as substrate. The specific activity was expressed as μmol· min^{-1} (mg protein)$^{-1}$ using an extinction co-efficient 9.6 $mM^{-1}cm^{-1}$[14].

2.2. SDS-PAGE and nano LC-MS/MS

Purified SdGST was electrophoretically separated by 12.5% sodium-dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) under non-reducing condition. GST was loaded at a concentration of 5 μg/well. The resolved antigen was electrotransfered onto nitrocellulose membrane (Millipore) by semidry blot (Bio-Rad). The protein blots were blocked with 2% BSA in PBS for 1 h at room temperature and cut into 5 mm strips. Each strip was washed and incubated separately with 5 mL of test serum (1:50) for 2 h in a flat rotary shaker at 4 ℃. After washing, each strip was incubated with peroxidase-conjugated anti-human IgG (1:500) for 2 h at room temperature in a flat rotary shaker at 4 ℃. The strip was washed and the color was developed with diaminobenzidine substrate (Sigma-Aldrich, USA). The Nano LC MS/MS analysis of SdGST was carried out at the center for genomic application (TCGA), New Delhi. Briefly, First the purified SdGST was digested with trypsin before apply to LC-MS/MS. Mass spectral data obtained from LC-MS/MS were submitted to database searching using MS/MS ion search (matrix science). Search parameter allowed a maximum of one miss cleavage, fixed modification is carbamidomethyl; the possible oxidation of methionine, peptide mass tolerance is ±2 Da and fragment mass tolerance is ±4 Da.

2.3. Blood sample collection

The study was carried out in villages of Khurda district of Orissa, India, which are known to be highly endemic for *W. bancrofti* infection[15]. Informed consent for participation was obtained before subjects were included in the study. Blood samples (5 mL) were collected by anti cubical veni-puncture between 20:30 h to 23:30 h from interested individuals in sodium heparin for cell culture experiments and a part of the sample was collected directly without any anti-coagulant for serum preparation for antibody assay. A thick film of 50 μL of collected blood was examined for parasitological detection of microfilaria with Giemsa stain. Based on clinical and parasitological criteria, donors were classified as (i) Chronic filariasis (CP), individuals exhibiting elephantiasis and/or hydrocele (ii) Asymptomatic microfilaraemic carriers (AS), microfilaraemic carriers without any clinical symptoms (iii) Endemic normals (EN), permanent residents of the regions who are circulating filarial antigen negative and free from infection as judged clinically and parasitologically. Sera were also collected from normal people of non-filarial region (NEN) with similar socio-economic background. These sera were used as non-endemic normals for serological comparisons for determining the positivity (>mean+3 SD). Detection of circulating filarial antigen (CFA) in filarial sera was carried out using Og4C3 test kit (JCU Tropical Biotechnology, Townsville, Australia) according to manufacturer's instruction. The Indian Council of Medical Research and the review boards of the human ethical committee of the Regional Medical Research Centre approved the study.

2.4. Enzyme-linked immunosorbent assay (ELISA)

IgG antibody isotypes to GST were determined in individuals living in areas endemic for filariasis by enzyme linked immunosorbent assay as described previously[16]. Briefly, ELISA plates were coated overnight at 4 ℃ with 2 μg/mL of purified SdGST in bicarbonate buffer pH 9.2. The plates were saturated with bovine serum albumin (0.5%) at 37 ℃ for 1 h and then incubated for 3 h at 37 ℃ with diluted serum samples (1/50 in Tween-20 (0.1%) in phosphate buffered saline, PBS). After washing with PBS-Tween, 100 μL of mouse monoclonals of anti human IgG1, IgG2, IgG3 and IgG4 (1:1 000 fold, Sigma, USA) were added to the wells and kept at 37 ℃ for 1 h and then at 4 ℃ overnight followed by a 2 h incubation with peroxidase conjugated antimouse Igs (1:2 000, Sigma) at room temperature. After washing with PBS-Tween, the presence of antibodies was detected with OPD substrate (o-phenelyne diamine containing H_2O_2). Adding a drop of 2 mol/L sulphuric acid stopped the enzymatic reaction and the absorbance was read at 492 nm using an ELISA reader (Bio-Rad). The antibody positivity was calculated in comparison to a panel of non-endemic normals (>mean OD 492 + 3 SD of the mean).

2.5. Immunization of mastomys with SdGST

Mastomys (n=3) were immunized intraperitoneally with SdGST (25 μg/animal) in PBS with equal volume of Freund's complete adjuvant and subsequent two injections were given 15 d apart in Freund's incomplete adjuvant. The last injection was given intravenously in saline as booster dose. Control group of animals received only saline emulsified

in Freund's adjuvant as described above. A weak after last dose of antigen, the animals were bled from retro-orbital plexus and immune sera were isolated.

2.6. Inhibition of GST activity by filarial sera

The effect of filarial sera and immunize sera on the GST activity was carried out following the method developed for schistosomiasis[17]. Briefly, purified SdGST (5 μg) was incubated with 20 μL of sera for 2 h at 37 ℃ and then 4 ℃ for 4 h. Sera used in this assay were pooled sera of 20 individuals of each filarial group. The immune sera are from three SdGST immunized animals. After incubation residual enzyme activity was measured as described in the purification section and the percentage inhibition of activity relative to control was calculated.

2.7. In vitro antibody dependent cellular cytotoxicity

ADCC assay was performed *in vitro* to determine the cytotoxic effects of anti-SdGST antibodies against *S. digitata* MF as described by Chandrasekhar *et al*[18]. Sera samples used in this assay were pooled from EN individuals and SdGST immunized *Mastomys*. Pre-immune and NEN sera samples served as negative control. PEC cells were collected from normal mastomys by washing peritoneal cavity and PBMC's collected from NEN individuals. Briefly, 50 Mf in 50 μL of RPMI-1640 were incubated with 50 μL of peritoneal exudates cell (2×10^3 cell) and 50 μL of sera in final volume of 200 μL. The sera were tested in triplicate in 96 well culture plate. The plate was incubated in 5% CO_2 incubator at 37 ℃ for 48 h and 72 h. Cellular adherence and cytotoxicity was determined under light microscope by considering the larval viability. Results were expressed as the ratio of immobile or dead parasite to the total number of parasite within each experiment.

2.8. Cytokine production and proliferation assays

Peripheral blood mononuclear cells (PBMC) were prepared by density gradient centrifugation on Ficoll-Hypaque from heparinized venous blood and re-suspended in RPMI-1640 supplemented with 10% FCS, 4 mmol/L glutamine and penicillin. PBMC were cultured in triplicate at a concentration of 2×10^6 cells/mL in a total volume of 1 mL. ConA (5 μg), SdGST (5 μg) was added separately in 96 wells (U-bottom) tissue culture plate. Control group of cells was cultured without any antigen. The plates were incubated in humidified environment (5% carbon dioxide, 80% humidity) at 37 ℃ for 72 h. A set of wells was used for harvesting culture supernatants for cytokine production and another set was used for T-cell proliferation assay. Then the culture supernatants were harvested, centrifuged and the supernatants were frozen at -70 ℃ for estimation of cytokine. Cytokine was quantified by sandwich ELISA using IFN-γ, IL-2, IL-4 (Peprotech Inc), IL-5 (e Biosciences) kits and was expressed in picograms per milliliter by interpolation from standard curves.

The proliferative response was measured by adding 3H-thymidine (1 μCi/well) for an additional 18 h before harvesting. The cells were harvested by using a cell harvester. Amount of thymidine uptake was measured using a β counter (Beckman coulter). The stimulation index (SI) of stimulated PBMCs was calculated using the formula, SI=mean CPM (counts per minute) of experimental wells/mean CPM of unstimulated culture wells. Individuals showing a SI>2 were taken as responders.

2.9. Immunization and challenge infection

S. digitata-Mastomys coucha model was developed in our animal facility as described previously[19]. Experiments were performed in three sets of mastomys. In the first set of experiment, immunization before implantation, *Mastomys* of either sex (n=8) were immunized by intra peritoneal route with GST antigen in two doses (75 μg each without adjuvant) at 15 d interval. Worms were implanted in the peritoneal cavity of the *Mastomys* 10 d after the last immunization. In the second set of experiment, immunization after implantation, *Mastomys* (n=8) were implanted with the parasites and then immunized twice by intra peritoneal route with GST antigen (75 μg/mastomys without adjuvant) at 7th and 22nd day of post implantation. In the third set of experiment, control animals (n=8) were implanted with parasites and received saline alone. ELISA determined the anti-GST antibodies before implantation in GST immunized experimental groups. In a separate set of experiments, *Mastomys* of either sex (n=4) were immunized by intra peritoneal route with tetanus toxoid (non filarial antigen) in two doses (75 μg each without adjuvant) at 15 d interval. Worms were then implanted in the peritoneal cavity of the *Mastomys* 10 d after the last immunization. Control group of animals (n=4) for the non-filarial antigen were implanted with parasites and received saline alone.

2.10. Statistical analysis

For animal experimentation, comparison of groups was done with the non-parametric Mann-Whitney U-test. P values<0.01 were considered significant. Comparison of T-cell proliferative response was done using Fischer's exact test. Unpaired t-test was used for comparison of antibody response, cytokine secretion among different group of filarial patients and cytotoxicity by different sera. P value<0.05 was considered as significant.

3. Results

GST purified from *S. digitata* (SdGST) by glutathione-agarose column exhibited 30-fold enhancement of enzyme activity over crude parasitic extract. SDS-PAGE analysis of purified SdGST revealed a prominent band at 26 kDa (Figure 1A). Antibody recognition pattern to SdGST was checked by ELISA and immunoblotting assay. On closer examination of immunoblotting analysis showed that anti SdGST IgG antibody recognized a distinct band with pooled sera of EN and CP. However the recognition of band intensity was very less in AS sera and sera from non-endemic normal individuals could not recognize the band (Figure 1B). Seroreactivity to SdGST for IgG isotypes was evaluated by ELISA in individual subjects. Anti-SdGST antibodies in sera of EN individuals were predominantly IgG1 and IgG3, whereas IgG2 isotype was predominant in AS individuals. Interestingly the anti SdGST IgG4 antibodies were significantly elevated in both EN and CP individuals (Figure 2).

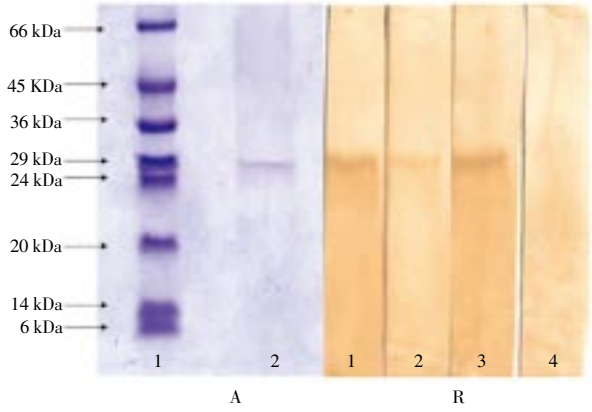

Figure 1. A. SDS-PAGE analysis of SdGST on 12.5% gel and stained with coomassie brilliant blue 250. Lane-1: molecular weight marker proteins. Lane-2: purified SdGST. B. Immunoreactivity of SdGST with sera from different clinical groups of filariasis.

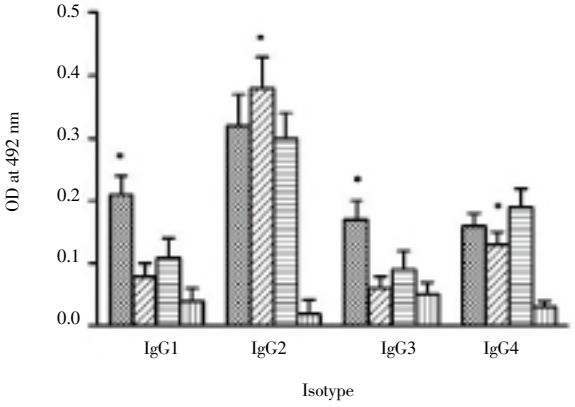

Figure 2. IgG subclass response to SdGST in the sera from different clinical groups. Data presented is mean ± SD value from 30 subjects from EN group and 18 each from AS and CP. EN (▩), AS (▨), CP(▤) and NEN (▥). *P<0.01 (Unpaired t test) compared to all other three groups.

To determine the amino acid sequence of SdGST the protein was subjected to nano-LC MS/MS and the mass spectral data analyzed through database searching of the Mascot Programme. The predicted peptide and their sequence are shown in Table 1. Basic alignment search toll was used to compare SdGST with other GST. This analysis showed that SdGST has 100% sequence identity and similarity with *D. immitis*. In contrast it showed 43% sequence identity with *Homo sapiens (H. sapiens)* and 75% with both *B. Malayi* and *W. bancrofti* (Table 2). Database also showed Phi-class of GST and did not show the existence of any other classes of GST. The sequences of GST of different parasites were derived from the database to generate the phylogeneic tree to show the relationship with SdGST. The GST of *W. bancrofti* and *B. Malayi* are more likely same as compared to *D. immitis* which formed a sister clade. SdGST being most similar with *D. immitis* GST also have common origin with human filarial parasite GST. However *H. sapiens* and *Rattus* formed a completely different clade with that of SdGST (Figure 3).

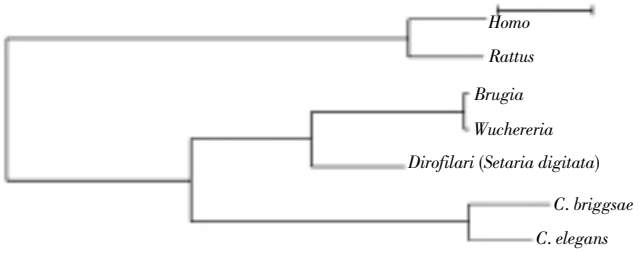

Figure 3. Phylogenetic tree of parasitic GST.
Closely related GST from other parasites is included for comparison.

To evaluate the functional property of antibodies against SdGST shown by the immune individuals, we perform a neutralization assay. Sera from endemic normals, chronic filariasis and SdGST immunized *Mastomys* significantly inhibited the GST activity up to 65%, 69% and 78% respectively. But sera from microfilaraemic groups showed a marginal inhibition upto 21%. In contrast NEN sera and control mastomys sera could not show inhibitory effect on the enzymatic activity of SdGST. The *in vitro* serum dependent cellular cytotoxicity assay showed that anti-SdGST sera from immunized *Mastomys* promoted adherence of PEC to MF and inducing 58% and 82% of cytotoxicity in 48 h and 72 h respectively (Figure 4). Similar results were obtained with sera from endemic normals, which induced 52% and 72% of death of MF in 48 h and 72 h respectively. However in control group (sera from NEN and normal *Mastomys*) 12%-27% cytotoxicity was observed with MF in 48 h and 72 h respectively.

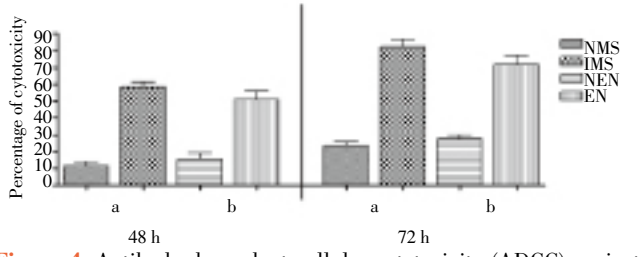

Figure 4. Antibody dependant cellular cytotoxicity (ADCC) against *S.digitata* MF by (a) immunized mice sera (IMS) and (b) immune individuals (EN) after 48 h and 72 h.

Isolating PBMC from different clinical groups of filariasis assessed the role of GST in T-cell stimulation. Proliferation of T-cell in response to SdGST (SI index>2) was more than 75% in endemic normals and chronic patients. A few (22.2%) asymptomatic microfilaraemic individuals showed T cell proliferative response to GST, which is significantly lower (P<0.01), compared to the values obtained with endemic normals or chronic patients. However SdGST unable to stimulate the T-cell from NEN sera and proliferation to ConA was almost similar in all the groups (Table 3).

GST specific secreted cytokines Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-5) were measured in T-cell culture supernatant of PBMC of various categories (Figure 5). IFN-γ production was significantly higher in EN group as compared to all other groups. However, PBMCs of different individuals from different clinical categories produced almost similar level of IL-2 to GST. Asymptomatic subjects (EN and CP) produced significantly more IL-4 compared to symptomatic subjects (CP). In contrast AS produced significantly more

Table 1

MS analysis of the tryptic peptide generated from GST of *S. digitata*.

Start – End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Peptide sequence
12 – 18	378.543 6	755.072 7	754.433 7	0.639 0	0	R.GLAEPIR.L(Ions score 20)
136 – 146	610.773 6	1 219.532 7	1 219.619 7	–0.087 0	1	R.DNGKNFILGDK.I(Ions score 32)
140 – 146	403.653 6	805.292 7	805.433 4	–0.140 7	0	K.NFILGDK.I(Ions score 30)
140 – 146	806.320 0	805.312 7	805.433 4	–0.120 7	0	K.NFILGDK.I(Ions score 56)

IL-5 to GST than EN group.

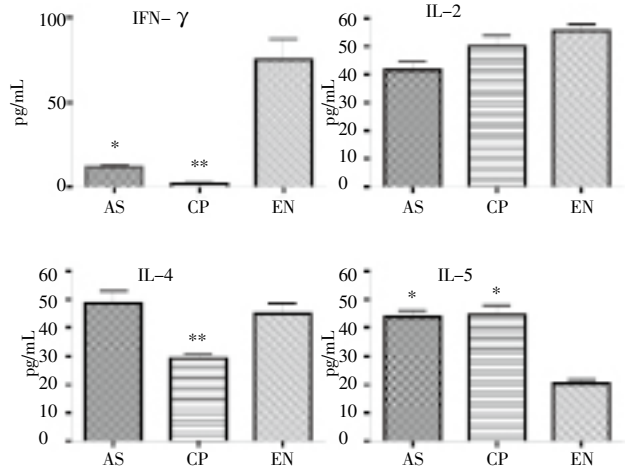

Figure 5. Cytokine responses in T-cells upon stimulation with SdGST in different clinical groups of filariasis.

AS (▩), CP (▤) and EN (▨). Statistical significance was determined using GraphPad Prism software by unpaired *t*-test with IFN-γ. **P*=0.002, ***P*=0.001, compared with EN group. IL-4 with ***P*=0.002 compared with EN group. IL-5 with **P*=0.001 compared with EN group.

Table 2

Identity/similarity of SdGST with other parasitic GST.

S. digitata GST	Identity (%)	Similarity (%)
D. immitis GST	100	100
B. malayi GST	75	88
W. bancrofti GST	75	88
C. briggsae GST	58	73
C. Elegans GST	57	73
R. norvegicus GST	46	61
H. sapiens GST	43	61

The *S. digitata* - *Mastomys* model was standardized in order to study the clearance of microfilaria following GST immunization. Microfilaria was detected in the peripheral blood on day 4 of post implantation. It was observed that microfilaria could persist in the circulation up to day 160 of post implantation with a peak on day 25 in the non-immunized control group of *Mastomys*. In group-I immunization with SdGST resulted in the Induction of anti-GST antibodies (mean ± SD) was 0.87 ± 0.22 at the time of implantation. The microfilaria (mean ± SD) count on day 25 was observed to be 13.3 ± 2.6 in immunized animals in contrast to the control group of Mastomys (36.2 ± 6.3) (Figure 6A). Reduction of Mf density (about 7%) was observed in immunized group *vs*. 180% increase in non-immunized control animals on day 25 as compared to day 7. In immunized animal Mf density was declined by about 52% on 45 d, 57% on 55 d and 78% on 75 d and a total clearance by about 90 d of post implantation. The microfilaria density was more than 100% till 85 d in non-immunized control animals. The effect of immunization with GST antigen was studied in animals with ongoing microfilaraemia (Figure 6B). Intraperitoneal immunization with GST antigen resulted in suppression of Mf density and a total clearance by 115 d. Anti-GST antibody level (mean OD ± SD) was observed to be 0.65 ± 0.18 and 0.09 ± 0.02 on day 35 in immunized and control group of *Mastomys* respectively. The antibody level was found to be maintained in immunized animals till day 115 (0.53 ± 0.14). The reduction in MF density was marked after day 35, resulting in a sharp decline. Significant reduction in MF density was observed about 32%, 60% and 71% of the original microfilaraemia by 55, 75 and 85 days respectively. Immunization of tetanus toxoid antigen (non filarial antigen) before implantation of worms could not induce any effect on microfilaria clearance or microfilaria density (data not shown).

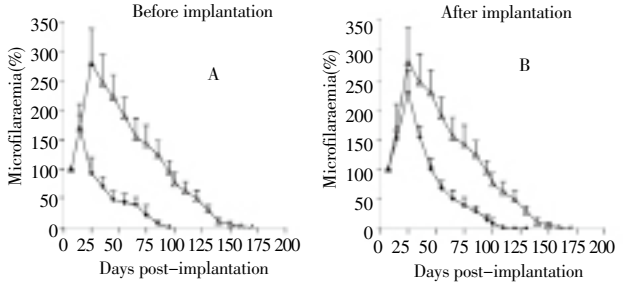

Figure 6. Effect of immunization with SdGST antigen on microfilaraemia in *S. digitata*-implanted *Mastomys coucha*.

(A) group I, animals immunized with 2 doses of GST antigen before implantation of worms (■, *n*=8); group II, normal microfilaraemic controls (△, *n*=8). The Mf density on day 7 was taken as 100% for each of the animals. group I *vs*. group II, *P*<0.01 on day 25, 35, 45, 60 and 75. (B) Immunization after implantation. group I *vs*. group II, *P*<0.01 on day 35, 45, 60 and 75.

4. Discussion

With the development in vaccination strategies and understanding host immune responses against helminthic diseases, it is now easier to think about vaccine against lymphatic filariasis when drugs are failing to combat the situation. In this connection GSTs could be consider a potential candidate since it plays a crucial role in parasite survival. GSTs are extensively investigated as vaccine candidate against several parasitic infections[10,20,21]. The present study describes the characterization of SdGST and evaluates its immunoprophylactic potential in experimental filariasis and as well as in human filariasis. Results from this study showed that adult parasite of *S. digitata* contain substantial GST activity and migrated as a major component of 26 kDa in SDS-PAGE analysis. The presence

Table 3

T cell proliferative response to ConA and SdGST in different spectrum of filariasis.

Group	*n*	ConA		GST	
		+*n*	positive(%)	+*n*	positive(%)
Endemic normals	55	54	98.2	42	76.4
Asymptomatic microfilaraemics	18	15	83.3	4	22.2*
Chronic filariasis	18	15	83.3	14	77.8

The stimulation index (SI) of stimulated PBMCs was calculated using the formula, SI=mean CPM of experimental wells/mean CPM of control wells. Individuals showing a SI>2 were taken as responders. *significantly lower (P<0.01, using Fischer's exact test) compared to EN and CP group.

of GST in parasite is critical because parasite GST behave like mammalian GST in neutralizing oxidative radical and counteracts host mediated oxidative stress. The GST of *W. bancrofti*, *Fasciola hepatica* and *Schistosoma mansoni* are all belongs to Phi class and our current study support these findings that SdGST also belongs to the same class[12,22,23]. Protein sequencing and blast search of SdGST showed that the purified GST was different from human GST having only 43% homology. It also showed a remarkable identity with other human filarial GST. So this heterologous purified native worm GST may be used to evaluate its protective potential against human lymphatic filariasis in context of cost, convenience and abundance. This is further supported by the immunoblotting analysis which showed that immune individuals carry significant levels of anti-SdGST antibodies compared to microfilaraemics which emphasizes antibody to SdGST are generated due course of natural infection. The intensity of the band decreases with the onset of microfilaraemia. Again EN and CP group are immunologically show similarity but at the two extreme end of filarial spectrum. Endemic normals are a group of individuals in an endemic area; carry high levels of antibody against parasite antigens, which are believed to be protective[24]. It is speculated that high antibody titer against GST in EN may have some role in keeping these individuals free from infection. In addition, the predominance of cytophilic antibodies like IgG1 or IgG3 to SdGST in putatively immune individuals (EN) may have a role in protection against human filariasis. Similar type of response was observed in EN individuals in response to rBmALT, which is a candidate vaccine antigen in filariasis[25]. Interestingly EN individuals also showed a significant increase in anti-SdGST IgG4 antibody similar to AS and CP individuals. So putatively immune individuals showed Th2 type of biased antibody response whereas microfilariaemic individual showed IgG2 antibody against SdGST, which is a Th1 type of response. Sera (NEN) collected from individuals from non-endemic region were negative for any isotype indicating the filarial specificity of GST.

The inhibition of enzymatic activity of SdGST by immunized sera and sera from EN and CP individuals signified the specificity of antibodies with this antigen whereas control sera were unable to neutralize the activity. However, absence of 100% inhibition of GST activity by these sera suggested that there is no correlation between titer of GST neutralization antibodies and protection against parasitic infection[20]. Yet immunization of *S. mansoni* GST gives protection by inactivating the GST activity and anti-*S. mansoni* 28 GST IgA antibodies can impair *in vitro* hatching of *S. mansoni* eggs[17]. Previously it has been shown that sera from *W. bancrofti* endemic area have the capability to neutralize parasite GST function[9,12]. So the result of the present study with the support of previously documented findings suggested that antibody-mediated neutralization of the GST enzymatic activity could render the parasite vulnerable to toxic products generated by immune attack on the tegument, as they lack other detoxification mechanism. It also emphasizes that the high tittered antibody raised against SdGST in immune individuals may have adverse effect on parasite survival and makes these people to remain uninfected Our *in vitro* results clearly show that anti-SdGST antibodies are participate in the killing of microfilariae. Thus binding of anti-SdGST antibodies to the parasite that promotes cell-mediated cytotoxicity is critical for the SdGST induced protection against lymphatic filariasis inection.

SdGST induced a strong proliferative response in endemic normals and chronic patients in comparison to asymptomatic microfilaraemics (hypo-responsiveness)[26]. Our result suggested that not only strong Th1 like response indicated by the production of IFN-γ but also a Th2 associated cytokine (IL-4) response in putatively immune individuals (EN). The mixed response of Th1 and Th2 showed by the PBMC of endemic normals upon stimulation with GST is in agreement with the report where GST used as a vaccine candidate in schistosomiasis[27] and hookworm infection[28]. PBMC from endemic normals were shown to display a mixed Th1 and Th2 response to *Onchocerca volvulus* antigen[29]. Immunity against adult worms (as shown by EN individuals) is apparently Th1 and Th2 type is supported by animal experimentation carried out using KO mice[30].

Active immunization with SdGST antigen either before or after implantation caused rapid clearance of microfilaria in *S. digitata* implanted *mastomys*. The circulating Mf in immunized group were found to get cleared very rapidly in comparison to control group animals, indicating that microfilaria clearance *in vivo* was significantly potentiated by antibodies to GST. Immunization of tetanus toxoid antigen (non filarial antigen) before implantation of worms could not induce microfilaria clearance and suppress the microfilaria density, suggesting microfilaria clearance was not due to the non-specific stimulation of the immune system. The antibody level was found to be maintained in immunized animals till day 115 and inversely correlated with MF density. It is speculated that induction of humoral response could be due to the antigenicity of SdGST, which reduces the microfilarial load in immunized group. Previous reports also support our findings that vaccination with parasitic GST reduced the adult worm burden in fascioliasis and filariasis[11,31]. Recently, a vaccine trial study with recombinant GST from *Nectar americanus* showed 32 and 39% reduction in adult worm burden following larval challenge[28]. Our results support and are in agreement with previous studies that parasite GSTs are potential immunoprophylactic candidate in filariasis.

In conclusion, results of this study showed that putatively immune individual carry antibodies to SdGST, which neutralize parasite GST activity and kill the microfilariae. Immunizations with SdGST clear microfilariae from circulation in experimental filariasis indicating its immunoprophylactic potential in filariasis. Further WbGST and SdGST resemble closely and considerably cross reactivity exist between the two species. So the heterologous GST can be used as vaccine candidate against lymphatic filariasis in future.

Conflict of interest statement

We declare that we have no conflicts of interest.

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