A study of a possible new vaccine candidate rWbL2 in Lymphatic filariasis in mastomys; an animal study.

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Abstract :

Lymphatic filariasis is a major health problem caused mainly by three lymph dwelling parasites; Wuchereria bancrofti, Brugia malayi and Brugia timori. Since long it has been cause of clinical morbidity in tropical and sub-tropical countries. Currently there is no vaccine available to prevent this infection. For complete elimination of filariasis, vaccine could complement the existing therapeutic and vector control methods. In the present study, in an attempt to explore the efficacy of a vaccine candidate, we have shown that the mastomys, which are fully permissible rodents for Brugia malayi infection when immunized with Wuchereria bancrofti recombinant SXP/RAL-2 family protein L2 (rWbL2) could induce 51.25% in situ cytotoxicity against Brugia malayi infective (L3) larvae. The protection was characterized by higher levels of WbL2- specific IgG1 and IgG2a antibodies and significant levels of IFN-γ, IL-4 and IL-10 cytokines The findings indicate that immunization of mastomys with rWbL2 could impart protection against filarial infection.

Key words : Wuchereria bancrofti, Brugia malayi, SXP/RAL-2 family protein WbL2, vaccine, mastomys

Introduction : Lymphatic filariasis (LF) considered as one of the neglected tropical diseases (NTD) is still a public health problem in India.It is endemic in 17 states and 6 union territories. Wuchereria bancrofti and Brugia malayi are the two important parasite species thatcauses the infection. The former accounts for almost 95% of the disease burden in the tropical countries, while the latter is responsible for most of the remaining 5% of cases.. The load of lymphatic filariasis in India is so high that it accounts for 40% of global prevalence of the infection[1]. The total disability adjusted life years (DALYs) lost in India due to this disease is around 2.06 million, resulting in an annual wage loss of US $811 million[2]. It is estimated that there are approximately 120 million cases of lymphatic filariasis around the world and over one billion people are at risk of getting infected with this disorder[3]. To deal with such a high burden of lymphatic filariasis, World Health Organization has already introduced the 'global programme to eliminate lymphatic filariasis (GPELF) by 2020' to limit transmission of filariasis by means of mass drug administration (MDA) and thus restricting the overall incidence of lymphatic filariasis[4].

However, there are many possible limitations in the presently available tools; specially MDA, as it requires several rounds of drug administrations, hence non-compliance and development of drug resistance to curative drugs may become a critical problem, thus affecting the eradication of disease from endemic and crowded areas[5]. The above limitations in mass drug delivery has been hindering to achieve the goal of eradication of transmission in the endemic and highly populated areas like India. Therefore, to tackle lymphatic filariasis, there is intense need of some preventive tools like developing vaccines against filariasis. Earlier studies also have shown the utilization of recombinant proteins as vaccine candidates to combat the lymphatic filariasis [6,7,8,9,10].

A nematode gene was identified that encodes a new member of the SXP/RAL-2 family protein. An SXP/RAL-2 family protein, WbL2 is a Wuchereria bancrofti L3 larval protein. The WbL2 gene is specifically expressed in the subventral pharyngeal glands and the protein is most likely secreted[11]. Another member of SXP/RAL-2 family, Ac-16 an immunodominant surface antigen of Ancylostoma caninum has already been successfully tested as a vaccine candidate, induced significant protection against hookworm infection in dogs[12]. In the present study, we have assessed and evaluated the immunogenicity and protective efficacy of WbL2 in fully permissible filarial animal model Mastomys coucha and further studied the humoral, cellular and cytotoxic responses associated with the induced protection due to immunization.

Materials and Methods :

Experimental animals and B. malayi parasites

Multi-mammate mastomys (Mastomys coucha) of 6–8 weeks of age, also known as swiss rats, bred and maintained in the animal house facility of our institute registered with Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India , were used in this study. The animals were maintained under standard laboratory conditions with free access to animal chow and drinking water ad libitum and all the surgical procedures were

performed under the strict aseptic conditions. All the experiments were approved by the Institutional Animal Ethics Committee.

B. malayi infective stage (L3) larvae used in this study were acquired using Baermann's technique[13] by the method described previously[5]. Four days old Aedes aegypti mosquitoes were fed with the blood of mastomys infected with B. malayi and dissected after two weeks to recover L3 stage larvae.

Recombinant protein Wuchereria bancrofti L2 (rWbL2) :

The Escherichia coli BL21 bacterial cells containing pRSET-B-WbL2 construct was grown at 370C to A600, 0.6 in LB medium containing suitable antibiotics. The expression of recombinant protein was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (1 mM; Merck Millipore, Bengaluru, Karnataka, India). The recombinant protein was purified using a nickel affinity chromatography column (Thermo Fisher Scientific, Mumbai, Maharashtra, India) and the protein content was estimated using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Mumbai). Endotoxin contamination was checked by a quantitative LAL chromogenic endotoxin quantitation kit and the endotoxin content was found to be within permissible limits (Thermo Fisher Scientific, Mumbai).

Immunization of experimental animals with rWbL2

Five mastomys (n = 5 in each group) each were divided under two groups, first one as: rWbL2 group (mastomys immunized with rWbL2 in alum adjuvant) and second one as Alum control group (mastomys administered with alum alone). Immunization of mastomys with rWbL2 consisted of three intraperitoneal doses of rWbL2 (15 µg / dose in 200 µl of alum adjuvant) administered at 15 days intervals followed by one booster dose. Animals in alum control group received four doses of alum adjuvant only. Ten days after the final dose of immunization, the sera were collected from each mastomys through caudal vein puncture and tested for the presence of anti-WbL2 antibody levels.

Analysis of anti-WbL2 antibody titres in the sera of mastomys

The levels of total anti-WbL2 Immunoglobulin (Ig)-G antibody and IgG antibody isotypes (IgG1, IgG2a, IgG3 and IgG4) were determined in the sera samples of mastomys using an indirect ELISA[14-15]. Immuno plates of 96 wells (Thermo Fisher Scientific, Mumbai) were coated with rWbL2 protein (100 ng /100 µl / well) in carbonate-bicarbonate buffer (100 mM, pH 9.5) and incubated overnight at 40C. The wells were washed once with PBS/T (0.05 M PBS containing 0.05% of tween 20, pH 7.2) and blocked by BSA (2% in PBS, 300 µl/well) for 1 h at 37°C. After washing thrice, the optimally diluted sera samples (diluted in PBS) were added and incubated for 1 h at 370C. After washing the wells for five times, the bounded antibodies were detected by addition of HRP conjugated goat anti-mouse IgG (1:10000) or IgG1 (1:1000) or IgG2a (1:15000) or IgG3 (1:5000) or IgG4 (1:15000) antibodies (diluted in PBS; Thermo Fisher Scientific, Mumbai). After incubation of 45 minutes at 37°C, the colour developed was recorded by measuring absorbance at 450 nm using spectrophotometer (Biotek India, Mumbai).

Depletion of anti WbL2 antibodies from sera

Anti-WbL2 antibodies in the sera of immunized mastomys were depleted by passing the sera over rWbL2 coupled to Cobalt IMAC resin (Thermo Fisher Scientific, Mumbai). 1 mg of his-tagged rWbL2 was coupled to the resin, washed and incubated overnight at 40C with about 200 µl of neat sera. Supernatant was collected by centrifuging the resin mixture for 2 min at 750 rpm. Depletion of anti-WbL2 antibodies were confirmed using an ELISA as described above.

In vitro antibody dependent cellular cytotoxicity assay (ADCC)

The cytotoxic effect of anti-WbL2 antibodies against B. malayi L3 larvae was determined by in vitro cytotoxicity assay by the method described previously [8,16-17]. Pooled sera samples from immunized mastomys before and after depletion of anti-WbL2 antibodies were used in this assay. The ADCC assay was performed by adding about 20 L3 larvae of B. malayi to a suspension of peritoneal exudates cells (PEC; 2 x 105 cells / well in 100 µl of RPMI medium) collected from normal mastomys. Sera samples (50 µl) were added to respective wells and the final volume of each well was adjusted to 200 µl by addition of RPMI medium in 96 well tissue culture plate (Thermo Fisher Scientific, Mumbai). After incubation (48 h at 37˚C in 5% CO2), larval viability was determined under a light microscope. The larvae were considered as dead if they appeared limpid and straight with no movements. Percentage of cytotoxicity was expressed as the ratio of number of immobile or dead larvae to the number of larvae recovered within the experimental period.

In situ cytotoxicity against L3 larvae in immunized mastomys

In situ cytotoxic response against B. malayi L3 larvae in immunized and control groups of mastomys was analysed by micropore chamber technique as

described previously [18]. Briefly, micropore chambers (Diffusion chamber with hole; Millipore India, Bangalore, Karnataka, India) containing about 20 live and infective larvae in RPMI-1640 medium were implanted into the peritoneal cavity of experimental mastomys under the effect of anaesthesia (ketamine). After 48 h of implantation, the mastomys were killed and the chambers were taken out from the peritoneum, washed in normal saline and the contents were removed onto a glass slide and examined microscopically for cell adherence and cytotoxicity. The percentage of cytotoxicity was expressed as mentioned above.

In vitro assessment of splenocytes proliferation and cytokines in culture superntant of splenocytes

The spleens were aseptically removed from the mastomys and minced in RPMI 1640 medium (supplemented with 80 µg/ml gentamicin, 25 mM HEPES, 2 mM glutamine and 10% foetal calf serum), pelleted and resuspended in erythrocyte lysis buffer (0.1% ammonium hydrochloride). Cells (0.2×106 cells / well in 200 µl of RPMI media) were plated in triplicates in 96 well flat bottom tissue culture plate (Thermo Fisher Scientific, Mumbai), then stimulated with rWbL2 (1 µg / well / 200 µl) or Concanavalin A (1 µg / well / 200 µl) (Con A; Sigma-Aldrich, Mumbai). Wells with media alone served as un-stimulated controls. After incubation (48 h at 370C in 5% CO2), cell proliferation was measured using Cell Titre 96 Aqueous non-radioactive cell proliferation kit (MTS assay; Promega, New Delhi). Cell proliferation expressed in terms of stimulation index (SI) was calculated by dividing geometric mean, GM absorbance of the cells stimulated by antigen/mitogen by the absorbance (GM) of the unstimulated cells.

Similar sets of cell cultures were placed in 24 well tissue culture plates and after the incubation for 72 h (at 370C in 5% CO2), culture supernatants were collected in separate micro-centrifuge tubes by centrifugation for the estimation of the release of interleukin (IL)-4, IL-10 and interferon (IFN)-γ cytokines using ELISA kits from Invitrogen (Mumbai) as per the manufacturer's instructions.

Statistical analysis

The statistical analysis was performed using SPSS 21.0 (IBM, India) software. The data were checked for normality assumptions. Comparison between independent means was analysed by Student's t test. P values ≤ 0.05 were considered to be significant.

Results :

High titres of anti-WbL2 antibody and isotype responses in the sera of immunized mastomys

To evaluate the immunoprophylactic effect of rWbL2, sera from mastomys immunized with rWbL2 was checked for the total IgG antibody levels by ELISA. Mastomys immunized with rWbL2 developed high levels of anti-WbL2 IgG antibodies in their sera. The sera of immunized animals showed positivity for anti-WbL2 IgG antibodies even at the highest dilution tested indicating that the end titres of anti-WbL2 antibodies in their sera were > 10,000 (Fig. 1a).

The isotype profile in the sera of mastomys immunized with rWbL2 showed significantly elevated levels of IgG1 ($p<0.05$) and IgG2a ($p<0.05$) isotype of antibodies as compared to the levels in the sera of control group of mastomys (Fig. 2b). Whereas, no significant change was observed in the levels of IgG2b and IgG3 antibodies in the sera of mastomys immunized with rWbL2 (Fig. 1b).

Figure 1a.

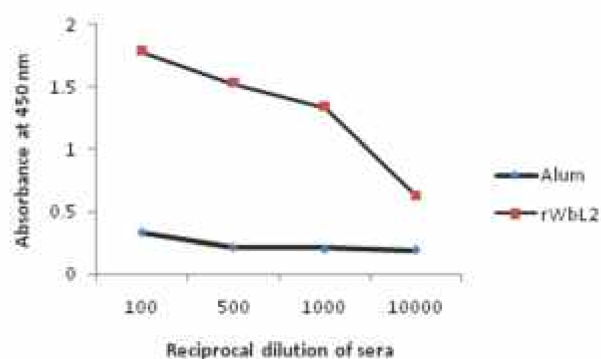

Figure 1b.

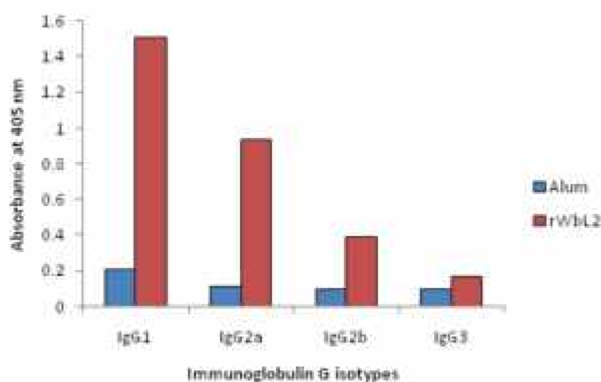

Figure 1: High titre of antibody response in the sera of mastomys immunized with rWbL2. a) Anti-WbL2IgG antibody titre in mastomys immunized with rWbL2. Each data point indicates mean of data from five animals. b) Levels of anti-WbL2 IgG isotypes in the sera of mastomys immunized with rWbL2. Bars shown are mean ± SD of data of different groups of animals (n=5 in each group). *p< 0.05 in comparison with Alum group as analysed by Mann Whitney U test.

Antibody dependent cellular cytotoxicity (ADCC) induced against infective larvae of B. malayi by the sera of immunized mastomys

Pooled sera from the immunized mastomys promoted the adherence of PEC to the infective (L3) larvae thereby inducing the significant cell mediated cytotoxicity (53.89%, p<0.05) against L3 larvae compared to the cytotoxicity induced by serum from control group of mastomys (12.93%) (Table 1). While, the depletion of antibodies from the sera of immunized animals significantly decreases the cytotoxicity (21.2%, p<0.05) induced by the antibodies (Table 1).

Table 1: In vitro antibody dependent cellular cytotoxicity induced against B. malayi L3 larvae by pooled sera of mastomys immunized with rWbL2

Number of *B. malayi* L_3 recovered followed by treatment with sera of different groups of mastomys immunized with			
	Alum	rWbL2	Anti-WbL2 Ab depleted sera
Live L3 larvae	18	11	16
	19	10	15
	17	9	13
Dead L3 larvae	3	12	5
	2	11	4
	3	12	3
% cytotoxicity (Mean ± SD)	12.93± 2.97	**53.89± 2.81***	**21.2± 2.53**

Peritoneal exudate cells (PEC) were incubated with the pooled sera from the mastomys administered with rWbL2 / Alum / Anti-WbL2 antibody depleted sera and L3 larvae in vitro. The total live and dead worms were counted in each well after 48h. The data shown is the number of larvae recovered from three different sets of experiments.

*p=0.027 in comparison with Alum and anti-WbL2 antibody depleted groups as analysed by Kruskal-Wallis test.

In situ cytotoxicity induced against infective larvae of B. malayi (L3)

Micropore chamber method was used to evaluate the immunoprophylactic efficacy of rWbL2. The microscopic observation of chambers implanted in the peritoneum of immunized mastomys showed the migration of hosts' immune cells into the chambers leading to their adherence and killing of the L3 larvae within 48 h of their implantation. Results showed that, the antibodies in the rWbL2 immunized mastomys were capable of inducing the significant cytotoxicity (51.25%, p<0.05) compared to the control group of mastomys (7.79%) (Table 2).

Table 2: In situ cytotoxicity assay against B. Malayi L3 .

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Groups of mastomys (n=5) immunized with	L3 larvae recovered			% cytotoxicity (Mean ± SD)
	Live	Dead	Total	
Alum	13	1	14	7.14
	15	2	17	11.76
	15	1	16	6.25
	13	1	14	7.14
	14	1	15	6.66
				7.79 ± 2.25
rWbL2	7	8	15	53.33
	8	7	15	46.66
	7	8	15	53.33
	8	9	17	52.94
	7	7	14	50
				51.25 ± 2.92*

In situ micropore chamber experiment was performed by surgically implanting 15-20 B. malayi L3 loaded in micropore chambers into the peritoneal cavity of each mastomys immunized with rWbL2 / Alum. After 48h, live L3 were recovered from the micropore chambers implanted in to the peritoneal cavity of mastomys immunized with rWbL2. *P=0.036 in comparison with Alum group as analysed by Mann Whitney U test.

Effect on the splenocytes proliferation and cytokine analysis

Cellular response was analysed to check the ability of the antigen to stimulate lymphocytes from mastomys immunized with rWbL2 after re-stimulation with the same protein. There was significant ($p<0.05$) proliferative response of spleen cells from animals immunized with rWbL2 (Stimulation index i.e. SI of 1.92 ± 0.04) as compared with the control group of mastomys treated with alum (1.07 ± 0.03) (Fig. 2a).

The release of anti-inflammatory cytokines IL-4 (70.2 ± 11.75 pg/ml) was increased significantly ($p<0.05$) and at the same time IL-10 was also farely raised (49.92 ± 4.75 pg/ml) in response to rWbL2 in the mastomys immunized with rWbL2 suggesting the rWbL2 induced mixed Th1-Th2 immune response (Fig. 2b). Additionally, we also studied the release of inflammatory cytokine, IFN- , was high in the culture supernatants of splenocytes from mastomys immunized with rWbL2 (Mean ± SD value of 36.7 ± 4.48 pg/ml) suggesting cell mediated immune response (Fig. 2b).

Figure 2a.

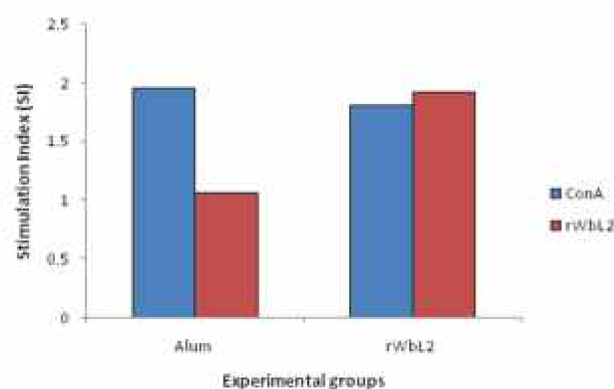

Figure 2b.

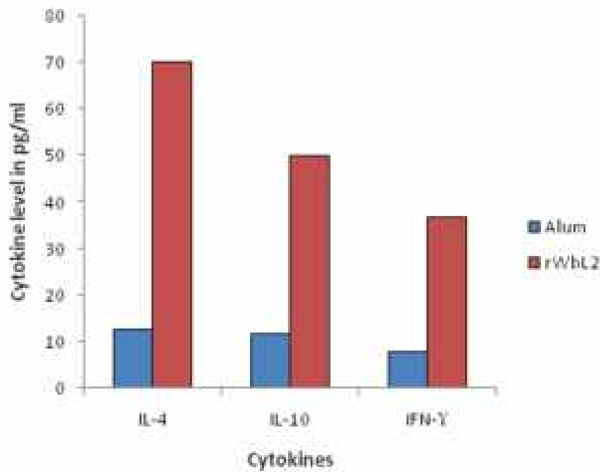

Figure 2: Effect of the immunization of mastomys with rWbL2 on splenocytes proliferation and cytokines profile. a) Splenocytes from mastomys administered with rWbL2 / Alum were cultured in vitro and re-stimulated with either ConA or rWbL2 and the effect on the splenocytes proliferation was checked by MTS assay after 48h. Each bar represents mean stimulation index (SI) ± SD. b) The levels of cytokines IFN-γ, IL-4 and IL-10 were measured in the culture supernatants of splenocytes after 72h by ELISA. Each bar represents Mean ± SD. n=5 mastomys per group; *p<0.05 in comparison with Alum group as analyzed by Mann Whitney U tests.

Discussion :

An SXP/RAL-2 family protein, WbL2 is a Wuchereria bancrofti L3 stage specific protein identified by differential display.. The similarity of WbL2 to other proteins from the SXP/RAL-2 family, such as from Ancylostoma caninum (Ac-16), Ascaris suum (As14), Acanthocheilonema viteae (Av-RAL-2), Setaria digitata (Sd-SXP-1), Brugia malayi (Bm-SXP-1), and Onchocerca volvulus (Ov17 and P36991) suggests that WbL2 might share some biological properties with these (apparently) nematode-specific proteins.[19] Not much information is available about biological function of these proteins, but few proteins have been explored as vaccine candidate for nematodes due to the protection they conferred upon immunization.

The member of SXP/RAL-2 family, Ac-16 an immunodominant surface antigen from the hookworm Ancylostoma caninum induced significant protection in dogs[12]. Immunization of mice with O. volvulus homologue, Ov17/Ov-RAL-2, induced 51 to 60% protection against L3 challenge.[20]. Moreover, the Ascaris homologue (As14) belongs to SXP/RAL-2 family of proteins has been shown to confer protective immunity against A. suum infection in mice[21]. SXP/RAL-2 proteins have also been identified as potential antimicrofilarial vaccines where immunization of gerbils with recombinant B.malayi SXP protein showed significant decline in worm density upon challenge[22].

Thus, W.bancrofti SXP/RAL-2 family protein rWbL2 can be important protein to be explored for prophylactic studies in lymphatic filariasis. In the present study, the vaccine potential of rWbL2 was assessed in another rodent model that is fully permissible for B. malayi infection and in addition, we also tested the humoral and cellular response associated with the protection induced by rWbL2. The mastomys which are fully permissible for B. malayi when vaccinated with rWbL2 developed significant titres of anti-WbL2 antibodies. The immunoglobulin antibody isotype profile for the rWbL2-immunized mastomys showed predominance of both IgG1 and IgG2a antibodies which are suggestive of a both cell mediated and humoral immune response. Increased IgG1 and IgG2a isotypes in rodents have the ability to fix complement and bind to protein antigens and have been shown to participate in ADCC reactions against invading pathogens [23,24].

Killing and clearance of filarial parasites from circulation is mainly achieved through the ADCC mechanism[16,17]. We observed that the sera from mastomys immunized with rWbL2 promoted adherence of peritoneal exudate cells to L3 larvae and induced significant killing of parasite (53.89% of cytotoxicity against L3). While reversal in the killing of parasite was observed after depletion of WbL2 specific antibodies from the sera of immunized mastomys. These results indicate that, anti-WbL2 antibodies may be playing a crucial role against the filarial infection.

Similar results were observed in in situ micropore chamber experiment. The micropore chambers removed from the mastomys vaccinated with rWbL2 showed accumulation of cells inducing significantly higher cytotoxicity of 51.25% against L3 larvae. These results suggest that rWbL2 is the promising vaccine candidate against filarial infection.

We observed significantly higher levels of WbL2 specific IFN-γ cytokine in the immunized animals. Also, level of IL-4 and IL-10 was found to be farely high. The immunized mastomys showed mixed Th1-Th2 immune response which is in correlation with the predominance of IgG1 and IgG2a antibody isotypes and significant levels of IFN-γ, IL-10, IL-4 cytokines.

Taken together the results of present study showed that rWbL2 could be a promising vaccine candidate against filarial infection or it can serve as one of the important vaccine candidate in cocktail vaccines in filariasis.

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