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Fecundity of adult female worms were affected when *Brugia malayi* infected Mongolian gerbils were immunized with a multivalent vaccine (rBmHAXT) against human lymphatic filarial parasite

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Abstract

A multivalent recombinant fusion protein prophylactic vaccine, rBmHAXT developed against lymphatic filariasis (LF) demonstrated over 57% protection against challenge infection in rhesus macaque model. Currently, we do not know if the rBmHAXT vaccination has any effect on adult worms and/or on the fecundity of adult female worms. Thus, the major focus of this study was to determine the effect of rBmHAXT vaccination on *Brugia malayi* infected mongolian gerbils. We performed two sets of experiments. In the first set of experiment, gerbils were infected with 100 *B. malayi* L3. After confirming the establishment of infection, four rounds of DEC treatment and rBmHAXT vaccination was given. Results showed that following vaccination with rBmHAXT, the microfilaria (Mf) count was significantly decreased in all vaccinated animals compared to controls. At the end of these experiments, we collected and counted the established adult worms. There was a 36% reduction in the recovery of adult female worms, which might account for the low Mf load in vaccinated animals. In the second set of experiments, animals were vaccinated first with rBmHAXT followed by surgically implanting adult male or female *B. malayi* parasites into the peritoneal cavity to determine the effect of vaccination on each sex of the parasite. Our results show that the rBmHAXT vaccination has no effect on male adult worms compared to controls. However, there was 40% reduction in the Mf load in vaccinated animals that were transplanted with adult female worms. These findings suggested that the rBmHAXT vaccination has potential damaging effect on the fecundity of adult female worms. Scanning electron microscopy studies showed cuticular damage on the surface of adult female worms. These studies thus show that the rBmHAXT vaccination in infected gerbils has partial microfilaricidal effect and potentially affect the fecundity of adult female worms.

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Declaration of competing interest

The authors have no conflicts of interest to declare.

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Keywords

Lymphatic filariasis; *Brugia malayi*; Multivalent vaccine; Filarial adult worm

1. Introduction

Lymphatic filariasis (LF) caused by *Wuchereria bancrofti*, *Brugia malayi*, and *B. timori* parasites is a neglected chronic tropical parasitic infection transmitted by mosquitoes. The disease is endemic in about 52 countries, with about 120 million people suffering from chronic LF infection and another 856 million people at risk of acquiring the infection (WHO, 2018). Currently, mass drug administration (MDA) is the major strategy adopted by the World Health Organization (WHO) to prevent disease transmission and to treat actively infected subjects with the filarial infections (Brady, 2014; Ramaiah and Ottesen, 2014). MDA program has been instrumental in the substantially limiting LF infection in several endemic regions in addition to reducing the intensity of soil transmitted helminth infections (Gyapong et al. 2018; Bockarie and Deb 2010). However, there are several limitations to the MDA program including lack of effectiveness of the drugs used in the MDA against established chronic infections and against the adult worms (Critchley et al., 2005; Sangshetti JN, 2017). There are reports of subject non-compliance to MDA that is potentially leading to the reemergence of LF infection in several parts of the world (Krentel et al., 2013; Nujum et al., 2012). There are also significant issues with the distribution of the MDA drugs in some of the endemic areas of the world that has also potentially contributed to the overall slow progress of the MDA in certain regions, especially since the MDA treatment needs to be repeated annually (Krentel et al., 2013; Nujum et al., 2012; Sunish et al., 2013). Vaccination-based control strategy has been highly successful in preventing several infectious diseases (F. E. Andre, 2008). However, there is no prophylactic vaccine available to date to control LF. Several pioneering work over the last 50 years on the immunology of human filarial infections clearly suggests that vaccine-based control is possible against human LF infections (Ottesen 1984; King and Nutman 1991; Ravindran et al. 2008; Sahu et al. 2008; Morris et al. 2012; Rajamanickam and Babu 2013; Babu and Nutman 2014). Several laboratories including our laboratory are currently working towards developing a prophylactic vaccine for LF and have identified several potential vaccine targets (Anugraha et al., 2015; Hartmann et al., 2014; Kalyanasundaram, 2018). In our laboratory, we identified and evaluated the vaccine potential of several vaccine candidates and demonstrated that a multivalent fusion protein vaccine gave better protection than monovalent or bivalent antigens (Chauhan et al., 2017; Dakshinamoorthy et al., 2013; Dakshinamoorthy et al., 2012; Joseph et al., 2012). A tetravalent formulation, recombinant *B. malayi* HAXT (rBmHAXT), containing; heat shock protein 12.6 (HSP) (Dakshinamoorthy et al. 2012), abundant larval transcript-2 (ALT-2) (Gregory et al. 2000), thioredoxin peroxidase-2 (TPX-2) (Gnanasekar et al., 2004), and tetraspanin large extracellular loop (TSP) (Gnanasekar et al 2008), was found to be a better vaccine formulation (Chauhan et al., 2018; Khatri et al., 2018) than a vaccine formulation with three antigens (rBmHAT) (Chauhan et al., 2017; Dakshinamoorthy et al., 2014). Comparison of various adjuvant formulations demonstrated that alum combined with glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE), a TLR4 agonist is a better adjuvant formulation for rBmHAXT (Chauhan 2018,

Dakshinamoorthy 2013b). Thus, vaccination trials with r*Bm*HAXT along with alum combined with GLA-SE gave over 88% protection in rodent models (Chauhan et al., 2018) and over 57% protection in rhesus macaque model (Khatri et al., 2018).

Acutely infected subjects rarely show any clinical symptoms (Babu and Nutman, 2014). However, chronically infected individuals show the classic lymph edema and other physical deformities, which helps in the diagnosis of the disease (Chakraborty et al., 2013). Therefore, in a mass vaccination program it will be difficult to selectively identify the acutely infected individuals, unless a mass screening for infection is performed before the vaccination campaign. Thus, when vaccinating infected individuals, a vaccine that has some detrimental effect on the adult worms or that possesses anti-fecundity effect will be significantly advantageous in a mass vaccination campaign. Those with active filarial infection actively contribute in disease transmission. If this vaccine can decrease the fecundity of adult female worms, it can aid in restricting the disease transmission. Effect of DEC on circulating Mf is transient, whereas the anti-fecundity effect of vaccine can stay for longer period. Currently, we do not know if r*Bm*HAXT vaccination has any effect on the adult filarial worms. However, based on previous studies the vaccine antigens are expressed on the surface of larval and adult parasites, which suggest that this may have some effect on the adult filarial worms (Gnanasekar et al., 2004; Maizels et al., 2001). Therefore, the major focus of this study was to evaluate the vaccine potential of r*Bm*HAXT against adult *B. malayi* worms in a *Meriones unguiculatus* (Mongolian gerbil) model.

2. Material and Methods

2.1. Experimental animals, parasites and adjuvant

The use of animals in this study was approved by the IACUC committee of the University of Illinois College of Medicine at Rockford. Four-week old male Mongolian Gerbils (*M. unguiculatus*) were purchased from Charles River Laboratories (Wilmington, MA). *B. malayi* male and female adult worms and infective third stage larvae (L3) were obtained from the NIAID/NIH Filariasis Research Reagent Resource Center (University of Georgia, Athens, GA) under NIAID supply contract AI#30022. The TLR4 agonist, GLA-SE combined with alum (AL019) adjuvant was purchased from the Infectious Disease Research Institute (IDRI, Seattle, WA).

2.2. Preparation of recombinant protein

r*Bm*HAXT was cloned and expressed as described previously (Khatri et al., 2018). Endotoxin was removed using an endotoxin removal column (Thermo Fisher Scientific, Rockford, IL). Endotoxin levels in the final purified preparations was < 0.3 EU/mg of protein.

2.3. Effect of r*Bm*HAXT on established *B. malayi* worm infection in gerbils

2.3.1. Establishing *B. malayi* infection in gerbils—To evaluate the effect of r*Bm*HAXT vaccination on established adult worm infection, twenty-five gerbils were challenged intraperitoneally with 50 *B. malayi* L3s. All the larvae were examined microscopically for viability and only the viable larvae were used for challenge. Three

months after challenge, one ml of sterile saline was injected into the peritoneal cavity of gerbils. After gentle massage, about 500 µl of peritoneal fluid was retrieved from each animal using a 21-gauge needle and the microfilariae (Mf) load in the peritoneal fluid was determined under a light microscope.

2.3.2. Experimental groups and treatment strategy—Out of 50 gerbils challenged with *B. malayi* L3, 17 gerbils became positive for *B. malayi* microfilariae and were randomly divided into three groups, Group 1 (n=6 gerbils) was treated with AL019 adjuvant alone (AL019 group), Group 2 (n=6 gerbils) received diethylcarbamazine (DEC) plus AL019 adjuvant (DEC plus AL019 group) and finally Group 3 (n=5 gerbils) received DEC plus rBmHAXT vaccine (DEC plus rBmHAXT group). Prior to each dose of vaccine, gerbils were treated three times i.p. with 6mg/kg of diethylcarbamazine (DEC) for three consecutive days (Fig. 1). This will mimic the MDA approach. The peritoneal fluid of each gerbil was checked the day following DEC treatment to determine the Mf load. After confirming that the Mf load has decreased substantially following DEC treatment in all infected gerbils, Group 3 animals were immunized four times at 2 weeks interval with 15 µg of rBmHAXT plus 10 µg of AL019 adjuvant after DEC treatment. Gerbils in Group 2 received 10 µg of AL019 only after DEC treatment and remained as controls for the vaccination. Blood samples were collected from the retro-orbital space of all animals before each immunization, two weeks after the last immunization and prior to euthanasia to determine the titer of IgG antibodies.

2.3.3. Monitoring the status of infection—Peritoneal cavity of gerbil was tapped at several time points before and after DEC treatment and immunizations to determine the Mf load. One month after the last dose of immunization, gerbils were euthanized, and peritoneal cavity was checked thoroughly to collect all the adult worms. Recovered, male and female adult worms were separated based on their morphology and counted to evaluate the number of adult worms established.

2.4. Effect of rBmHAXT vaccination on surgically transplanted filarial adult worms

2.4.1. Experimental groups and immunization protocol—This study was carried out to evaluate the effect of rBmHAXT vaccination on adult male or female worms. For this experiment, eight gerbils were immunized four times with 15 µg of rBmHAXT plus 10 µg of AL019 adjuvant at 2 weeks interval. Control group of gerbils (n=5) received AL019 only. Blood samples were collected from the retro-orbital space prior to each immunization, two weeks after the last immunization and prior to euthanasia to determine the titer of IgG antibodies.

After confirming that all the vaccinated gerbils have developed significant titers of IgG antibodies, the eight rBmHAXT immunized gerbils were divided into 2 groups (Group 1 and Group 2; n=4 each). Similarly, the five control gerbils (AL019 group) were also divided into 2 groups (Group 3; n=3 and Group 4; n=2). Five live male *B. malayi* adult worms were surgically implanted into the peritoneal cavity of each animal in Group 1 and Group 3 and five live female *B. malayi* adult worms were surgically implanted into the peritoneal cavity of each animal in Group 2 and Group 4.

2.4.2. Surgical implantation of live adult worms into the peritoneum of gerbil

—Fresh live male and female worms were obtained from University of Georgia, NIAID/NIH Filariasis Research Reagent Resource Center. The worms were first washed with RPMI media and their viability were checked. Fecundity of female worms were determined under a microscope for the release of Mf in vitro. Damaged or sluggish worms were removed and only intact and highly active adults were used for the surgical implantation.

Briefly, after anesthetizing the animals with a combination of ketamine (50 mg/kg) and xylazine (5 mg/kg) intramuscularly, the abdominal skin was shaved, and scrubbed with betadine. Approximately 10 mm incision was made on the skin, abdominal muscle and peritoneal membrane. The worms were then carefully inserted into the peritoneal cavity of each gerbil and the incision on abdominal muscle and skin was closed separately with 1–3 sutures and with liquid silicone sheet (Scar Guard Repair Liquid, Scarguard Labs, NY) respectively. Animals were monitored for 48h post-surgery to assess their complete recovery.

2.4.3. Monitoring the status of surgically implanted adult worms in

vaccinated animals—One month after placing the worms into the peritoneal cavity, animals were tapped and the Mf load in the peritoneal fluid was determined. All animals were euthanized on day 30 after the infection and the implanted adult worms were recovered and observed for damage or death (Fig. 2).

2.5. Scanning electron microscopy

Scanning electron microscopy of adult worms recovered from the peritoneal cavity of gerbils was performed to determine any damage to the surface of the adult worms. Briefly, adult worms recovered from the peritoneal cavity were washed with phosphate-buffered saline (PBS) and then fixed with 2.5% glutaraldehyde solution (Sigma–Aldrich) for 3 h. After incubation, worms were rinsed three times with PBS, and then fixed with 1% osmium tetroxide (Sigma–Aldrich) for 1h. Worms were washed again and dehydrated in an ethanol series (20, 40, 60, 80 and 100%) twice for 15 min each. Samples were then air dried overnight and mounted on aluminum stubs, sputter-coated with gold and observed under scanning electron microscope (SEM; EPIC SEM Hitachi SU8030) for any changes in the cuticle.

2.6. Levels of Total IgG and IgG isotypes in the sera of experimental animals

Levels of anti-*rBmHAXT* IgG, IgG1, IgG2a, IgG2b, IgG3, IgM and IgA antibodies were determined in the sera of each animal using an indirect enzyme-linked immunosorbent assay (ELISA) with cross-reacting mouse monoclonal isotype antibody reagents purchased from Sigma-Aldrich (St Louis, MO) as described previously (Chauhan et al., 2018). Currently there are no antibody reagents available for gerbils.

2.7. Antibody-dependent cell-mediated cytotoxicity (ADCC) assay

The ADCC assay was performed as described previously (Dakshinamoorthy et al., 2014). Approximately 200 live *B. malayi* Mf were incubated at 37°C with 5% CO₂ in duplicate wells together with 2×10⁵ peritoneal cells from normal control gerbils and 50 µl of test sera

sample. After 72 h incubation, viability of the *B. malayi* Mf was determined. The percentage of Mf death was expressed as the ratio of the number of dead Mf to the total number recovered from each well multiplied by 100.

2.8. Statistical analysis

GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA) was used to plot all the graphs and statistical analysis of the data was carried out in SPSS v 26.0 (IBM corporation, Armonk, NY) analyze the data. Data was analyzed for normality assumptions using Shapiro-Wilk Test. The data with significance value >0.05 was normally distributed data. Followed by analysis using suitable parametric or non-parametric statistical test. A probability (p) value of < 0.05 was considered statistically significant. Visualization images were prepared using [Biorender.com](https://biorender.com).

3. Results

3.1. Immunization with rBmHAXT reduces Mf load in *B. malayi* challenged gerbils

In the first set of experiments, we challenged 50 gerbils intraperitoneally with *B. malayi* L3. When we examined the peritoneal fluid 90 days after infection, 17 of the gerbils showed the presence of Mf in their peritoneal fluid. The Mf load ranged from 30,000 to 34,000 Mf per ml of peritoneal fluid (Table 1). We then gave three doses of DEC to the 12 animals that were positive for the Mf. Analysis of the peritoneal fluid confirmed that the Mf count substantially dropped after the first three doses of DEC treatment compared to the AL019 controls (Fig. 3). At this point (on day 94), we gave the first dose of the rBmHAXT vaccine to one group of infected gerbils. When we checked the peritoneal fluid on day 109, Mf load started showing an increase in the peritoneal cavity of all the animals including all DEC treated animals, suggesting that the effect of DEC is only transient. However, the Mf load was substantially low in the peritoneal cavity of vaccinated animals. Another three doses of DEC were given on day 109 and when checked the Mf load returned to near zero on day 113 in all DEC treated animals compared to AL019 controls. At this point, we gave the second dose of the vaccine. On day 128 we checked the Mf count again the count was high in DEC treated control animals compared to the vaccinated animals. We then gave the third dose of DEC on day 128. As expected, the Mf count was near zero on day 131 in all DEC treated animals compared to AL019 controls. On day 132, we gave the third dose of the vaccination. When checked again for Mf on day 147, the DEC treated control animals had very high levels of Mf in their peritoneal fluids, whereas, the Mf load was low in vaccinated animals. Last dose of DEC was given on day 147 and when checked as expected the Mf load was near zero on day 150 in all animals that were given DEC. The last immunization dose was given on day 151. On day 166, all animals were sacrificed to count the worm establishment. Before collecting the worms, we determined the peritoneal Mf count in each animal. Our results show that the Mf count in AL019 control and DEC treated control were very high and was nearly the same. However, the Mf count in vaccinated animals were low, although the differences were not significant ($p=0.09$; Fig. 3; Table 1).

Thus, our results show that after four rounds of rBmHAXT immunization, there was a progressive decrease in the Mf load in rBmHAXT-vaccinated animals (Table 1). Mf load in

vaccinated animals was 5080 ($\pm$ 5053) per ml of peritoneal fluid after the last dose of *rBmHAXT* ($p = 0.131$) compared to mean Mf counts of 34500 ($\pm$ 12045) per ml of peritoneal fluid prior to the start of immunization (Table 1). However, the Mf load in DEC plus AL019 treated gerbils was 37,280 ($\pm$ 38,292) per ml of peritoneal fluid even after the four rounds of DEC treatments, which was higher than DEC plus *rBmHAXT* treated animals (Table 1). There was no significant difference in the Mf load between the DEC plus AL019 group and the AL019 control group (35,500 $\pm$ 34,925) (Table 1).

All the vaccinated animals developed significantly ($p = 0.001$) high titer of *rBmHAXT* specific IgG antibodies (Fig. 4A). To determine the pattern of antibody responses, we measured the levels of antigen specific IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA antibodies in the sera of vaccinated and control gerbils (AL019 and DEC+AL019 groups). All the isotype of antibodies that we evaluated were significantly ($p < 0.001$) increased in the *rBmHAXT* vaccinated animals compared to the control animals (Fig. 4B).

3.2. Immunization with *rBmHAXT* affects the fecundity of *B. malayi* female adult worms

In the first set of experiments, there was significant reduction in the Mf load. Therefore, we designed a second set of experiment to determine if the *rBmHAXT* vaccination has any effect on the male worms or female worms separately. In these experiments, we first vaccinated the gerbils and once they developed sufficient titers of antibodies, we surgically implanted live adult male or female *B. malayi* worms into the peritoneal cavity.

Our results show that all the *rBmHAXT*-vaccinated animals developed significantly ($p = 0.0001$) high titers of *rBmHAXT*-specific IgG antibodies (Fig. 5). The levels of isotypes specific antibodies were also significantly ($p < 0.0001$) high in the *rBmHAXT*-vaccinated animals compared to the AL019 control animals (Fig. 5). One month after placing the adult female *B. malayi* worms in the peritoneal cavity, we collected the peritoneal fluid to determine the presence of Mf and to determine the Mf load in each animal. Our results show that in control animals, the Mf load was 6500 ($\pm$ 2262) per ml of peritoneal fluid (Table 1). However, the Mf recovery from *rBmHAXT* vaccinated animals was low 2550 ($\pm$ 1894) ($p = 0.1$). On day 30 after surgical implantation, all animals were euthanized, and the implanted worms were collected and observed under light microscope to determine their viability. There were no significant differences in the recovery of adult male worms between the two groups (Table 1). However, there was about 36% decrease ($p = 0.06$) in the recovery of female worms from the vaccinated group of animals compared to the controls (Table 1). Since our recovery was not 100%, we were not sure if this decrease in the recovery of male and female worms was an effect of the vaccination or not. When we calculated the average number of Mf per adult female worm recovered from the peritoneal cavity of each group of animals, we found that in control animals, there was 1962 Mf per adult female worm recovered. However, in *rBmHAXT*-vaccinated animals, there was only 1075 Mf per adult worm recovered. This suggested that the female worms in vaccinated animals released only fewer Mf.

The reduction in the number of Mf may be due to fewer Mf released from the female worms or due to a microfilaricidal effect by the protective antibodies in the sera of vaccinated animals. To test the later, we performed an ADCC assay using sera from *rBmHAXT*

vaccinated animals. Our results using the ADCC assay showed that the sera samples from vaccinated animals efficiently killed significant numbers of *B. malayi* Mf in vitro ($64.7\% \pm 4.8$) compared to the sera from AL019 control animals ($20\% \pm 0.1$; Fig. 6A). The Mf recovered from the wells supplemented with sera from vaccinated animals had several cells attached on their surface (Fig. 6B, supplementary video).

Although the vaccine appeared to have no lethal effect on the adult parasites, we wanted to find out if the effector cells and molecules generated following the vaccination have damaged the cuticle of the parasite. We performed a scanning electron microscopy analysis on the parasites (Fig. 7). Our results show that worms recovered from control animals had smooth cuticular surface and there were no cells found attached to the cuticle. However, on the worms recovered from the vaccinated animals, we found several cells (mainly macrophages and lymphocytes) attached to the cuticle and there was clear damage to the cuticular surface (Fig. 7). We also found significant mucus like materials adhered to the surface of the worms (more specifically the female worms) and this appeared to trap the parasites and prevent their normal movements.

4. Discussion

This is the first study evaluating the effect of *rBmHAXT* vaccination on male and female adult worms of *B. malayi* in a gerbil model. The rationale for evaluating the effect of the vaccine against adults is based on the findings that the vaccine antigens are expressed on the surface of larval and adult parasites (Gnanasekar et al., 2004; Maizels et al., 2001). Therefore, we hypothesized that the vaccine-induced immunity will be effective against microfilariae and the adult parasite as well.

Patients who are acutely infected with LF do not show any clinical symptoms of the disease (Babu and Nutman, 2014). Therefore, during a prophylactic vaccination campaign it will be difficult to diagnose and isolate acutely infected patients from the pool of subject for vaccination. At this time, we do not know if vaccination with *rBmHAXT* have any effect on the already established parasites in acutely infected individuals. Therefore, in this study we evaluated if vaccination with *rBmHAXT* in infected gerbils has any effect on the adult worms and/or the microfilariae. Our results show that, the *rBmHAXT* vaccination has some microfilaricidal and anti-fecundity effect on the female adult worms.

The canonical host immune responses to lymphatic filariasis is a strong Th2 type response with diminished Th1 response to the antigens (Babu and Nutman, 2014). The microfilariae are notorious for their ability to impair immune responses in the infected individuals (Babu et al., 2007; Semnani et al., 2003). The immunosuppressive effect induced by the LF parasite also results in poor immune responses to vaccination, and allergens in the infected individuals (Labeaud et al., 2009). DEC treatment reversed this immunosuppressive state in the humans (Piessens et al., 1981). Therefore, one of the main reasons for treating the infected gerbils with DEC was to remove the microfilariae and allow the gerbils to be immunocompetent so they can respond to the vaccination. One of our preliminary studies showed that the infected animals do not develop sufficient antibodies following vaccination with *rBmHAXT* (data not shown). Therefore, we decided to give the DEC treatment before

immunization. A second reason for the treatment of infected gerbils with DEC was to mimic the MDA protocol before vaccination. Our results show that all animals were positive for Mf before DEC treatment. Following each dose of DEC treatment, the Mf count significantly decreased to nearly zero Mf in all animals. However, within two weeks after stopping the DEC treatment, all the animals in the AL019 plus DEC group showed high levels of Mf in their peritoneal cavity suggesting that the DEC treatment does not clear the adult worms. This was confirmed at the completion of the study where we recovered seven adult worms (4 females and 3 males) in the DEC + AL019 group. The findings also show that even four doses of DEC treatment was ineffective in preventing the appearance of Mf, especially when the drug effect is gone. Following DEC treatment, we gave four rounds of *rBmHAXT* immunization. All the vaccinated animals in both the sets of studies developed high titer of *rBmHAXT*-specific IgG antibodies. The levels and type of antibody response was similar to our previous reports in mice (Chauhan et al., 2017) and in macaques (Khatri et al., 2018), where a balanced Th1 and Th2 response was elicited by the vaccine. These findings suggested that the vaccine was still immunogenic in the infected gerbils. There was an inverse correlation between the titer of anti-*rBmHAXT* antibodies and the Mf load. As the titer of antibodies increased, the Mf load decreased in the vaccinated animals. However, the Mf load showed a steady increase in the DEC + AL019 control group. These findings suggested that vaccination with *rBmHAXT*, potentially reduced the number of microfilariae in vaccinated animals.

This decrease in Mf load can be due to two potential reasons; one, it can be a direct effect of the vaccination on the female adult worms affecting their fecundity and viability resulting in reduced number of Mf produced by the female worms. The second possibility might be that the vaccination has potential direct effect on the Mf. DEC is known to decrease the Mf load (Ramzy et al., 2002), but has no significant effect on the fecundity of adult female worms (Fernando et al., 2011; Meyrowitsch et al., 2004; Sunish et al., 2002). Our results confirm these earlier reports in that even four rounds of DEC administration had no significant effect on the fecundity of adult female worms. Therefore, the decreased Mf load observed in the vaccinated animals could be attributed to the reduced fecundity of the female worms following the vaccination. Another possibility is a direct effect of the vaccination on microfilariae.

To test this, we performed an ADCC assay using sera from vaccinated animals. Our results confirmed that the protective antibodies developed in vaccinated animals could kill significant numbers of Mf. Our group and others previously reported that the antigens that are used in the construction of the *rBmHAXT* vaccine are expressed on the surface of all larval stages including microfilaria. Thus, the findings in this study confirm that *rBmHAXT* vaccination has partial microfilaricidal activity and may be important in transmission blocking. The Mf count in vaccinated animals was consistently lower at all the time points that we tested. To determine if this was due to adult worms dying in vaccinated animals, we euthanized all the animals on day 15 after the last dose of immunization and recovered the worms from the peritoneal cavity. Our recovery of adult worms from infected animals were not very efficient. In some cases, we found Mf in the peritoneal cavity but could not recover female or male worms. Therefore, we could not confirm if the poor recovery of Mf in vaccinated animals were due to the vaccine effect or due to the poor recovery of female

worms. Nevertheless, our data suggest that the vaccination has some effect on the adult worms. To further confirm this, we surgically implanted adult male and female worms separately into the peritoneal cavity of animals vaccinated with r*Bm*HAXT. We chose to conduct these studies with single sex infections to separate out the effect of vaccination on male and female worms. Our results on the surgical transplant studies showed that the r*Bm*HAXT vaccination might have some effect on the adult female worms including potential anti-fecundity effect. When we calculated the mean Mf load per female adult worm recovered, we found that there was about 45% reduction in the Mf load per female worm in the vaccinated animals. Thus, the female worms in vaccinated animals appeared to release fewer Mf.

Currently r*Bm*HAXT is one of the leading prophylactic vaccines for LF (Kalyanasundaram, 2018) that has successfully undergone preclinical trials in rodents and macaque and demonstrated significant protection against challenge infections (Chauhan et al., 2018; Khatri et al., 2018). Although the current study did not prove that the r*Bm*HAXT vaccine has therapeutic effect, there appears to be some anti-fecundity effect on female adult worms. Our results also showed that the vaccination has anti-microfilaricidal effect. Thus, in conclusion, findings in this study suggest that vaccination with r*Bm*HAXT could generate protective antibodies in subjects who already carry the lymphatic filariasis infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Use of animal in this study was approved by the Animal Care Committee of the University of Illinois, Rockford. The study followed the National Institutes of Health guidelines for the care and use of laboratory animals. This article does not contain any studies with human participants performed by any of the authors.

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Highlights

- *rBmHAXT*-vaccination has partial macrofilaricidal effects on adult female worms.
- The fecundity of adult female worms was affected following *rBmHAXT* vaccination.
- Vaccination with *rBmHAXT* has microfilaricidal effects in infected jirds.
- Balanced Th1/Th2 immune response were generated in *rBmHAXT*-vaccinated animals.

Figure 1.
Schematic diagram of the experimental groups with time-line showing the DEC treatment and vaccination schedule used in the study.

Figure 2. Schematic diagram showing the protocol used for immunization and surgical transplantation of adult male and adult female worms.

Fig. 3. Effect of *rBmHAXT*-vaccination on the peritoneal microfilariae (Mf) counts of gerbils. The Mf load in the peritoneum of each gerbil was checked by tapping after each DEC cycle and before every immunization cycle. Each data point indicates the Mf count per ml of peritoneal fluid. $n=5$ gerbils for AL019 group and $n=6$ for DEC+AL019 and DEC +*rBmHAXT* groups.

Fig. 4. Titer of antigen-specific IgG antibodies in the sera of gerbils.

B. malayi infected gerbils were immunized (s/c) with four doses (15 µg each dose) of rBmHAXT in AL019 as adjuvant after treating three times (i.p.) with DEC (6mg/kg). Blood samples were collected and the levels of rBmHAXT-specific (A) IgG antibodies and (B) IgG1, IgG2a, IgG2b, IgA, and IgM antibody isotypes were analyzed in all the groups of animals using ELISA. Each data bar indicates the mean absorbance value for each group $\pm$ S.D. $n=5$ gerbils for AL019 group and $n=6$ for DEC+AL019 and DEC+rBmHAXT groups. $*p < 0.05$ when compared to AL019 group as analyzed by one-way ANOVA followed by Tukey's post hoc test except for the levels of IgM antibodies analyzed using Kruskal-Wallis test followed by Bonferroni correction for multiple analysis.

Fig. 5. Titer of antigen-specific IgG antibodies in the sera of vaccinated gerbils.

Gerbils were immunized (s/c) with four doses (15 μ g each dose) of *rBmHAXT* in AL019 as adjuvant. Blood samples were collected and the levels of *rBmHAXT*-specific IgG antibodies and IgG1, IgG2a, IgG2b, IgA, and IgM antibody isotypes were analyzed in all the groups of animals using ELISA. Each data bar indicates the mean absorbance value for each group $\pm$ S.D. $n=8$ gerbils for *rBmHAXT*+AL019 groups and $n=5$ for AL019 group. * $p < 0.0001$ when compared to AL019 group as analyzed by Mann–Whitney U test.

Fig. 6. Antibody-dependent cell mediated cytotoxicity (ADCC) assay.

Approximately 200 *B. malayi* Mf were incubated for 72 h at 37°C with 0.2 million peritoneal cells and 50 µl of sera samples from each animal. Mf death in each well was monitored under a light microscope. (A) Each bar represents the percent microfilaricidal activity using the sera from respective group (Mean ± S.D values). (B) Representative images of Mf recovered from respective wells at 100x magnification. $n=4$ sera samples. * p 0.005 when compared to AL019 group as analyzed by Kruskal-Wallis test.

Fig. 7. Scanning electron microscopy of recovered adult worms from experimental animals. Worms recovered from the peritoneum of experimental animals were processed for SEM and images were scanned to identify damage to the worm surface. (A) Worm recovered from the control group showed no visible damage to the outer surface and there were no cells attached to the worm surface. (B-D) Worms recovered from the *rBmHAXT* plus AL019 vaccinated group showing cuticular damage at several places (white arrows) and cells attached on the surface of worms (black arrows). We also observed mucus-like material attached to the surface of the worms (black stars).

Table 1

Effect of rBmHAXT vaccination on *B. malayi* infected animals

Microfilariae (Mf) load prior to each immunization (per ml peritoneal fluid)												Adult worms recovered	
Groups	Animal No.	0	DEC-1	1	DEC-2	2	DEC-3	3	DEC-4	4	Female	Male	
AL019	1	7000		7600		23000		1500		14000			
	2	65000		100000		72100		41000		95500	1		
	3	15000		3800		2800		100		5000			
	4	44000		27500		37900		4000		28000	1		
	5	32000		18000		29700		20000		35000			
	Mean Mf load $\pm$ S.D.	32600 $\pm$ 24620		31380 $\pm$ 37548		33100 $\pm$ 26414		13320 $\pm$ 16492		35500 $\pm$ 34925	Total	2	
DEC + AL019	1	7000	0	1000	0	500	0	100	0	800			
	2	43000	0	35000	120	12600	0	135000	120	89000	1	1	
	3	50000	100	20800	0	12500	0	9000	0	32300			
	4	15000	0	2400	0	7900	0	2000	0	2300	1		
	5	28000	0	12000	0	15000	0	18000	0	62000			
	6	37500	80	19000	0		0	Animal died			2	2	
DEC + rBmHAXT	Mean Mf load $\pm$ S.D.	30083 $\pm$ 16632	30 $\pm$ 46.9	15033 $\pm$ 12754	20 $\pm$ 48.9	9700 $\pm$ 5749	0 $\pm$ 0	32820 $\pm$ 57550	20 $\pm$ 48.9	37280 $\pm$ 38292	Total	4	
	1	32000	0	6000	0	6600	0	2500	0	3200			
	2	24000	0	200	0	4200	0	7500	0	5000			
	3	19000	0	0	0	0	0	0	0	0			
	4	39000	0	0	0	0	0		Animal died				
	5	41000	0	11000	0	5800	0	35000	0	13500	1	1	
DEC + rBmHAXT	6	52000	50	18000	0	7800	0	13000	0	3700			
	Mean Mf load $\pm$ S.D.	34500 $\pm$ 12045	8.3 $\pm$ 20.4	5866 $\pm$ 7409	0 $\pm$ 0	4066 $\pm$ 3360	0 $\pm$ 0	11600 $\pm$ 13997	0 $\pm$ 0	5080 $\pm$ 5053	Total	1	
												1	