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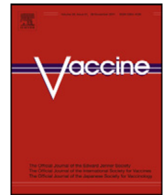

Multivalent fusion protein vaccine for lymphatic filariasis

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ABSTRACT

Lymphatic filariasis affects approximately 3% of the whole world population. Mass drug administration is currently the major control strategy to eradicate this infection from endemic regions by year 2020. Combination drug treatments are highly efficient in controlling the infection. However, there are no effective vaccines available for human or animal lymphatic filariasis despite the identification of several subunit vaccines. Lymphatic filariasis parasites are multicellular organisms and potentially use multiple mechanisms to survive in the host. Therefore, there is a need to combine two or more vaccine candidate antigens to achieve the desired effect. In this study we combined three well characterized vaccine antigens of *Brugia malayi*, heat shock protein 12.6 (HSP12.6), Abundant Larval transcript-2 (ALT-2) and tetraspanin large extra cellular loop (TSP-LEL) as a multivalent fusion vaccine. Putative immune individuals carry circulating antibodies against all three antigens. Depletion of these antigen specific antibodies from the sera samples removed the ability of the sera to participate in the killing of *B. malayi* L3 in an antibody dependent cellular cytotoxicity (ADCC) mechanism. Vaccination trials in mice with a bivalent [HSP12.6 + ALT-2 (HA), HSP12.6 + TSP-LEL (HT) or TSP-LEL + ALT-2 (TA)] or trivalent [HSP12.6 + ALT-2 + TSP-LEL (HAT)] vaccines using DNA, protein or heterologous prime boost regimen showed that trivalent HAT vaccine either as protein alone or as heterologous prime boost vaccine could confer significant protection (95%) against *B. malayi* L3 challenge. Immune correlates of protection suggest a Th1/Th2 bias. These finding suggests that the trivalent HAT fusion protein is a promising prophylactic vaccine against lymphatic filariasis infection in human.

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1. Introduction

Lymphatic filariasis caused by *Brugia malayi*, *Wuchereria bancrofti* and *Brugia timori* affects more than 120 million people living in 72 different countries [1]. Chronic infections are associated with severe lymphatic pathology characterized by lymph edema and lymphadenitis. Human infection occurs when mosquitoes transmit the third stage larvae (L3) of the parasite. Thus, strategies that can kill L3 can prevent lymphatic filariasis infection in the human. In endemic regions, certain individuals are naturally immune to lymphatic filariasis [2]. These individuals also called endemic normals (en) carry circulating antibodies against several antigens of L3. We recently showed that these circulating antibodies can participate in the killing of L3 in an ADCC mechanism [3,4]. By screening a phage display cDNA expression library of *B. malayi* L3 with en sera samples, we identified several antigens of L3 as potential vaccine candidates [5–8]. Similarly, our group and others have reported several subunit vaccine candidates with varying degrees of protection in experimental animals [3,9–14]. Among these, three vaccine antigens; Abundant Larval transcript [ALT-2] [5,9], small Heat Shock Protein 12.6 [HSP12.6] [4] and Tetraspanin Large extracellular loop [TSP-LEL] (unpublished data) were identified as leading vaccine candidates.

L3 stages are multicellular organisms that have evolved multiple mechanisms to evade host immune responses [15,16] for their survival. Thus, it is important to target more than one critical antigens of the parasite to get the desired vaccine-induced protection. This notion was confirmed by our previous studies, where we show that combining two antigens as a multivalent vaccine can synergistically increase the degree of vaccine-induced protection [8,17]. Similar findings were reported by other groups as well [18–20]. Another advantage of using multivalent vaccine is that it allows generation of multiple peptides with varied MHC restriction. Since human population express a vast repertoire of MHC, a multivalent vaccine can generate a better and broader immune response in a human population than a single subunit vaccine that has limited MHC restriction [21,22]. Therefore, in this study we attempted to combine the three antigens (ALT-2, HSP12.6 and TSP-LEL) of *B. malayi* as a multivalent

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fusion vaccine and evaluated its vaccine potential *ex vivo* in human and in a mouse model.

2. Materials and methods

2.1. Animals and parasites

B. malayi L3 were obtained from the NIAID/NIH Filariasis Research Reagent Resource Center (University of Georgia, Athens, GA) and Balb/c mice were purchased from Charles River laboratory (Wilmington, MA). Use of animals in this study was approved by the Animal Care Committee of the University of Illinois Rockford.

2.2. Collection of human blood samples

Blood samples were collected after proper consent from endemic normal (en) subjects residing in and around Sevagram, Maharashtra, India. en subjects are asymptomatic, non-microfilaraemic individuals with no circulating parasite antigens in their blood [3–5]. Sera samples from healthy non-endemic normal (nen) subjects were collected at Rockford, IL after proper consent. Use of human subjects in this study was approved by the Institutional Review Board (IRB) of the University of Illinois, College of Medicine at Rockford and the IRB committee of Mahatma Gandhi Institute of Medical Sciences, Sevagram, Maharashtra, India.

2.3. Construction of multivalent DNA vaccine

DNA sequences were codon optimized (Genscript, Piscataway, NJ) for optimal expression in mammalian cells. To prepare multivalent construct, *Bmhsp12.6* and *Bmalt-2* genes were cloned into *pVAX*™*200-DEST* vector as described earlier [4]. No stop codons were added to these constructs for continuous transcription of both the genes. *Bmtsp-LEL* DNA was then cloned into *pVAXBmhsp12.6 + Bmalt-2* plasmid using forward primer 5′-CGC**GAATTC**ACCATGGTCCTGGAG-3′ containing EcoRI restriction site and the reverse primer 5′-GC**TCTAGA**TCAGTCCTTCTGGCTAG-3′ containing XbaI restriction site and stop codon. Bivalent DNA constructs *Bmhsp12.6 + Bmalt-2* (*ha*), *Bmhsp12.6 + Bmtsp-LEL* (*ht*) and *Bmtsp-LEL + Bmalt-2* (*ta*) were prepared using respective primers.

2.4. Construction and expression of rBmHAT multivalent fusion protein

pRSETA *Bmhsp12.6 + Bmalt-2 + Bmtsp-LEL* (rBmHAT) was constructed in the same manner as above. The primer sequences were *BmHSP12.6* (forward primer 5′-CG**GGATCC**ATGGAA GAAAAGGTAGTG-3′ and reverse primer 5′-CCC**TCGAGT**GCTTTCTTTTTGGCAGC-3′). *BmALT-2* (forward primer 5′-CCC**TCGAGA**TGAATAAACTTTTAATAGCAT-3′ and reverse primer 5′-GGG**TACCCG**CGCATTGCCAACCC-3′). *Bmtsp-LEL* (forward primer 5′-GGG**GTACCC**CGGCAAGGATCAATTTAAAA-3′ and reverse primer 5′-CG**GAATTC**TCA ATCTTTTTGAGATGAAT-3′). Bivalent constructs (HA, HT and TA) were also cloned individually into pRSETA vectors using appropriate primer pairs. Recombinant fusion proteins were expressed in *Escherichia coli* BL21 (pLysS), purified and endotoxin removed by Pierce High Capacity Endotoxin removal resin column (Thermo Fisher Scientific, Rockford, IL).

2.5. Immunization of animals

Six weeks old Balb/C mice were randomly divided into 8 groups with 10 mice per group. Each set of mice were immunized at 15 days interval with two doses of 100 μg of endotoxin free DNA (intradermally) followed by two doses of 15 μg of protein (subcutaneously) suspended in Imject® Alum (Thermo Fisher Scientific). Vaccination protocol is schematically presented in Supplement Fig. 1A. The following groups were used (a) HSP12.6, (b) ALT-2, (c) TSP-LEL, (d) HSP12.6 + ALT-2 (HA), (e) HSP12.6 + TSP-LEL (HT), (f) TSP-LEL + ALT-2 (TA), (g) HSP12.6 + ALT-2 + TSP-LEL (HAT) and (h) controls that received *pVAX* vector and alum. The experiments were repeated three times with all the groups.

A second set of experiment was performed with the trivalent HAT vaccine. Groups of 10 mice were divided into 6 groups and immunized at two weeks intervals with (a) 100 μg of endotoxin free codon optimized *hat* DNA suspended in PBS, (b) 15 μg of rBmHAT proteins in Imject® Alum, and (c) Heterologous prime boost vaccination (primed with two injections of *hat* DNA at 2 weeks interval, and boosted with 2 injections of rBmHAT proteins also given at two weeks interval). Vaccination protocol is schematically presented in Supplement Fig. 1B. There were three control groups (d) *pVAX DNA*, (e) Alum and (f) *pVAX vector* plus Imject® Alum. The experiments were repeated three times with all the groups.

2.6. Evaluation of antibody responses in the sera of human and mice

Levels of anti-BmHSP12.6, anti-BmALT-2 and anti-BmTSP-LEL IgG antibodies were determined in the sera of human subjects (en and nen) and mice (immunized or control) using an indirect ELISA as described previously [3]. Antibody specificity was confirmed after separating the recombinant proteins on a 12% SDS-PAGE gel, transblotting to nitrocellulose sheet and probing with respective antibodies [3].

2.7. Antibody-dependant cellular cytotoxicity (ADCC) assay

An *in vitro* ADCC assay was performed as described previously [3,4] to determine if serum antibodies can participate in the killing of L3. Briefly, ten (10) L3 of *B. malayi* were incubated with 50 μl of pooled ($n = 10$) sera samples from en subjects and 2×10^5 PBMC from healthy volunteers in a 96 well culture plate at 37 °C and 5% CO_2 for 48 h. In the mouse studies, 10 L3 were incubated with 2×10^5 peritoneal cells (PEC) from normal Balb/C mice and 50 μl of pooled ($n = 10$) sera samples from immunized mice. Larval viability was determined under a light microscope as described previously [3]. Parasites that were clear and straight with no movements were counted as non-viable; if they still showed no movement and remained straight for the next 8 h at 37 °C, they were counted as dead. The live larvae remained active. ADCC was estimated as percent larval death, calculated using the formula: number of dead larvae × 100/(total number of larvae used for ADCC).

In another study, we depleted total IgG, anti-BmHAT, anti-BmHSP12.6, anti-BmALT-2 or anti-BmTSP-LEL antibodies from mouse and human sera samples [4]. Briefly, 200 μl of pooled sera samples were incubated overnight at 4 °C with rBmHSP12.6, rBmALT-2 and/or rBmTSP-LEL that were coupled to IMAC resin and flow through was collected. Total IgG was removed by incubating sera samples with Protein A gel beads (Thermo Fisher Scientific). Depletion of respective antibodies in the sera samples was confirmed by an indirect ELISA as described previously [3]. The antibody depleted sera was then used in ADCC assays.

2.8. Challenge studies in mice using micropore chamber technique

Vaccine-induced protection was determined by implanting a challenge dose of twenty (20) L3 into the peritoneal cavity of immunized mouse in a micropore chamber as described previously [4,17,23]. 48 h after implantation, contents of each chamber were examined microscopically for larval death.

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