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Review Article

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Development of filarial vaccine by targeting stage specific proteins

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ABSTRACT

Brugia and Wuchereria are the mosquito born nematodes and causes major tropical disease lymphatic filariasis. More than 100 million people are infected and at risk of acute lymphangitis and elephantiasis. Vaccine against filariasis may be developed by targeting stage specific proteins like 2nd stage larval(L2) protein transglutaminase and 3rd stage larval(L3) protein abundant larval transcript-1 and -2 (ALT-1 and ALT-2). This review is based on research on targeting ALT-1 and ALT-2 protein as vaccine. The ALT-1 and ALT-2 (antigens) were developed using mRNA of larva. The peptide chains of targeting proteins were sequenced and cDNA library developed. These cDNA libraries help in development of genes of desired protein. Newly constructed genes were expressed in host (bacterial vector) and isolated by downstream processing with the help of IMAC. These isolated proteins were infected in lab animals (susceptible rodents i.e. jirds) and immune response studied. The antibody titer measured by taking spleen of test animal. The animals immunized with ALT-1 or TGA proteins shows upto 76% reduction in parasitic survival. Stage specific proteins are therefore strong candidates for a future vaccine against human filariasis.

Keywords: Filaria**,** Brugia malay, Elephantiasis, Antifilarial vaccine, Wuchereria

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INTRODUCTION

The thread-like, parasitic filarial worms *Brugia malayi* and *Wuchereria bancrofti* cause lymphatic filariasis and live almost exclusively in humans. These worms lodge in the lymphatic system, the network of nodes and vessels that maintain the delicate fluid balance between the tissues and blood and are an essential component for the body's immune defense system. They live for 4-6 years, producing millions of immature microfilariae (minute larvae) that circulate in the blood

Vaccines against filariasis must generate immunity to the infective mosquito-derived third-stage larva (L3) without accentuating immunopathogenic responses to lymphatic-dwelling adult parasites. Two highly expressed genes designated as abundant larval transcript-1 and -2 (*alt-1* and *alt-2*). ALT-1 and ALT-2 share 79% amino acid identity across 125 residues, including a putative signal sequence and a prominent acidic tract. Expression of *alt-1* and *alt-2* is initiated midway through development in the mosquito, peaking in the infective larva and declining sharply following entry into the host. Humans exposed to *Brugia malayi* show a high frequency of immunoglobulin G1 (IgG1) and IgG3 antibodies to ALT-1 and -2, distinguishing them from adult-stage antigens, which are targeted by the IgG4 isotype. Immunization of susceptible rodents (jirds) with ALT-1 elicited a 76% reduction in parasite survival, the highest reported for a single antigen from any filarial parasite. ALT-1 and the closely related ALT-2 are therefore strong candidates for a future vaccine against human filariasis.

The parasites have a complex life cycle in which mosquito-borne infective third-stage larvae (L3) invade the human

body, mature to adult worms, and produce large numbers of newborn larvae (microfilariae) which must transit the mosquito vector in order to develop to L3 [1]. Overt disease has a major immunopathologic component, and a prominent risk of vaccination with filarial antigens is exacerbation of pathology [2,3,4]. The target of immunopathological reactions, however, is thought to be the long-lived adult worm and not the infective larva

EXPERIMENTAL SECTION

A Brugia malayi parasite was obtain from Laboratories and maintained by feeding *Aedes aegypti* mosquitoes with microfilariae in blood. Mosquitoes were maintained for up to 12 days and crushed to recover infective larvae by baermannization. Jirds are infected with around 300 infective larvae intraperitoneally, and peritoneal adult worms and microfilariae can be recovered 3 or more months later.

Genomic cloning:
A Uni-ZAP XR cDNA library of the infective L3 of *B. malayi* was obtained and used as a template for generation of cDNA by PCR amplification using primers flanking the cDNA inserts. PCR parameters were 95°C of denaturation for 30 s, 55°C of primer annealing for 30 s, 72°C of primer extension for 3 min and cycled for 30 cycles. A final extension of 5 min was performed at 72°C before storing the samples at 4°C. PCR was performed with PfuTurbo DNA polymerase (Stratagene, La Jolla, Calif.) to enhance the fidelity, sensitivity and yield of PCR product. The forward primer for PCR was T3 primer with sequence 5′AATTAACCCTCACTAAAGGG3′ whereas the reverse primer was T7 promoter primer with sequence 5′GAAATACGACTCACTATAGGG 3′. PCR products were purified using the QIAquick PCR Purification kit (Qiagen, Valencia, Calif.) and size fractionated to select products of >300 bp length, using CHROMA SPIN columns (Clontech, Palo Alto, Calif.). The PCR products were digested with EcoRI and HindIII enzymes and ligated to similarly digested phage display vector T7Select 1-1 cloning system (Novagen, Madison, Wisc.). The library was packaged in vitro, titered and amplified as per the procedures outlined in T7Select system. Length and frequency of insertion was verified by PCR amplification of randomly selected clones.

BIOPANNING-The strategy used for biopanning the cDNA library of *B. malayi*, to select EN specific clones that display *B. malayi* gene products on the surface of T7 bacteriophage. Wells of a high binding microtiter plate (Pierce Chemicals, Rockford, Ill.) was coated overnight at 4°C with 1:100 dilutions of a pooled serum sample (from 10 individuals) from NEN, MF, CP or EN individuals. After washing the wells with phosphate buffered saline containing 0.1% Tween 20 (PBST), nonspecific sites were blocked with 5% bovine serum albumin (BSA) for 1 h at 37°C. For iterative screening, 100 µl of T7Select library (containing 10^{11} PFU/ml) was first added to wells coated with NEN sera and incubated for 1 h at room temperature. Unbound phages were removed from the wells and transferred to wells coated with CP sera. After 1 h of incubation at room temperature, unbound phages were removed and transferred to wells coated with MF sera and incubated for 1 h at room temperature. Unbound phages were again removed from the wells and transferred to wells coated with EN sera. After a final incubation for 1 h at room temperature, the unbound phages were discarded this time by washing the wells five times with PBST. The bound phages were then eluted with 200 µl of T7 elution buffer (TBS in 1% sodium dodecyl sulfate [SDS]) and amplified by infecting *Escherichia coli* host BLT5403. The amplified phages were then subjected to another three rounds of selective screening as above to enrich the clones that are highly specific for EN sera.

SEQUENCING AND ANALYSIS - After four rounds of biopanning, the final enriched EN specific clones were plated and single pure plaques were isolated. The cDNA inserts in these plaques were amplified by PCR using primers flanking the inserts. PCR primers were T7SelectUP with sequence of 5′GGAGCTGTCGTATTCCAGTC3′ and T7SelectDown primer 5′AACCCCTCAAGACCCGTTTA3′. PCR conditions were denaturation for 1 min at 94°C, primer annealing for 1 min at 50°C and primer extension for 1 min at 72°C for a total of 30 cycles. QIAquick columns (Qiagen) were then used to purify the PCR products after a final extension for 5 min at 72°C. Ends of the amplified PCR products were converted into blunt ends and cloned into pST Blue-1 Vector (Novagen). The nucleotide sequence of selected cDNA inserts in pST-Blue was determined at the DNA core facility of the University of Illinois Chicago. Sequences were analyzed by BLAST (www.ncbi.nlm.nih.gov) searches with the GenBank database. Further analysis by multiple sequence alignment was performed using the Clustalw (www.ebi.ac.uk) program.

PLAQUE LIFT HYBRIDIZATION ANALYSIS- To determine the abundance and frequency of the clones, a plaque lift hybridization analysis was performed. Briefly, DNA probes were prepared by PCR amplifying the genes using

insert specific primers and labeling with horseradish peroxidase (HRP) (Amersham). EN specific plaques were plated at a dilution of 10^{-6} and the plaques were transferred to five different nitrocellulose nylon membrane (Hybond N+) discs (Amersham) by replica plating for 5 min. The blots were then denatured on filter papers saturated with 0.5 M NaOH and rinsed twice with 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Following this the blots were transferred to hybridization buffer containing the probes and hybridized for 4 h at 42°C. After completion of the hybridization, blots were washed twice with primary wash buffer (6 M urea, 0.4% SDS and 0.5× SSC) at 42°C for 20 min followed by two secondary washes (2× SSC) at room temperature for 5 min. An ECL kit was then used to detect the hybridization signals (Amersham). Spots in each blot were then counted using SigmaScan Pro software and later confirmed by manual counting.

Expression, antibody production, and western blotting:
A recombinant construct of BmALT-2 in T7 expression vector was maintained in XL-1 Blue (Stratagene). For expression, the recombinant plasmid was transformed into BL21 (DE3) pLysS (Invitrogen, Carlsabad, Calif.) to minimize toxicity due to the protein. When the cultures reached an optical density of 0.7 at 600 nm, 1 mM isopropyl-1-thio-β-d-galactopyranoside was added to the cultures to induce gene expression, and the cultures were incubated for an additional 3 hrs. Total proteins were separated in SDS-12% polyacrylamide gel electrophoresis, and the presence of histidine-tagged protein was confirmed using an anti-Xpress antibody (Invitrogen). Subsequently, the histidine-tagged recombinant proteins were purified using a TALON metal affinity resin (Clontech) as per the manufacturer's recommendations. The purified rBmALT-2 was passed through a polymyxin B-agarose column (Detoxi-gel; Pierce) to eliminate endotoxin (if any) in the preparation before use in protection studies. Analysis of the final purified rBmALT-2 protein by LAL assay confirmed that the endotoxin levels were comparable to background levels (1 ng/ml).

Elisa:
Forty human sera from Sulawesi, Indonesia, were selected for testing. Recombinant Bm-ALT-1 was coated at 1 µg/ml, a concentration determined to be optimal in a pilot experiment. Isotype-specific monoclonal antibodies were used as described previously [5]. Recombinant Bm-33, an aspartyl protease inhibitor [6], was expressed as a fusion protein with maltose-binding protein (MBP) in the pMAL expression vector. rBm-33 and bacterially expressed MBP were each used to coat enzyme-linked immunosorbent assay (ELISA) plates at 1 µg/ml, and anti-MBP responses were subtracted from those measured for Bm-33/MBP fusion proteins.

Immune response study in animal (jirds or mice or both):
Male outbred Mangolian gerbils (jirds) weighing 35 to 40 g or male BALB/c mice weighing 15 to 20 g were purchased from Charles River Laboratories (Wilmington, Mass.), and five mice or jirds each were used per group. For immunization, mice or jirds were injected intraperitoneally with 10 µg of endotoxin-free rALT-2 protein (single band at 14 kDa) in Imject Alum (Pierce) followed by two booster doses at 2-week intervals. Control animals received equal amounts of adjuvants alone. Serum samples were collected from tail vein every 2 weeks to determine the titer of anti-ALT2 antibodies using an ELISA. Group of mice or jirds injected similarly with the adjuvant alone remained as negative controls.

Because the parasites do not develop into adults in mice, we used a micropore chamber method to determine the degree of protection conferred after immunization with ALT-2. Briefly, two weeks after the last booster dose, a micropore chamber (Millipore Corporations, Bedford, MA) containing 20 L3 of *B. malayi* was implanted into the peritoneal cavity of each mouse. The micropore chamber consisted of a Plexiglas ring (diameter, 13 mm; catalog no. PR0001401; Millipore) covered on either side with polycarbonate membranes (5-µm pore size). Chambers were sealed with cement (Millipore) after introduction of the larvae and checked for leakage. Strict aseptic conditions were followed for the surgical procedure. At 48 h after implantation, animals were sacrificed and the chambers were removed carefully from the peritoneum. Numbers of live and dead larvae in each chamber were then determined under a Nikon inverted microscope.

Male jirds (*Meriones unguiculatus*) were immunized with 75 µg of rALT-1 in CFA subcutaneously or with CFA alone (six jirds per group). At weeks 32 and 33, boosts were given of 25 µg of rALT-1 in incomplete Freund's adjuvant (IFA) or IFA alone, and at week 45 a final boost of 7.5 µg in IFA was given. Two weeks later, all jirds were challenged with 300 larvae of *B. malayi* introduced intraperitoneally. After 4 weeks, jirds were euthanized, and parasites were recovered from the peritoneal cavity and testes. Parasite recoveries and counting were performed without knowledge of the experimental status of each animal.

Collection of peritoneal fluids:
After removing the micropore chamber, the peritoneal cavity of mice was washed with 5 ml of cold sterile phosphate-buffered saline. The fluid was centrifuged at 200 × *g* to remove the cells and supernatant was stored at −70°C. There was minimal contamination of blood in these wash fluids as determined by the presence of erythrocytes. Presence and titer of rBmALT-2 specific antibodies in the peritoneal wash fluid was then determined by an immunoblot analysis.

DISCUSSION

Stage-specific gene expression:
Expression of *alt-1* and *alt-2* at different points of the filarial life cycle was assessed by RT-PCR. Primers designed to span the introns defined above were used with the stage-specific cDNA libraries prepared by the Filarial Genome Project. From these, *alt-1* expression was shown to be strictly L3 specific and *alt-2* largely so, although trace levels of amplification was evident in other stages. To provide greater detail, freshly prepared first-strand cDNA was taken at daily intervals during development of parasites from the microfilarial stage to the infective larva in *A. aegypti* vector mosquitoes. This showed that both *alt-1* and *-2* are switched on between 5 and 6 days following uptake into the mosquito vector and remain expressed for the duration of tenure in the insect. Similarly, parasites were recovered following infection of the rodent host *M. unguiculatus* (the jird or Mongolian gerbil). Here, *alt-1* expression terminated abruptly on transfer into the jird; although brief periods of transcription were detected between days 4 and 8, no subsequent expression could be detected. *alt-2* transcription was less rigorously controlled, with expression continuing for 3 days postinfection and recurring at intervals over the following 3 weeks

Antibodies to recombinant ALT-1 protein reacted specifically with a doublet of 20 kDa in soluble extract of L3 on Western blots, but no reactivity was detectable towards extracts of microfilariae and adult stages. Thus, at the protein level, ALT-1 and -2 are effectively L3 specific. This concords with the larva-specific expression of the related Di20/22L proteins in *D. immitis* [7,8] and of the secreted larval acidic protein of *O. volvulus* [9], both of which are released from larvae once they are cultured under mammalian conditions. Although ALT proteins have not been identified on the surface of *B. malayi* larvae, immunoelectron microscopy has revealed intense staining with anti-Bm-ALT-1 antibody in the larval glandular esophagus, implying that this product may also be stockpiled ready for release within the mammalian host.

Human recognition:
The prominence of the *alt* transcripts suggests that exposed humans may be serologically reactive to the ALT proteins. We tested sera from 40 patients resident in a *B. malayi*-endemic area of Indonesia, drawn equally from the two categories of amicrofilaremic and microfilaremic. The former group will contain both parasite-free individuals and subjects with subpatent infections; the latter group all have detectable blood-borne microfilariae. In patients with filariasis, it is well established that the overwhelming proportion of antibodies to crude adult and microfilaria-stage antigens are of the IgG4 isotype [10,11,12].

Protective immunization:
B. malayi infects only certain host species, among them the jird, which has been used as a model for human filariasis [13]. We immunized jirds with four doses of ALT-1 and challenged them with 300 live L3. Four weeks later, 76% fewer live parasites were recovered from the immunized group versus the adjuvant-only controls. This difference was significant ($P < 0.05$) by Whitney-Mann nonparametric statistics. The data reported here indicate greater than 70% protection in jirds against a challenge infection; this is substantially better than any previous filarial recombinant antigen reported and is in the range achieved by vaccination with radiation-attenuated larvae, 44 to 91% [14]. Of the previously tested antigens, paramyosin has yielded disappointing results [15,16], while heat shock protein 70, myosin, and 1-type IV collagen have recently been shown not to stimulate protective immunity [17]. Thus, ALT-1 and ALT-2 offer the best vaccine candidate yet found for filariasis.

Immunization of jirds (*M. unguiculatus*) with ALT-1. Groups of six jirds were immunized with either ALT-1 in CFA or CFA alone, boosted with ALT-1 in IFA or IFA alone, and challenged with 300 *B. malayi* L3. After 28 days, the number of parasites recovered

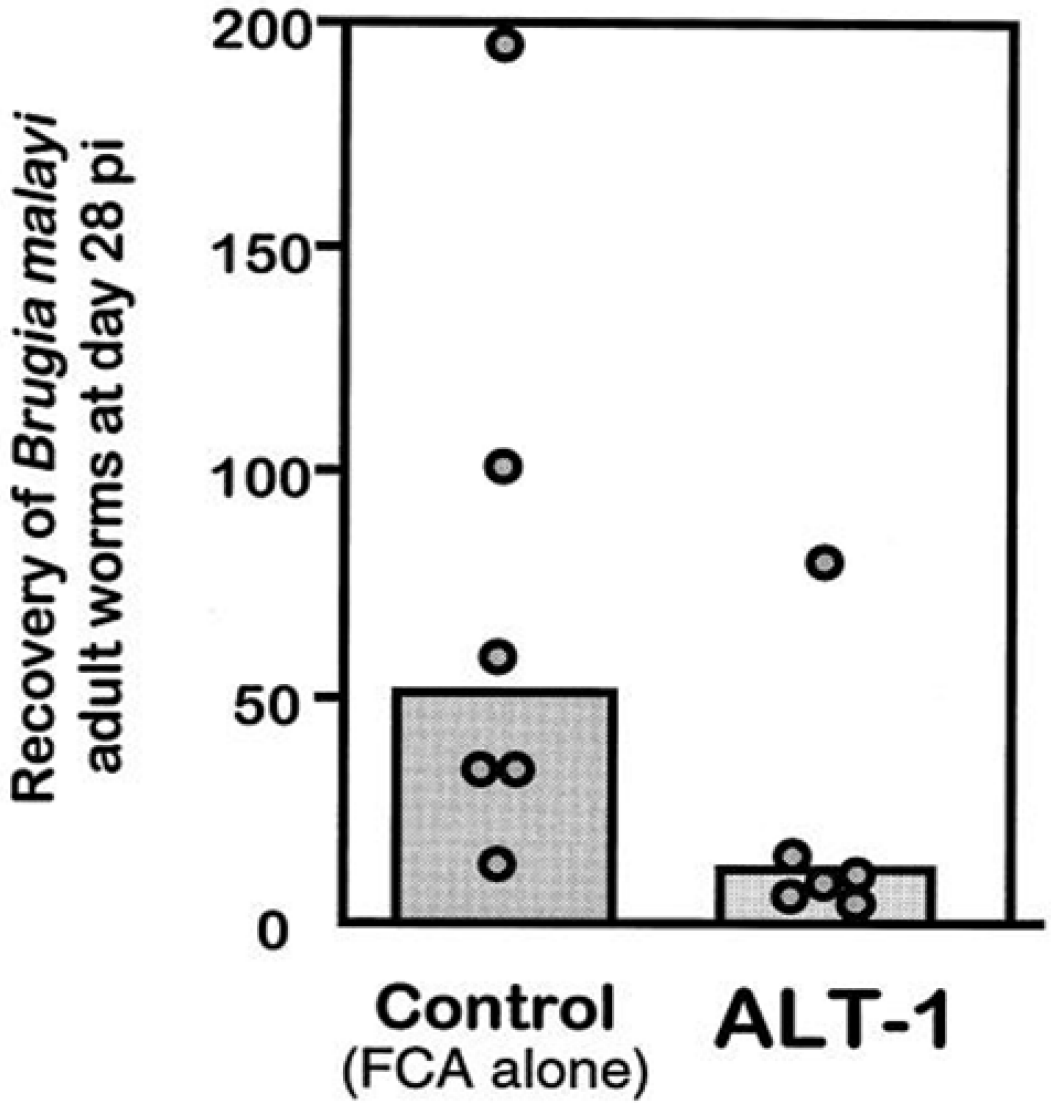

Fig. 1. Immunization of jirds (*M. unguiculatus*) with ALT-1.[21]

CONCLUSION

Vaccination against helminth parasite organisms has proved problematic, both in identifying likely vaccine antigens from the wide repertoire of antigens expressed and with respect to the immunopathological responses to many of these antigens [2,18]. We report here a new approach, selecting highly expressed, stage-specific products such as the ALT proteins, which we show are not present in the mature adult stage. This, coupled with the fact that ALT proteins are parasite-specific products unrelated to any host constituent, renders less likely any adverse consequences of immunization. There is evidence from two other filarial species to associate ALT recognition with immunity [8,19,20], but the data given here provide the first demonstration of protective immunity in a susceptible host. The high level of sequence similarity between ALT sequences from *B. malayi* and *W. bancrofti* suggests that there will be immunological cross-protection between these two species, one of which (*W. bancrofti*) is responsible for >90% of human infections but does not infect laboratory animals [21].

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