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***Brugia pahangi*: Immunization with early L3 ES alters parasite migration, and reduces microfilaremia and lymphatic lesion formation in gerbils (*Meriones unguiculatus*)**

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Abstract

Previous studies have shown that intradermally (ID) injected *B. pahangi* L3s migrate through various tissues and into the lymphatics of gerbils in a distinct pattern. Excretory/secretory products (ES) produced at the time of invasion of *B. pahangi* are likely to be important in this early migration phase of the parasite life cycle in their rodent host. Hence, early L3 ES was collected from 24 hr *in vitro* cultures of *B. pahangi* L3 larvae and used in immunization experiments to investigate the effect of immunity to early L3 ES on worm migration, survival and development of *B. pahangi*. Immunization of gerbils with ES in RIBI adjuvant produced antibodies to numerous ES proteins eliciting a strong humoral response to ES and indirect fluorescent antibody (IFA) assay using anti-ES serum recognized the ES proteins on the surface of *B. pahangi* L3 larvae. Following ES immunization, gerbils were challenged either ID or intraperitoneally (IP) with 100 L3s of *B. pahangi* and euthanized at 3 or 106 days post inoculation (DPI). Immunization with early ES slowed the migration of ID inoculated L3 at 3DPI and significantly altered the locations of adult worms at 106 DPI. Immunization did not induce protection in any treatment group. However, immunized animals had significantly fewer microfilariae per female worm suggesting the antigens in ES are important in microfilariae development or survival in the host. The number of lymphatic granulomas was also significantly reduced in ES immunized animals. It is important to note that microfilariae serve as a nidus in these granulomas. Our results shows immunization with early *B. malayi* L3 ES alters the worm migration, affects circulating microfilarial numbers and reduces lymphatic granulomas associated with *B. pahangi* infection in gerbils.

Keywords

Filariasis; *Brugia pahangi*; immunization; excretory/secretory products (ES); parasite migration; fecundity; parasite granulomas

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1. Introduction

Lymphatic filariasis, caused by the filarial nematodes *Brugia malayi*, *B. timori*, and *Wuchereria bancrofti*, affects approximately 120 million people in tropical and subtropical regions of the developing world and it has been estimated that 2 billion people are at risk (Molyneux et al., 2005; WHO, 2010; WHO, 2011). These infections are initiated when an infected mosquito feeds on a host and infective third stage larvae (L3) emerge from the mosquito proboscis onto the skin surface in a drop of hemolymph (Ewert and Ho, 1967). The L3 then migrates presumably into the bite wound created by the mosquito and through connective tissues of the skin, into the lymphatics and subsequently to the lymph nodes (Scott, 2000). Earlier studies by Ah and his coworkers (1973) demonstrated that vector derived *Brugia pahangi* L3 are capable of extensive rapid migration through a variety of tissue types. In order to quantitatively study this early migration, an *in vivo* intradermal inoculation model has been established using *B. pahangi* infections in gerbils (Chirgwin et al., 2006; Porthouse et al., 2006). In this model, L3s are required to move through the connective tissues of the skin prior to establishment in the lymphatics mimicking the natural early L3 migration pattern more closely than infections induced by either subcutaneous and intraperitoneal (IP) inoculations. Although it is presumed that molecules secreted by the L3 facilitate this migration and are involved in parasite establishment (Scott, 2000) little is known regarding the specific parasite factors involved.

The excretory-secretory (ES) products of the filarial parasite, *B. malayi* are potentially important for pathogenesis and immunodiagnosis of helminth infections (Kaushal et al., 1982). Proteomic analysis of ES products of the L3 and adult male, adult female and microfilarial stages of *B. malayi* identified functional proteins which are stage specific and postulated to have an important role in immune evasion and immunomodulation (Bennuru et al., 2009). Several anti-oxidant proteins such as superoxide dismutase, glutathione peroxidase and thioredoxin peroxidase are shown to be present *B. malayi* L3 ES which can detoxify the reactive oxygen and nitrogen intermediates produced by immune cells, thereby facilitating evasion of parasite inside the mammalian host (Hewitson et al., 2008). Filarial parasites can also suppress the host immune response by releasing protease inhibitors and host cytokine mimics. In *B. malayi*, three cysteine94 protease inhibitors (Bm-CPI-1, -2 and -3) have been characterized (Gregory and Maizels, 2008) and Bm-CPI-2 is expressed in abundance in *B. malayi* ES and nematode homolog of macrophage migration inhibitory factor (MIF-1) is highly expressed in *B. malayi* ES (Gregory and Maizels, 2008; Hewitson et al., 2008). Immunity to these *B. malayi* ES proteins promises to have a significant impact on the establishment of filarial infections in mammalian hosts. Recently it was shown that mice when primed IP with live *B. pahangi* L3 larvae or cuticle from molting L3–L4 larvae or ES of *B. pahangi* L3 conferred significant protection against *B. pahangi* when challenged IP (Dash et al., 2011).

Though previous reports have demonstrated the potential importance of these ES proteins on parasite biology, still the role of ES to early parasite migration remains unanswered. In an attempt to further address this issue we induced an immune response to early ES proteins secreted by L3s during their first 24 hours outside the vector. The hypothesis was that antibody or other immune responses to these proteins would inhibit or alter larval migration and development. In this study we performed intradermal (ID) inoculation which mimics the natural infection pathway and served as a more natural challenge in that L3s are required to migrate through the tissues of the skin prior to establishment in the lymphatic system. Intraperitoneal (IP) inoculation was also performed and served as further controls for parasite development without tissue migration.

Our results show that an immune response to vector derived L3 ES reduces the rate of early larval migration and the subsequent establishment and location of adult worms. Immunity to early ES did not confer significant protection against migrating L3s or the subsequent establishment of adult parasites. However, microfilarial numbers were significantly reduced. Similarly, the development of granulomatous lymphatic lesions, intralymphatic thrombi, was also significantly reduced at the time of patency in ES immunized gerbils.

2. Materials and Methods

2.1. Parasites

Third stage *B. pahangi* infective larvae (L3) were collected from infected *Aedes aegypti* mosquitoes using techniques previously described (Klei, et al., 1990). In brief, 12 days following an infected blood meal, mosquitoes were crushed in Roswell Park Memorial Institute-1640 medium (RPMI) (Hyclone, Logan, UT) and L3 collected in a Baermann apparatus.

2.2. Excretory/Secretory Products (ES)

Seven hundred fifty viable L3s were aliquoted into 15 ml conical tubes and washed 3 times with RPMI supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml of Amphotericin B. Final aliquots were resuspended in 2.5 ml RPMI in 24 well tissue culture plates (BD Falcon, Franklin Lakes, NJ) at a concentration of 750 L3/well. Plates were incubated for 24 hours at 37°C in an atmosphere of 5% CO₂. The culture medium containing ES was collected under a stereomicroscope using pipettes to ensure larvae were not collected and was stored at -20 °C. ES was thawed on ice and concentrated 100× using YM-3 Centriprep centrifugal filter units (Millipore, Billerica MA) with a 3 kDa molecular weight cutoff. ES was centrifuged at 4°C at 2850 × g until approximately 700 µl ES in RPMI was retained. The RPMI was exchanged with cold phosphate buffered saline, pH 7.6 (PBS) containing 1% general protease inhibitor cocktail (Sigma, St. Louis, MO) by centrifuging as above. The ES was collected and supplemented with an additional 1% protease inhibitor. Protein concentration was determined by Lowry's method using the DC Protein Assay (BioRad, Hercules, CA). Lipopolysaccharide (LPS) activity in concentrated ES was measured using the Pyrotell Limulus Amebocyte Lysate assay (Associates of Cape Cod, East Falmouth, MA). The minimum LPS concentration considered as positive for the presence of LPS was 0.25 endotoxin units/ml.

2.3. Animals, Experimental Design and Necropsy

Eight week old male Mongolian gerbils (*Meriones unguiculatus*) were purchased from Charles River Laboratories (Wilmington, MA) and maintained on standard rodent chow and water *ad libitum*. The Institutional Animal Care and Use Committee (IACUC) at the Louisiana State University (LSU) approved the animal experimental protocols. Two *in vivo* experiments were conducted to test the effect of anti-ES response on early larval migration and survival (Experiment 1) and parasite maturation with in the gerbils (Experiment 2). The summary of treatment groups is shown in Table 1. In both experiments, 40 gerbils were divided into 4 groups each containing 10 gerbils. Gerbils were immunized with ES in RIBI adjuvant (Sigma, St. Louis, MO) or adjuvant alone. Gerbils in each immunization group received multiple injections until 100 *B. pahangi* L3s were inoculated ID within the dermis of the lower leg or into the peritoneal cavity (IP) as previously described (Chirgwin et al., 2006; Porthouse et al., 2006). The syringe was flushed with RPMI to check for any remaining L3. The numbers of larvae injected were recorded. The ID inoculations serve as a more natural challenge in that L3s are required to migrate through the tissues of the skin prior to establishment in the lymphatics. Also, the pattern of L3 migration following this injection route has been established in gerbils (Chirgwin et. al., 2006; Porthouse et. al.,

2006). The IP inoculations, in which the majority of parasites do not migrate and remain in the peritoneal cavity, served to determine if anti-ES effects seen in the ID infected groups were related only to migration.

2.3.1. Experiment 1—In Experiment 1, necropsies were performed at 3 to 5 DPI in order to measure the effects of anti-ES immune responses on early migration and parasite survival. Previous studies have shown that by 3 DPI, most larvae are located in the left popliteal lymph node following ID inoculation, indicating that larvae are actively migrating away from the injection site (Chirgwin et al., 2006; Porthouse et al., 2006). Necropsies were performed on 3–5 DPI due to time constraints. Any subsequent citations pertaining to 3–5 DPI are termed 3 DPI for convenience.

On days 3, 24, and 71, gerbils were immunized with 50 µg LPS-free ES in PBS suspended in 300 µl of 1:1 RIBI adjuvant: LPS-free physiological saline. Controls were immunized with 300 µl of 1:1 RIBI adjuvant:LPS-free physiological saline, according to the manufacturer's protocol (Sigma, St. Louis, MO). Each inoculation was given in multiple intramuscular and subcutaneous sites. Animals were bled for sera on days 0 and 86. Gerbils were challenged with 100 L3s in 200 µl of RPMI on day 88 either ID in the left hind limb above the knee or 100 L3s IP.

At 3 DPI, all gerbils were euthanized and worm recoveries performed. The skin and muscle at the injection site of all ID infected gerbils were removed. Right and left popliteal lymph nodes (RPOP and LPOP), right and left renal lymph nodes (RRLN and LRLN), ileo-lumbar vessels (ILV), right and left spermatic cord lymphatics (RSPCD and LSPCD), right and left sub-inguinal and iliac lymph nodes (RSUB and LSUB) and right and left testes (RTEST and LTEST) were gently teased in PBS under a stereomicroscope. In addition, the peritoneal cavity was washed with RPMI as well as the viscera and the carcass soaked for 1 hour in PBS. Following teasing, all tissues were left to soak for 1 hour to allow larvae to emerge. Tissues were examined twice, with hourly soaks in between, and all visible L3s recovered. Necropsy of gerbils with IP infections, worms were collected by washing the peritoneal cavity with RPMI as well as by soaking the viscera and the carcass for 1 hour in PBS.

2.3.2. Experiment 2—The second experiment (Experiment 2) was performed to assess the role of anti-ES immune responses on adult parasite establishment and lesion formation. The immunization schedule, ES dosage, adjuvant and challenge groups in this experiment were same to those of Experiment 1, except the necropsies on infected animals were performed at 106–110 DPI. Microfilariae were counted in 0.5 ml blood at 101 DPI by the modified Knott's method (Chirgwin et al., 2003). All necropsies were not performed at 106 DPI due to time constraints. Any subsequent citations pertaining to 106–110 DPI are termed 106 DPI. ID infected animals were injected with approximately 0.1 ml 1% Evan's blue dye in saline in each hind footpad and testes prior to necropsy to better visualize spermatic cord intra-lymphatic thrombi (ILT), adult parasites, and lymphatic dilation (Klei et al., 1982). ILT were counted in the SPCD and categorized as small, medium or large in size as previously described (Jeffers et al., 1987). Lymphatic dilation scores ranging from 0–4 were determined for each ID infected animal (Klei et al., 1982). The criteria for scoring was as follows: 0, no change; 1, 10–20% of the spermatic cord length of infected animals dilated; 2, 30–50% dilation; 3, 50–80% dilation; 4, >80% dilation. Tissues were teased for adult worms, which were subsequently sexed. In addition to the tissues examined in Experiment 1, the heart and lungs were teased in both IP and ID infected animals. For ID and IP infected animals, the peritoneal cavity was washed with RPMI as well as the viscera and the carcass soaked for 1 hour in PBS.

2.3.2.1 Parasite Growth: In order to determine the effects of anti-ES immune responses on migrating parasite growth, the lengths of adult female parasites recovered from lymphatic sites of ES+BP+ID animals (n=26) and AJ+BP+ID animals (n=20) in experiment 2 were measured. Worms were mounted onto slides in glycerin and scanned using an HP Scanjet 7400c equipped with an HP Scanjet XPA slide adapter. Length measurements to the nearest 0.01 millimeter were accomplished using Image Pro Plus 5 standardized with a stage micrometer.

2.4. ES Characterization and Antibody Response to ES

SDS-PAGE was performed to profile ES proteins and to compare the profile with that of somatic *B. pahangi* L3 antigen (SL3A). Protein electrophoresis was conducted using a 4–20% Tris-HCL polyacrylamide gradient gel (BioRad, Hercules, CA) and a Mini Protean II apparatus (BioRad, Hercules, CA) as previously described (Edmonds, 2001). Eight micrograms of ES or 25 live *B. pahangi* L3s were mixed with loading buffer and denatured as previously described (Laemmli, 1970; Edmonds, 2001). Electrophoresis was carried out at 20 mAmp. Gels were stained with ProteoSilver system (Sigma, St. Louis, MO) following manufacturer's protocol. Photo-imaging was carried out on a FluorChem 8800 photoimager (Alpha Innotech, San Leandro, CA).

Pre-immunized, ES immunized but pre-challenge, and AJ immunized but pre-challenge serum from both experiments, pooled serum from subcutaneously induced *B. pahangi* infections of >1 year (chronic) and from infections of 35 days duration (acute) were analyzed for anti-ES antibodies using Western blots. Antigens used in Western blots included ES and SL3A to assess immunogenic proteins in each extract and somatic antigens of L3 shared with ES. ES was used in ELISAs to assess the relative level of the antibody response.

2.4.1. ELISA—Immulon I B flat bottomed, 96 well polystyrene microwell plates (Dynex Tech., Chantilly, VA) were coated with 0.75 µg of ES from Experiment 1 and 2 in coating buffer (0.02M sodium carbonate buffer, pH 9.6). After antigen was bound overnight at 4°C, antigen and coating buffer were removed and plates washed 3× for 5min with 200µl of PBS-0.05% Tween 20 (PBST). Plates were blocked for 1.5 hours at room temperature with 100µl of 3% fish gelatin-PBS and washed again. Ten individual samples (either ES immunized or adjuvant immunized from Experiment 1 and 2) were used as the primary antibody. Primary antibody was diluted 1:100 in 1% fish gelatin-PBST (Ab buffer) and incubated in triplicates with ES for 1hr at 37°C. After washing, 50µl of goat anti-mouse IgG antibody conjugated to alkaline phosphatase (IgG-AP) (Jackson ImmunoResearch, West Grove, PA), or rabbit anti-mouse IgG1, IgG2a, IgG2b, or IgG3-AP (Rockland, Philadelphia, PA) diluted 1:1000 in Ab buffer were added to plates and incubated 1hr at 37°C. After washings, 100µl of BluePhos microwell phosphatase substrate was added to each well according to the manufacturer's protocol (KPL, Gaithersburg, MD). Reactions were stopped after 20min with 100 µl of 1× BluePhos Stop Solution (KPL, Gaithersburg, MD). OD values were read on a MRX TC Revelation ELISA reader standardized with PBST. The minimal OD value considered accurate was either 2.5% of the coefficient of variance or 0.005 OD, whichever is greater, as determined by the manufacturer.

2.4.2. Western Blot Analysis—Eleven and a half micrograms of ES and SL3A from 80 live *B. pahangi* L3s were separated using SDS-PAGE as described above. Unstained electrophoretically-resolved proteins were transferred to nitrocellulose using a Mini Trans Blot electrode (BioRad, Hercules, CA). Protein transfer was carried out at 25 mAmp overnight followed by 225 mAmp for 1 hour at 4°C. Western blot was carried out as previously described (Edmonds, 2001). All sera were diluted 1:1000 in 1% fish gelatin-TBS

(Ab buffer). Chronic, acute, pre-immunized, and adjuvant pre-challenge serum were diluted 1:250 for each type of antigen. Anti-ES serum was diluted 1:1000 for ES and 1:250 for SL3A. IgG-AP was diluted 1:10,000 in Ab buffer and incubated for 1 hr at 37°C. Anti-mouse antibodies were used due to a lack of suitable commercial gerbil reagents. These have been reported and used by others (Hartmann et al., 2006). These reagents recognize gerbil antibodies although isotype matching of gerbil immunoglobulins remains undescribed. Conjugates were developed with 3 ml of BCIP/NBT 1-component phosphatase substrate (KPL, Gaithersburg, MD). Double-distilled water was used to stop the reaction development. Photo-imaging was carried out on a FluorChem 8800 photoimager (Alpha Innotech, CA).

2.4.3. Indirect Fluorescent Antibody (IFA) assay—A positive anti-L3 gerbil serum was produced for use in this experiment. Five thousand L3s were harvested from mosquitoes as described previously (Klei, et al., 1990). L3s were washed three times in PBS with 1% general protease inhibitor and crushed with a glass tissue homogenizer. Five gerbils were immunized three times at two week intervals each with 1000 crushed L3s suspended in 150 µl PBS and 150 µl RIBI adjuvant. Serum was collected two weeks after the final immunization and pooled.

L3s were harvested as stated above and fixed in 4% paraformaldehyde on ice for 1 hour. Larvae were washed with PBST and resuspended in PBST at a concentration of 2 L3/µl. Fifty µl of fixed L3s were added to 96 well flat bottomed microtiter plates (Costar, Corning, NY). Each well was added either with 50 µl of positive anti-L3 serum or pooled anti-ES or pooled adjuvant serum at final serum dilution of 1:10. Samples were incubated at 4°C for 20 hours. Three washings with PBST were carried out by gently aspirating the fluid with a multichannel pipette. Subsequent to the last wash, the L3s were resuspended in 50 µl PBST. Horse anti-mouse IgG antibody conjugated to biotin (Vector Labs, Burlingame, CA) was diluted 1:100 in PBST. Fifty µl of the diluted conjugate was incubated with the 50 µl of worms for a final concentration of 1:200 biotinylated antibody for 1 hour at 37°C. Wells were washed and resuspended as described above. Streptavidin conjugated to Alexa fluor 488 (Invitrogen, Carlsbad, CA) was diluted 1:250 in PBST. Fifty micro liters of the diluted conjugate was incubated with the 50 µl of worms for a final concentration of 1:500 streptavidin conjugate for 1 hour at 37°C. Wells were washed 5 times and resuspended as described and examined on a Leica DM 4000B fluorescence microscope (Wetzlar, Germany) equipped with an Evolution QEi monochromatic camera (MediaCybernetics, Silver Spring, MD). Worms were examined for the presence or absence of an antibody reaction on the surface.

2.5. Statistical Analysis

Statistical analysis was performed using SigmaStat software (Chicago, IL). When data passed normality tests, pair wise multiple comparisons were made using the Student-Newman-Keuls method. One Way Analysis of Variance on ranks was used when normality tests failed. Differences were considered statistically significant at $P = 0.05$.

3. Results

3.1. Composition of L3 ES used in experiments 1 and 2 was complex and similar

SDS-PAGE was used to demonstrate the protein profile of ES and compare it with that of SL3A. The overall profiles of the mixtures were similar. Some small differences in the protein profiles were, however, observed between ES used in Experiment 1 and ES used in the Experiment 2 (Fig. 1). Fewer distinct bands were observed in Experiment 2 ES although more bands were observed in the lower molecular weight range. Nonetheless, the general

pattern of bands appeared to be the similar. The protein profile of SL3A was different from that of the ES used in both experiments (Fig. 1).

3.2. L3 ES contains numerous specific antigenic proteins

ES used in Experiments 1 and 2 and SL3A were transferred to nitrocellulose after separating by SDS-PAGE and blots were incubated with pooled pre-challenge serum from Experiments 1 and 2, pooled pre-immunized serum as well as pooled serum from gerbils with acute and chronic infections.

Some nonspecific binding of antibody to ES was observed when blots were incubated with all types of serum. This is particularly distinct in band at 62kDa (Fig 2, dotted light arrow). Pre-challenge serum from ES immunized animals in both experiments detected multiple similar protein bands in homologous ES. However, there are also some differences. There were 3 bands observed in Experiment 1 ES at 97, 40 and 33 kDa (indicated by light arrows) not visible in Experiment 2 ES (Fig. 2 lanes; A1, A2). In both experiments, anti-ES serum recognized heavy bands at 14 kDa and 20 kDa (indicated by dark arrows). Some bands detected in ES with pre-challenge serum were recognized weakly by acute and chronic infection serum indicating that the immune response in ES immunized animals markedly differ for that of unimmunized animals with existing acute and chronic lymphatic infections.

In ES used in Experiment 2, fewer bands were observed in ES from the same experiment when blots were incubated with acute or chronic infection serum. Identical reactions were seen with both Experiments 1 and 2 ES when blots were incubated with using control serum (adjuvant and pre-immunized). As expected, acute and chronic serum recognized different proteins in SL3A when compared to that of anti-ES serum (Fig. 3). Nonspecific reactions were not seen with any serum. Anti-ES, acute, and chronic infection serum recognized a 14 kDa band (indicated by light arrow).

3.3 Increase in ES specific IgG response

In both the ES immunization experiments, a significant IgG response to ES was observed in ES immunized gerbils. ES immunized animals in experiment 1 had a mean OD value of 1.074 ± 0.261 compared to the adjuvant controls of 0.163 ± 0.031 . In experiment 2, antibody responses to ES immunized animals had a mean OD value of 1.026 ± 0.283 compared to the adjuvant controls of 0.268 ± 0.075 .

3.4. Antibodies to ES recognize the surface of infective L3s

The L3s was examined for an antibody reaction in an IFA assay using pooled anti-ES serum from Experiments 1 and 2. Anti-L3 serum served as a positive control and serum from adjuvant immunized gerbils served as a negative control. 1:10 dilution of positive control anti-L3 serum produced an intense green patchy fluorescence spots along the entire surface, presumably the cuticle, of L3s. Accumulations of fluorescence were not seen at any orifice of the L3s examined. Staining of internal organs was not seen in the intact L3 used in these assays (Fig. 4A). A similar staining pattern was seen when the L3 larvae were stained with 1:10 dilution of anti-ES serum, (Fig. 4B). No fluorescence staining was observed using serum from animals immunized with adjuvant alone (Fig. 4C).

3.5. Immunization with L3 ES reduces early migration rates but does not confer protection

Total L3 recoveries were significantly greater in animals immunized with ES and challenged ID (ES+BP+ID) as compared to ID controls which were immunized only with adjuvant (AJ+BP+ID) (Fig. 5A). Although not significant, greater numbers of L3s were also found in ES immunized animals challenged IP (ES+BP+IP) as compared to IP controls (AJ+BP+IP) (Fig. 5A). Recoveries were significantly higher in IP infected animals of both treatment

groups (ES+BP+IP and AJ+BP+IP) when compared to ID inoculated animals (ES+BP+ID and AJ+BP+ID) (Fig. 5A) as expected in this model (McCall et al., 1973).

In both ID groups (ES+BP+ID and AJ+BP+ID), the total percent recoveries of L3s from different sites showed that a majority of larvae migrated away from the injection site, skin and muscle, of the left leg by 3 DPI (Fig. 5B). Most of these larvae were located on the left side of the animals' bodies in both treatment groups. At 3 DPI, tissues that harbored the most larvae in ES immunized and ID challenged animals (ES+BP+ID) were the LPOP, ILV, skin, and muscle. Within the ID controls (AJ+BP+ID), the larvae were found in highest numbers in the LPOP, LRLN, muscle, LSUB and LSPCD. Significantly more larvae were recovered from LSUB in AJ+ BP ID vs. ES+BP+ID animals and from the ILV in ES+BP +ID compared to AJ+BP+ID animals (Fig. 5B).

Larval recoveries from different tissues following ID challenge (ES+BP+ID and AJ+BP +ID) were also calculated according to proximity to the intradermal challenge site (Fig. 5C). Tissues proximal to the injection site included the skin and muscle surrounding the site and LPOP which is the initial lymph node that filters lymph from the challenge site. Other lymphatic sites considered distal to the injection site included RRLN, LRLN, RSUB, LSUB, RSPCD, LSPCD, RTEST, and LTEST. The RPOP was not considered since no larvae were recovered in this location in either treatment group. In addition, the ILV was not considered due to ambiguity as to whether its location is proximal or distal to the challenge site. As expected, based on previous observations (Chirgwin et al., 2006; Porthouse et al., 2006) in both treatment groups, the majority of larvae were found proximal to the challenge site at 3 DPI (Fig. 5C). However, there was significantly greater percentage of larvae recovered proximally in ES immunized animals challenged ID (ES+BP+ID) compared to ID controls (AJ+BP+ID). Conversely, in ID controls (AJ+BP+ID), significantly more larvae were recovered distally to the injection site.

3.6. Immunization with L3 ES alters long term parasite migration

A similar number of adult parasites were recovered in ID challenged animals in both treatment groups (ES+BP+ID and AJ+BP+ID) at 106 DPI (Fig. 6A). As for IP infected animals, 19% fewer adult parasites were recovered in ES immunized animals (ES+BP+IP) compared with controls (AJ+BP+IP), though this reduction was not statistically significant.

The majority of adult parasites recovered from ID infected animals were located in the heart and lungs, SPCDs, and ILV of both groups (ES+BP+ID and AJ+BP+ID) (Fig. 6B). A preponderance of worms was recovered on the right side of the body (RPOP, RSUB, RRLN, and RSPCD) in ES+BP+ID gerbils. Furthermore, increased worm recoveries from AJ+BP +ID controls were noted on the left side of the body (LPOP, LSUB, LSPCD, and LTEST). The only significant difference was observed in the LTEST. Because of these trends, the mean numbers of parasites for each treatment were grouped according to the right and left side of the gerbil body (Fig. 6C). Significantly more parasites were recovered on the left side; the side L3 larvae are inoculated, compared with the right side in AJ+BP+ID controls, as expected. This was not the case in ES+BP+ID as no significant difference in worm numbers was seen between the right and left side recoveries and significantly more adult worms were recovered on the right side of the body in ES+BP+ID animals than on the right side of the body in AJ+BP+ID animals (Fig. 6C).

3.7. Immunization with L3 ES does not affect adult female parasite growth but does reduce microfilaremia

The lengths of adult female parasites recovered were measured in millimeters and reported as mean $\pm$ S.D. There was no significant difference between ES immunized animals

challenged ID (ES+BP+ID) (42.88 ± 5.76) and adjuvant immunized animals challenged similarly (42.95 ± 5.54).

Although there was no statistically significant differences in the mean numbers of microfilariae/500 μ l of blood between the ES immunized and adjuvant immunized animals, more microfilariae were noticed in AJ+BP+ID (413 ± 430) and AJ+BP+IP (110 ± 218) controls when compared to ES+BP+ ID (172 ± 314) and ES+BP+IP (57 ± 115) groups (Fig. 7A). In ID infected animal groups, when mean MF produced per female worm was calculated by dividing the number of MF by total number of female parasite recovered, significantly more MF were produced per female parasite recovered in AJ+BP+ID controls (20.17 ± 17.37) compared with ES+BP+ID animals (9.64 ± 14.12) (Fig. 7B).

3.8. Immunization with ES reduces the formation of intralymphatic thrombi (ILT)

The role of ES immunization on the development of ILT was examined. There were significantly more ILT produced per female parasite in AJ+BP+ID control animals compared to ES+BP+ID animals (Fig. 8). In order of frequency of occurrence, small, medium, and large sized ILT were observed for both treatment groups. No differences were detected in degree of lymphatic dilation between treatment groups.

4. Discussion

Excretory/secretory products (ES) are likely to serve multiple vital roles in the early migration and establishment of tissue migrating nematode L3s. These include but are not limited to, aiding in parasite migration (Ghosh and Hotez, 1999; Tsuji et al., 2003; Tsuji et al., 2004; Bethony et al., 2005), immunomodulation perhaps by modulating cytokine signaling (Gomez-Escobar et al., 2005), and/or suppressing B and T cell proliferation (Harnett and Harnett, 1993). Anti-ES immunity has been demonstrated to be protective against *B. malayi* (Gregory et al., 2000) and in animals with other nematode infections (Tsuji et al., 2003; Tsuji et al., 2004; Bethony et al., 2005). Antibody to ES has also been demonstrated to inhibit migration of *Necator americanus* L3 through hamster skin *in vitro* (Goud et al., 2005). Therefore, functional inhibition of essential components in ES by their specific antibody can be expected to alter the outcome of infection. To test this hypothesis, gerbils were immunized with early L3 ES followed by ID challenge with infective *B. pahangi* larvae. The effects of anti-ES on tissue migration and early establishment were investigated by performing necropsies at 3 days following ID inoculations of infective L3 larvae. Necropsies at a later time measured the significance of L3 ES on later migrations and development of a patent infection. Any effect of the immunizations on IP infections was related to inhibitions of parasite factors not important to tissue migration.

Some differences in the protein profiles of the ES used in Experiments 1 and 2 were seen in silver stained SDS-PAGE gels and differential band recognition by acute and chronic serum. Protein profile of ES from experiment 1 and 2 was different from that of somatic L3 antigen (SL3A) which is in accordance with previously published data on proteomics analysis of ES and somatic extracts of *B. malayi* where 118 proteins were identified only in ES and not in somatic worm extracts (Bennuru et al., 2009). The reason for difference in protein profile between ES and SL3A is not clear and how or why some proteins are only found in the ES. However it is possible that these proteins are expressed in relatively low levels in somatic protein preparations but are upregulated in the ES products.

ES immunized gerbils in both experiments showed an increase in antibody response to ES at the time of challenge. Anti-ES serum when used in an IFA assay was able to recognize ES proteins in the surface region of L3s, presumably and cuticle indicating of presence of antibodies to major ES antigens in anti-ES serum. In spite of the presence of these

antibodies, no significant worm reductions were noted in immunized gerbils challenged either ID or IP in either experiment, indicating that the induced immune response and these antibodies to early ES proteins collected in the first 24 hrs of culture of infective L3 larvae do not provide considerable protection against *B. pahangi* infection in gerbils.

The reason for the lack of protection induced by ES in our *B. pahangi*-Mongolian gerbil model could be that the ES were collected from L3 larvae in culture for a period of 24 hrs only. There is a report where IP priming of mice with live *B. pahangi* L3 larvae or L3-L4 cuticle or ES collected over a period of 7 days conferred significant protection against L3 when challenged IP (Dash et al, 2011). Since in our study only early ES secreted during the first 24 hrs of L3 culture were used for immunization, it could be possible that a variety of major ES proteins which confer protection were absent or were present in lower concentration in the ES preparations used in the current experiments. It is also possible that these essential protective proteins were present but altered during the collection process destroying the relevant epitopes required for protection. It is likely that immune responses to the ES proteins as well as proteins of other developmental stages of the parasite are required to induce a significant level of protection. No measure of cellular immunity was made in this study but it is possible that an important aspect of cellular immunity was not induced in these ES immunization experiments.

Although no significant protection was observed in either experiment, it is important to note that larval recoveries in Experiment 1 were actually increased in ES immunized animals, particularly in the ID infected individuals at 3DPI (Fig. 5A). This probably occurred due to decreased larval migration, facilitating the recovery of L3 that concentrated in the area around the injection site. In control animals, the larvae were more scattered and therefore more difficult to recover. When mean worm recovery were represented as region relative to inoculation sites, proximal and distal region, compared to controls, significantly more worms were recovered in proximal region and less worms were recovered in distal region of ES immunized animals suggesting that ES immunization limits the worm migration (Fig. 5C). It is likely that this reduction in migration rate was induced by an early local inflammatory response seen in tissues as early as 3 hrs post L3 inoculation (Porthouse et al., 2006). It is known that larvae also migrate to tissues other than those examined at necropsy, such as the heart and lungs (Ah and Thompson, 1973). Since larvae in control animals migrated faster than in immunized animals, it is possible that more larvae migrated to those tissues such as heart and lungs not examined at necropsy 3DPI.

While protection was not observed in immunized animals (Fig. 6A), alterations in migration patterns were seen in ID infected individuals at 106 DPI. From our earlier work, we know that when gerbils are infected with *B. pahangi* L3 larvae ID in the dermis of the lower left leg, a majority of worms are recovered on the left side of the gerbil (Chirgwin et al., 2006). This was changed in ES immunized animals where slightly more worms were recovered on right side of gerbil compared to left side and as expected in control animals significantly more worms were recovered on left region compared to right region (Fig. 6C). This difference in the final location of adult worms suggests a drastic change in the migration of worms from left to right region in the ES immunized animals. Since *Brugia* spp. requires extensive tissue migration prior to parasite establishment, it is probable that proteins involved in processes such as tissue destruction are produced and secreted by invading L3s (Scott, 2000). It is possible that antibody-mediated neutralization of these proteins could partially explain the change in the migration pattern of worms found in immunized animals at 106 DPI.

The levels of circulating microfilariae were lower in ES immunized gerbils challenged ID and IP in Experiment 2 although this reduction was not significant (Fig. 7A). More

importantly, significantly fewer circulating microfilariae per adult female worm were seen in ES immunized animals compared to adjuvant controls following ID infections (Fig. 7B). Previous studies in this model demonstrated that immunizations with somatic *B. pahangi* antigen reduced the number of circulating microfilariae and increased granulomatous lesions without a concomitant reduction in adult worms (Klei et al. 1982). Similar results of decreased microfilaremia and increase in lymphostatic disease were seen in *B. malayi* infected ferrets immunized with lyophilized microfilariae (Crandall, et al. 1994). Immunizations with ES proteins have been demonstrated to reduce female nematode fecundity in other host parasite systems. Dogs immunized with ASP proteins show reduced adult hookworm fecundity as determined by a reduction in egg shedding (Bethony et al., 2005). Similarly, significant reductions in parasite fecundity was also seen in hamsters immunized with ASP-2 produced by *Ancylostoma ceylanicum* (Mendez et al., 2005). The reasons for the reduction seen in circulation microfilariae in the current study are unclear but could be due the killing of microfilariae released by the female worms or by a direct immune mediated effect on the female parasites reducing in the fecundity of the worms.

A reduction in the granulomatous response as indicated by the numbers of ILT was seen in ES immunized gerbils in Experiment 2. This observation is contrary to results of previous experiments which demonstrate that immunization with filarial antigens increases granulomatous lesions (Klei et al. 1982, Crandall et al. 1994). The reduction in ILT seen in the current experiment may be indirectly related to the reduced numbers of microfilariae in circulation seen in the ES immunized animals. The uteri of the female worms in the current study were not examined for developing microfilariae and thus direct evidence for a reduction in fecundity is not available. Nonetheless it is known that microfilariae contribute to ILT formation. This is evident from previous experiments where microfilariae were found as the nidus of ILT in microfilaremic gerbils (Vincent et al., 1980; Jeffers et al., 1987). If there were more microfilariae in the SPCD, then it is likely that more ILT will be formed, while fewer microfilariae will result in fewer ILT. It is also possible that some unknown phenomena such as an alteration in the immunomodulation of ILT formation was induced by the ES immunization were responsible for these observations.

In conclusion, our findings suggest that antibodies to early L3 ES collected during the first 24 hrs of *in vitro* *B. pahangi* L3 larvae cultures are not sufficient to induce protection against *B. pahangi* infection in gerbils. Nevertheless, the immune response to early ES showed reduction in early migration rates and changes in worm migration pattern and reduced microfilarial numbers and lymphatic lesion formation. Further analysis of the biological functions of these ES products in the host-parasite interaction will bring a clearer picture on how this complex mixture of products functions in the host-parasite interaction.

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Index Descriptors and Abbreviations

BP	<i>Brugia pahangi</i>
ES	Excretory/secretory products
L3	infective third stage larvae
ID	intradermal
IP	intraperitoneal

AJ	adjuvant
intraperitoneally (ES+BP+IP)	ES immunized animals challenged with L3s intradermally (ES+BP+ID)
intraperitoneally (AJ+BP+IP)	Adjuvant immunized animals challenged with L3s intradermally (AJ+BP+ID)
RPOP and LPOP	Right and left popliteal lymph nodes
RRLN and LRLN	right and left renal lymph nodes
ILV	ileo-lumbar vessels
RSPCD and LSPCD	right and left spermatic cord lymphatics
RSUB and LSUB	right and left sub-inguinal and iliac nodes
RTEST and LTEST	right and left testes
ILT	intralymphatic thrombi
SL3A	<i>B. pahangi</i> somatic L3 antigen
chronic	<i>B. pahangi</i> infection of > 1yr
acute	<i>B. pahangi</i> infection of 35 days duration
PBST	PBS-0.05% Tween 20
Ab buffer	1% fish gelatin-PBST
IgG-AP	goat anti-mouse IgG antibody conjugated to alkaline phosphatase
AP	alkaline phosphatase
ALT	abundant larval transcript protein
anti-ES1	anti-ES from Experiment 1
anti-ES2	Experiment 2

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Evaluation of immunization of gerbils with 24 hrs early excretory/secretory products (ES) of *Brugia pahangi* L3

Immunity to early L3 ES does not confer protection against *B. pahangi* infection.

But reduces early worm migration rates and alters worm migration pattern in long term.

Reduces the fertility of female worms.

Reduces the number of lymphatic granulomas associated with *B. pahangi* infection.

Fig. 1. Protein profile of ES antigens

Silver stained SDS-PAGE gel, lanes show Experiment 1 ES (ES1), Experiment 2 ES (ES2) and somatic L3 antigen (SL3A).

Fig. 2. Western blots using ES

ES used in Experiments 1 (A1, B1, C1, D1, & E1) and 2 (A2, B2, & C2) were run on a SDS-PAGE gel and proteins were transferred to nitrocellulose membrane. Blots were incubated with various pooled serum including anti-ES pre-challenge serum from Experiment 1 (A1) and Experiment 2 (A2), acute infection serum (B1, B2), chronic infection serum (C1, C2), adjuvant serum (D1), and preimmunized serum (E1).

Fig. 3. Western blots using somatic L3 antigen (SL3A) and serum from both experiments
Blots were incubated with anti-ES prechallenge serum from Experiment 1 (A1) and 2 (A2), acute infection serum (B1), chronic infection serum (C1), adjuvant serum from Experiment 1 (D1), and preimmunized serum from Experiment 1 (E1).

Fig. 4. IFA assay using anti-ES serum

B. pahangi L3 larvae were incubated either with positive anti-L3 serum (4A) or pooled anti-ES serum (4B) or pooled negative adjuvant serum (4C) at a serum dilution of 1: 10. Slides were photographed at 20X.

A

B

Fig. 5. Experiment 1: Anti-ES immunization reduces worm migration rates

Animals were immunized with either ES in RIBI adjuvant (ES immunized) or with RIBI adjuvant alone (AJ immunized) and challenged ID or IP.

5A. Total worms recovered at day 3 post infection (3DPI). 5B. Total worms recovered at 3DPI showed as specific sites. Specific sites examined included the skin and muscle surrounding the injection site. Lymph nodes examined were the left popliteal (LPOP), left and right subiliac and inguinal (LSUB and RSUB), and left and right renal (LRLN and RRLN) nodes. Other lymphatic sites included the left and right spermatic cord (LSPCD and RSPCD), ileo-lumbar vessels (ILV), and the left and right testes (LTEST and RTEST). The RPOP was not included because no larvae were found in this site in their immunized group. 5C. Total worms recovered at 3DPI showed as grouped sites. Sites were grouped according to proximity to the injection site. Proximal site include skin, muscle and LPOP. Distal sites are RRLN, LRLN, RSUB, RSPCD, LSPCD, RTEST, and LTEST. * denotes statistical significance ($P < 0.05$) between experimental and control groups. Error bars represent SEM.

A

C

Fig. 6. Experiment 2: Anti-ES immunization alters worm migration

Animals were immunized with either ES in RIBI adjuvant (ES immunized) or with RIBI adjuvant alone (AJ immunized) and challenged ID or IP. 6A. Total worms recovered at day 106 post infection (106 DPI). 6B. Total worms recovered at 106 DPI (106 DPI) showed as specific sites. Specific sites examined included lymph nodes: left and right popliteal (LPOP and RPOP), left and right subiliac and inguinal (LSUB and RSUB), and left and right renal (LRLN and RRLN) nodes. Other lymphatic sites included the left and right spermatic cord (LSPCD) and (RSPCD), ileo- lumbar vessels (ILV), and the left and right testes (LTEST and RTEST). Non-lymphatic sites were the heart and lungs and the peritoneal cavity (Per Cavity). 6C. Total worms recovered at 106 DPI (106 DPI) showed as location in gerbil, left or right side. The gerbil body is divided sagittally. Tissues located on the right side include RPOP, RRLN, RSUB, RSPCD and RTEST and on the left side include LPOP, LRLN, LSUB, LSPCD, and LTEST. * denotes statistical significance ($P < 0.05$) between experimental and control groups. Error bars represent SEM.

A

B

Fig. 7.

Mean microfilarial counts per female parasite recovered in anti-ES immunization experiment 2. Animals were immunized with either ES in RIBI adjuvant and challenged ID with *B. pahangi* L3s (ES+BP+ID) or RIBI adjuvant alone and challenged similarly (AJ+BP+ID). 7A. At 101 DPI, 500 µl blood was collected and microfilariae were counted. 7B. Microfilariae per female worm calculated by dividing the number of MF by total number of female parasite recovered at 106 DPI. * indicate statistical significance ($P < 0.05$) between experimental and control groups. Error bars represent SEM.

Fig. 8. Total intralymphatic thrombi (ILT) produced per worm in the SPCDs in anti-ES immunization experiment. Animals were immunized with either ES in RIBI adjuvant and challenged ID with *B. pahangi* L3s (ES+BP+ID) or RIBI adjuvant alone and challenged similarly (AJ+BP+ID). At 106 DPI, adult worms and ILT were counted in the SPCD lymphatics and ILT to worm ratio was calculated. * indicate statistical significance ($P < 0.05$) between experimental and control groups. Error bars represent SEM.

Table 1

Summary of treatment groups to test the effects of ES immunization on early larval migration (Experiment 1)^a and parasite establishment (Experiment 2)^b

Treatment Group	Immunization	Inoculation	Location of Inoculation
Immunized and challenged intradermally (ES+BP+ID)	ES in AJ ^c	BP ^d	ID ^e
Immunized and challenged intraperitoneally (ES+BP+IP)	ES in AJ	BP	IP ^f
Adjuvant control and challenged intradermally (AJ+BP+ID)	AJ ^h	BP	ID
Adjuvant control and challenged intraperitoneally (AJ+BP+IP)	AJ	BP	IP

^aNecropsy performed in each treatment group at 3DPI

^bNecropsy performed in each treatment group at 106DPI

^cES in AJ: Gerbils were immunized with ES in RIBI adjuvant

^dBP: 100 *Brugia pahangi* L3s

^eID: intradermal challenge

^fIP: intraperitoneal challenge

^hAJ: Adjuvant controls