Granuloma formation around *Brugia* Larvae

triggered by host responses to an E/S antigen

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Abstract

In previous studies using a murine model of *Brugia malayi* infection, granuloma formation was found to be a most important host protective mechanism. Previous studies also showed that *in vitro* cytoadherence is a surrogate for the formation of anti-filarial granulomas *in vivo* and this function requires “alternatively activated” host cells and a source of anti-filarial antibody. In this manuscript we show that antibodies against L3 excretory/secretory (E/S) products can facilitate *in vitro* cytoadherence. We generated a set of hybridomas reactive with filarial E/S products and screened them for their ability to mediate *in vitro* cytoadherence. One clone (1E9) that produced high titer of IgM antibodies were used in subsequent assays. To identify the antigen(s) recognized by the IE9 clone, we screened a T7 bacteriophage displayed cDNA expression library of *B. malayi* L3 with IE9 clone as the bait. The screening procedure identified two filarial antigens (TCTP and BmALT-2) that bound to 1E9. Immunization of mice showed that the cohort immunized with BmALT-2 cleared a challenge infection with infective *B. malayi* L3 in an accelerated manner, whereas cohorts immunized with TCTP cleared larvae with the same kinetics as unimmunized mice. These data confirm that the antigenic target of granuloma-mediated killing of *B. malayi* L3 is possibly BmALT-2. Our findings also confirm previous studies that BmALT-2 is a potential vaccine candidate for filarial infection. Our data reinforce the work of others, and also provide a possible mechanism by which immune responses to BmALT-2 provide host protection.
**Introduction**

Normal, immunocompetent mice quantitatively eliminate infections with *Brugia malayi* infective larvae. In contrast, inbred strains deficient in certain components of the immune system permit the larvae to grow to maturity (11). This dichotomous outcome has permitted us to analyze the mechanism of mammalian host protection against large, extracellular pathogens. In previous publications, we have shown that normal, immunocompetent mice form large multicellular host immune cell aggregates called granulomas around infected larvae (11). Mice that are deficient in T lymphocytes (such as TCR$^{αβ}$ knockout mice) (15), B1 B lymphocytes (such as CBA/N mice) (9) or both (such as SCID mice) (8) fail to form such granulomas. This, and other aspects of the kinetics of formation of granulomas have led us to propose that granuloma formation is one, if not the most important, mechanism by which mammals defend themselves against large extracellular-dwelling pathogens.

A mutant mouse strain that has been particularly helpful in dissecting the mechanism of granuloma formation has been the secretory IgM knockout mouse (secIgM$^{-/-}$ mouse) (2, 3). In this strain, cellular influx to the site of infection (the peritoneal cavity in our model) is similar to normal, immunocompetent mice; in addition, leukocytes at the site of infection become alternatively activated as they do in immunocompetent mice. However, in the absence of circulating IgM, granulomas do not form and worms are not eliminated with normal kinetics (10). This observation alerted us to the critical role of circulating anti-filarial antibodies, particularly of the IgM isotype in granuloma formation. However, the identity of the filarial antigens responsible for eliciting the requisite antibodies was not revealed in the previous studies.
In the course of these studies, we found that adherence of alternatively activated macrophages and eosinophils to infective larvae provides an *in vitro* surrogate for granuloma formation *in vivo*. This rapid *in vitro* test permits us to quickly assay the ability of cells or sera to mediate host protection.

In this communication, we describe our efforts to determine the identity of the candidate antigens against which host response is directed. We show that antibodies directed against a filarial protein known as *B. malayi* abundant larval transcript-2 (BmALT-2) (5, 6) are capable of promoting *in vitro* cytoadherence of alternatively activated macrophages to filarial larvae. Further, immunization of mice with BmALT-2, even in the absence of adjuvants, results in elimination of infective larvae with accelerated kinetics. These observations suggest that BmALT-2 can promote granuloma formation around the larva. Our findings also support and extend previous studies showing that BmALT-2 is a potential vaccine candidate for lymphatic filariasis (6, 16).
**Materials and methods**

**Mice:** C57BL/6J and BALB/cByJ mice were obtained from the Jackson Laboratories (Bar Harbor, ME). B6; 129S4-Igh-6 \(^{tm1Che} \)/J (secIgM\(^{-/-}\)) (2, 3) mice were obtained initially from the Jackson Laboratories. They were subsequently housed and bred at the AAALAC accredited University of Connecticut Health Center vivarium.

All mice were maintained under specific-pathogen-free (SPF) conditions in microisolator cages. They were given lab chow and sterile water *ad libitum*. The integrity of our secIgM\(^{-/-}\) colony was periodically confirmed by the absence of serum IgM levels in randomly selected mice as determined by sandwich ELISA.

**Infectious larvae:** *B. pahangi* L3 were harvested at TRS Inc., Athens, GA, the University of Georgia (Dr. John McCall) or the University of Louisiana (Dr. Thomas Klei) from infected *Aedes aegypti* mosquitoes and transported in RPMI supplemented with antibiotics as described previously (18).

**Experimental infection:** Mice were injected with approximately 50 *B. pahangi* L3 intraperitoneally in 500 µl of RPMI using 1 ml syringes fitted with 5/8” 25G needles. For challenge infections, 50 L3 of the same species were injected intraperitoneally into mice previously sensitized with 25 L3 two months earlier.

**Worm yields after experimental infection:** Following peritoneal lavage, intestines were removed and soaked in phosphate-buffered saline (PBS). The scrotal sacs were everted, and carcasses were placed in PBS for further soaking. Carcasses were then rinsed and soaked in PBS.
Viable worms in the peritoneal lavage, intestinal washes and carcass soaks were enumerated under a dissecting microscope.

Larval Culture and preparation of cuticles. Methods relating to culture of larvae have been previously published (12, 14). Briefly, larvae were cultured in α-MEM supplemented with 10% FBS (Gibco BRL Inc; Cat# 12571), at 37 °C with 5% carbon dioxide and 95% humidity. The medium was supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), 10 µg/ml gentamycin, 2 µg/ml ceftazidime and 2 µg/ml ciprofloxacin. Ascorbic acid was added to a final concentration of 75µM on day 5 of culture. Duration post culture was calculated from the time the larvae were first introduced into a 37 °C environment. Cuticles were picked out on day 7 or 8 of culture using a micropipette under direct visualization (14).

Protection through priming with cuticles and E/S products: Three cohorts of BALB/cByJ mice were primed with various worm fractions on the same day. The first group received 25 live L3 B. pahangi larvae per mouse; the second received 25 cuticles obtained from the L3-L4 molt during in vitro culture per mouse; and the third received E/S products collected from the culture of 25 worms per mouse. We chose this priming dose because earlier studied showed that priming with 25 L3 is sufficient to engender accelerated clearance of a challenge infection. A group of five mice injected with 500µl RPMI each served as controls. After a month, these four sets of mice were challenged with 50 live L3. All mice were euthanized ten days after the challenge and the numbers of worms surviving in the peritoneal cavity were determined.

Antibody-mediated Passive protection of secIgM-/- mice: Pooled serum was prepared from three groups of mice: Group 1 was a cohort of BALB/cByJ mice primed with E/S products from 25 L3; Group 2 was a cohort of BALB/cByJ mice injected with 25 L3; and Group 3 were
control BALB/cByJ mice that received RPMI. All mice were euthanized on day 12 and serum collected.

Another three groups of secIgM -/- mice received an injection of 50 *B. pahangi* L3. Twelve days after the injection, sec IgM -/- mice were passively transferred with 250 μl of pooled serum from one of the three immunized groups described above. Six days after serum transfer, all secIgM -/- mice were euthanized and percentage worm burdens were determined. In the protection experiments that used 1E9 antibodies, we injected 125μl of 1E9 monoclonal antibodies (mAb) (which is roughly equivalent to similar amounts of immunoglobulin in 250μl of serum) into secIgM-/- mice 12 days after they received L3s.

**Generation of hybridomas:** Non-secreting myeloma (NSO) cell line first described in 1981 (7) were a gift from Dr. Matthew Scharff at the Albert Einstein College of Medicine, Bronx, NY. These cells were grown in high glucose DME medium containing 10% fetal bovine serum (heat inactivated) (FBS), 10% NCTC-109, 1% non-essential amino acids, and 1% penicillin/ streptomycin.

One BALB/cByJ mouse was injected with 25 *B. malayi* L3. One month later, the mouse was euthanized and splenocytes were harvested and were mixed with NSO cells at a ratio of 4:1. Splenocytes and NSO cells were fused using PEG4000 (ATCC, VA). The fused cells were suspended in HAT medium (Invitrogen, Carlsbad, CA) and plated at a concentration of 2.5×10^6 myeloma cells/ ml. Two weeks later, supernatants from the wells containing visible clones were assayed for antibody titer. Clones from several positive wells were pooled for the cytoadhesion assay. Out of the 50 wells tested, 24 wells tested positive. The supernatants from these 24 wells were then individually tested to narrow down the positive clones to six wells. Limiting dilutions
were performed on these six clones. Several clones showed immune reactivity, but we chose, one clone, 1E9 for further study.

A cytometric bead array (BD Biosciences, San Jose, CA) was used to determine the type of mouse immunoglobulins generated from each hybridoma supernatants. As per the instructions of the manufacturer, supernatant was diluted to 10 fold and 50 µl of diluted supernatant and the detector antibodies were incubated together with the beads for 2 hours at room temperature. Standards were prepared as per the instructions. The beads were then washed and analyzed on a flowcytometer using the template provided by the manufacturer.

Antibody was purified from the supernatants of the hybridoma by a process of lyophilization, dialysis and affinity purification. Affinity purification was performed using Kaptiv M column (Tecnogen, Piana di Monte Verna (CE) - ITALY) as per the manufacturer’s instructions. For experiments requiring passive immunization of mice with 1E9, we injected 50 µl of 2mg/ml stock of 1E9 intraperitoneally into each mouse.

**Phage display library screening:** To identify the cognate antigen of the 1E9 antibody, we screened the phage-display cDNA expression library of *B. malayi* L3 as described previously (4). Briefly, phage display cDNA expression library of *B. malayi* L3 was prepared by ligating PCR amplified cDNAs from a *B. malayi* cDNA library into T7. The T7 bacteriophage DNA was then introduced into BLT5403 bacterial cell where it was packaged and amplified. The phage particles display different *B. malayi* L3 proteins on their capsid. In this study, we used the 1E9 clone to screen these phage expressed L3 proteins by a biopanning procedure already described (4). Briefly, high binding 96 well titer plates were coated with the 1E9 antibody. The phage particles were then added to this plate and allowed to bind. Unbound phage particles were washed off and the bound phages were eluted. Additional four rounds of amplification and
biopanning were performed to enrich 1E9 specific phage clones. cDNA inserts from the final phage clones were then PCR amplified and sequenced. Nucleotide sequence obtained were BLAST searched against *B. malayi* EST database to identify genes encoding proteins that bound to 1E9 clone.

**In vitro Cell adherence assay:** PECs were recovered from secIgM⁻/⁻ mice 14 days post-infection. The cells were washed with complete RPMI (RPMI containing 100 U/ml penicillin, 20 μg/ml gentamicin, 4 μg/ml ciprofloxacin and 2 μg/ml ceftazidime) and resuspended at 20 x10⁶ per ml (12). Sera were collected from naïve or infected wild type C57BL/6 or secIgM⁻/⁻ mice and used immediately or frozen at -20 C as 20% stocks in complete RPMI. Sera were thawed immediately before use. Replicate serum samples were heated at 56⁰C for 30 min. to inactivate heat labile complement components. *B. pahangi* L3 larvae were washed extensively in complete RPMI by serial passage through fresh growth medium in 6 well polystyrene cluster dishes. 5 L3 that had been cleaned by 3 serial passages through RPMI were added per well of a 96 well flat bottomed plate containing 50μl of complete RPMI. One million PECs were added in a volume of 50μl. Sera from 20% stocks were added at final concentrations ranging from 2-10% before adjusting the final volume of the well to 200μl. The plates were incubated at 37⁰C for 1 hour before scoring. Each condition was tested in duplicate (10). An independent observer, always blinded to the experimental conditions, scored the larvae for cell adherence on a scale ranging from zero to three: 0 if no cells were attached, 1 if few cells were attached in a single layer for even a part of the worm, 2 if several layers of cells were partially covering the worm and 3 if the worm was completely covered by several layers of cells.

**Immunization with rBmTCTP and rBmALT-2:** To determine whether both these antigens were host-protective, we immunized mice with these two proteins. Three groups of
mice were injected intraperitoneally each with 1E9 conjugated beads soaked in either 10µg of rBmALT 2, 10µg of rBmTCTP, 10µg of E4 (a Leishmanial antigen) and a fourth group was injected with E/S products. Three weeks after the priming injections, mice were challenged with 50 L3. On day 10 after challenge, mice were euthanized and worm burdens determined.

**1E9 binding to live larvae:** *B. pahangi* larvae were cultured in a 6-well dish with heat inactivated serum containing medium for at least 24 hours. We reasoned that serum proteins would adhere to larve blocking non-specific binding between antibodies and larvae. Viable larvae were transferred to a 24-well dish containing either 1:100 dilution of 1E9 or SP6. Sp6 is a IgM-Kappa hybridoma against TNP that we used as isotype control. After an hour, the larvae were washed by passing three times through α-MEM. Then they were incubated in 1:3000 dilution of goat anti-mouse FITC (Sigma Aldrich, St. Louis MO) or rhodamine conjugated isotype control. The larvae were washed again and mounted using Immu-mount (Thermo Scientific, Waltham MA) on a slide and observed under a fluorescent microscope.

**Statistical Methods:** All values are reported as mean ± standard error of mean. Unpaired two tailed t-tests were used in comparing worm burdens between groups.
Results

Both cuticles and E/S products can prime mice to reject challenge infection with accelerated kinetics: Mice primed with cuticles (0.83±0.45%) or E/S products (8.75±1.6%) had lower worm burdens and thus eliminated a significantly higher percentage of the larvae than unprimed mice (29.17±5.5%) (Figure 1). The percentages of remaining worm burdens in the cuticle primed mice and in the E/S primed mice were comparable to those seen in the mice primed with live L3 (0.42±0.27%).

Serum from E/S primed mice protects IgM deficient mice: Even though cuticles primed mice as effectively as E/S products, we chose to work with the latter for all subsequent experiments, since they are presumably composed of soluble proteins and therefore more easily handled. In an experiment similar to one that has been described in detail by Rajan et al. (10), we reconstituted secIgM-/- mice with serum from primed BALB/cByJ mice. These IgM deficient mice are normally incapable of eliminating an L3 infection. However, reconstitution with serum from L3 primed wild type mice, results in clearance of the infection.

In this experiment, we tested the ability of antibodies against E/S products to enable secIgM-/- mice to eliminate worms following passive-transfer reconstitution. A group of BALB/cByJ mice was primed with E/S products from 25 L3. Two other groups were injected with either L3 or with RPMI. Three groups of secIgM-/- mice were infected with 50 B. pahangi L3 intraperitoneally. Twelve days later, the secIgM-/- mice were passively transferred with serum from one of the three immunized groups described above. Six days after the transfer, mice were euthanized and percentage worm burdens were determined. The group which received
serum from the E/S product-primed mice showed a much higher degree of worm clearance than
the group which received serum from naïve mice (Figure 2). This clearance was comparable to
that observed in the mice which received serum from L3 primed mice. This showed that
antibodies directed against E/S products can effectively compensate for the absence of IgM
antibodies in the host. Furthermore, this finding also confirmed our conclusion from the previous
experiment that E/S products contain host-protective antigens.

The host-protective antigen is specific to the L3 stage: In order to determine if
protective antigen(s) are present in the E/S products of other life-cycle stages of the parasite, we
primed different groups of mice with culture supernatants from various life-cycle stages. We
cultured L3, L4, adult or microfilariae in serum free culture medium for seven days. At the end
of the seven days, we collected the culture supernatants and primed different sets of BALB/cByJ
mice with these E/S products. One month later, we challenged these mice with 50 L3. Ten days
after challenge, mice were euthanized and worm establishment determined. An additional group
of control mice were injected with 0.5ml RPMI and another control group was injected with 25
L3.

We found that only E/S products obtained from the cultures of L3 conferred protection
comparable to that seen in the mice which received live larvae for priming (Figure 3). The other
groups that received E/S products from the culture supernatants of L4, adult or microfilariae had
worm burdens that were not significantly different from those seen in the unprimed group.

Generation of anti-E/S Hybridomas: BALB/cByJ mice were primed with L3. One
month later, their spleens were harvested and the splenocytes were fused with cells from a non-
secreting myeloma cell line (NSO cells) using PEG 4000 (Polyethylene glycol). The cells were plated into four 96 well dishes. Two weeks after colonies started appearing, supernatants from these wells were screened using an *in vitro* cytoadhesion assay. With this screening procedure, pooled supernatants from 24 wells tested positive for cytoadhesion. The wells were then tested individually and we narrowed the positive clones to six wells. Limiting dilution was subsequently performed on the cells from these wells. Clones obtained through the limiting dilution were individually screened for cytoadhesion. Five of the wells had viable clones. These were named 1E9, 1C3, 2C2, 2D6 and 2G6 according to the well numbers. The first of these (1E9) gave the strongest and most consistent results (Figure 4). This clone had the best growth and antibody production when compared to the other clones. We chose to further characterize this clone for protective efficacy, isotype analysis and identification of cognate antigen(s). 1E9 was characterized as an IgM kappa antibody.

**1E9 protects Sec IgM−/− mice against infection with L3:** SecIgM−/− mice which do not possess circulating IgM are unable to clear an infection of L3. If we reconstituted these mice with primed serum, they are able to eliminate an infection. As described above, IgM deficient mice that received serum from mice primed with E/S products were also able to clear L3. Therefore, we wanted to test whether antibodies in the sera of E/S product primed mice that may function similarly to 1E9 antibodies might be responsible for this result. We challenged three sets of five secIgM−/− mice with 50 L3. On day 12 after challenge, we reconstituted these mice with primed serum, naïve serum or purified 1E9 antibodies. On day 18 after challenge, we euthanized the mice and quantitated the worms in the peritoneal cavity.

We found that the mice which had been reconstituted with naïve serum still harbored a large percentage of the injected worms (12.8%±3.5%) while the mice reconstituted with primed
serum eliminated all of the worms (1.3%±1.5%) (Figure 5). The mice reconstituted with 1E9 cleared a significant number of the worms (4.5±3.2%). This was significantly different from that seen in the naïve serum reconstituted group (p < 0.05). Thus, 1E9 antibodies are protective in this model.

Phage display library screening using 1E9: Purified 1E9 mAB was screened against a B. malayi cDNA phage display library in bacteriophages T7 as described in Materials and Methods. Phage that bound to 1E9 were amplified and sequenced. It was found that 1E9 recognized peptides in two known B. malayi proteins, ALT 2 and TCTP. To confirm this result, we performed dot blots on purified ALT2 and TCTP proteins to test the reactivity of 1E9 to these proteins. We found that 1E9 recognized both ALT 2 and TCTP in contrast to control proteins such as E4 (a Leishmanial protein), a tick salivary protein (obtained from Dr. S.K. Wikel, UConn Health Center, Farmington, CT), OVA and BSA (Figure 6). 1E9 mAbs also recognized E/S products as shown in column 5.

ALT 2 immunization protects against a challenge infection with L3: To determine whether both antigens recognized by 1E9 could be host-protective, we immunized mice with these proteins. 1E9 conjugated beads were mixed with ALT 2, TCTP, or E4. Four groups of mice were injected with these beads or with E/S and challenged with 50 L3 three weeks after the priming injections. We did this to create a depot effect for the antigens. We gave a single injection of the antigens because when we prime mice with live larvae, we do so once and get a second set rejection of worms at a subsequent time. Based on this, we wanted to give a single injection, so that we could compare the efficacy of immunization with
our larval priming, which is our "gold standard". However, we reasoned that if we give a single
injection of an aqueous solution of BmALT-2, it would be cleared very quickly in contrast to the
situation with larval immunization. In order to more closely mimic the production and release of
BmALT2 by larvae where production begins on day two after transition to the mammalian
environment and persists at least until the L3-L4 molt (7 days into the infection), we
immobilized BmALT-2 on 1E9 coated beads. The mice were euthanized on day 10 after the
challenge and the worm burdens were quantitated. The mice which had been injected with E4
mixed beads still harbored a significant percentage of the worms (18.4%±3.2%) (Figure 7). The
mice which had received E/S had completely eliminated the worms (0%±0%). The mice which
had been injected with TCTP beads retained a large worm burden (17.2±2.6%). However, the
mice which had been injected with the ALT 2 conjugated beads had eliminated a large
percentage of the worms (8.4%±0.7%). This worm burden was significantly lower than the
groups injected with E4 or with TCTP (p<0.05). The protection achieved by the injection of ALT
2 beads was intermediate between that achieved by E/S and that seen in the E4 or TCTP injected
mice.

1E9 specifically adhered to surface of live larvae. B. pahangi larvae were cultured for
2-3 days and then were divided into three groups of five each. Two groups of larvae were
incubated with 1E9 and one group was incubated with the isotype control (Sp6) antibodies. One
group of larvae incubated with 1E9 and the group incubated with Sp6 were incubated with
fluorescently labeled goat anti-mouse IgM antibody. The other group of 1E9 incubated worms
were incubated with isotype control secondary antibody. Several different concentrations of
antibodies were used however for the purpose of this paper the single best concentration is
reported. These results were replicated once. Surface fluorescence was noted on the worms incubated with 1E9 followed by anti-mouse IgM (Figure 8) whereas the ones labeled with the control antibodies did not. This shows that 1E9 specifically binds to the surface of L3 larvae.

1E9 recognizes a surface epitope on L3s as well as E/S products. We showed earlier (Figure 6) that 1E9 recognizes epitopes on E/S products. We now sought to determine whether it also recognizes the surface of infective larvae. *B. pahangi* L3 granulomas harvested from wild type C57BL/6 mice were fixed and embedded in plastic. Thin sections were cut and stained with 1E9 followed by silver enhanced colloidal gold conjugated anti-mouse IgM. We observed that the antibody stained predominantly around the outer surface of the larva (Figure 9). This shows that 1E9 antibodies recognize antigens present in E/S and on the surface of L3.
Discussion.

Over the past several years, our lab has been attempting to determine the mechanism by which inbred strains of mice, which we use as model mammalian hosts, respond to experimental filarial infections. The outcome of these studies indicates that an important host defense mechanism is the formation of multicellular aggregates of leukocytes called granulomas around incoming infectious larvae. Our studies have indicated important roles for T cells (for the recruitment of a robust population of leukocytes and for activating macrophages along the alternative pathway of activation), B lymphocytes (particularly the B1 subset), and macrophages and eosinophils (for participating in the granulomas). We have demonstrated the crucial importance of anti-filarial antibodies of the IgM isotype in binding to the surface of the larvae and facilitating the adhesion of activated macrophages to the larvae. We have further shown that the in vitro cytoadherence assays is a good surrogate for in vivo granuloma formation. These results, while indicating the ultimate mechanism of host defense, have not helped to identify the target antigen involved.

In the current study, we sought to identify the filarial antigens that elicited certain specific murine immune response. It is clear that whole, live L3 are an excellent source of antigens to prime mice. However, L3s contain several hundred to several thousand potential antigens. In order to narrow down and limit the diversity of antigens in our starting material, we first sought to determine the antigens that are subcomponents of live L3 that might be equally effective in priming mice. The serum free culture system developed in our lab served as a convenient system to obtain two components of L3 that could, conceivably contain the putative protective antigen(s). Since there is little, if any, L3 death during the first seven days of culture,
the supernatants of our culture system should only contain molecules that have been actively secreted or excreted by the worms (E/S products). Furthermore, the L3 cuticle is cleanly shed from the worm during the L3-L4 molt and does not contain any cells from the body of the worm. We first attempted to determine whether either of these two components of live larvae could prime mice.

Our results showed that both of these fractions contain protective antigens. This finding considerably narrows down the pool of candidate protective antigens to those present on either the surface of the worm or in the E/S products. Sera from mice that have been injected with live L3 show reactivity against E/S products on a dot blot and also against the surface of the cuticle on immunoflourescent labeling (data not shown). Thus, in a normal immune response against L3, antibodies seem to be formed against antigens present in E/S products and/or the cuticle. This is consistent with our hypothesis that these fractions of the worms contain antigens that are target of the host protective response in an actual infection. Though not always statistically significant, we have consistently noted that worm burdens are higher in mice immunized with E/S products compared to live larvae. The greater efficacy of larvae as priming agents may be explained by the mode of delivery of antigen – a bolus with the E/S products versus a sustained release with the larvae.

Evidence exists that certain antigens are shared between the E/S products and the surface of the worm. There are two hypotheses to explain this sharing of antigens. It has been suggested that the surface coat of the cuticle is produced by the excretory-secretory system. This would mean that molecules that cover the surface of the cuticle are released from the E/S pore and subsequently coat the surface. These same molecules may be released into the surroundings of the worm and form a part of the E/S products. A second explanation for the sharing of antigens is
that there is a constant turnover of surface proteins and these shed proteins constitute a portion of E/S products. E/S products are readily available at the site of infection to host APCs.

Based on this reasoning, we sought to focus on one of these two sources of candidate protective antigens for further study. Of the two, we selected E/S products as this represents a collection of soluble antigens, and is therefore, conceivably easier to sub-fractionate. We approached the problem of antigen identification by making monoclonal antibodies against ES products, and screening the hybridomas that we generated in our in vitro cytoadherence assay. Of the several hybridomas that were active in this assay, 1E9 was the most consistently effective. As shown here 1E9 recognizes antigens both in the ES products as well as on the cuticle.

When we screened for the potential cognate antigens of this antibody using a T7-based cDNA expression system, we found that it reacts with two antigens of filarial origin, ALT 2 and TCTP. The ALT 1 and ALT 2 antigens are the most highly expressed genes in L3. They are part of a gene family that is expressed in all filarial parasites. There is a high level of sequence similarity between ALTs of B. malayi and W. bancrofti.

Priming mice with ALT 2 helped mice to eliminate a challenge infection of L3 with accelerated kinetics, whereas priming with TCTP did not. However, it is worth noting that the protection induced by ALT 2 although significant, was less than that with E/S. This gives us reason to believe that ALT 2 may be just one of the protective antigens present in E/S albeit an important one. Since 1E9 also binds to the surface of the larvae, it is likely that ALT 2 is present on the cuticle of larvae as well as in the E/S products. ALT 2 that is released from the larvae may coat the surface resulting in the deposition of ALT 2 on the cuticle. This sharing of antigens between the E/S and the cuticle is important for protection, because only soluble E/S antigens would be accessible to the host APCs during the efferent phase of the immune response.
However, for the response to be protective, it would be necessary for antibodies to be directed against epitopes on the surface of the larvae. So it is likely that while antibodies are produced in response to the ALT 2 present in E/S, they recognize ALT 2 present on the surface of the larvae and bind to it. This in turn would facilitate binding of activated cells to the surface and this process initiates granuloma formation around the larvae.

We find it striking that, two very different approaches to the identification of post-protective antigens of filarial origin should have resulted in the identification of BmALT-2. Gregory et al. (6) sought to identify proteins that are abundantly expressed by infectious larvae. Subsequently they found that one of these abundantly expressed proteins, BmALT-2, is host protective. Similarly, Gnanasekar and coworkers (4) have been interested in identifying the host protective antigens. They too have, independently, discovered that BmALT-2 is host protective.

Sera from immune individuals were noted to react to BmALT2 and it appeared to be the immuno-dominant antigen in these individuals. We have approached the problem by first determining the mechanism of host protection, and then identifying antigens that might participate in this mechanism. Our independent and separate approach has resulted in the identification of the same antigen. This finding leads us to conclude that BmALT-2 is a major host protective antigen. In the ultimate goal of developing vaccine candidates for human lymphatic filariasis, BmALT-2 may be the ideal candidate. L3 larvae are the infective stage of the larvae and transmission blocking vaccines must target this stage. Recent studies have shown promising results in JIRDS and mice using ALT-2 as the candidate antigen (1, 17). Single antigen vaccination with ALT-2 has been shown to be more efficacious than other vaccine candidates or combinations of candidate antigens (17). Our experiments differ from the protein vaccination studies in that ALT-2 was administered by multiple injections with alum as an
adjuvant. We avoided the use of adjuvant because it would have been a sharp departure from our usual immunization protocol (which does not involve an adjuvant) and would have made comparison difficult amongst our experiments.

It is interesting to note that Ramachandran et al. (13) have found that endemic normals react to purified BmALT-2 more strongly than asymptomatic microfilaremics. One is tempted to speculate that the reason that asymptomatic microfilaremics harbor high levels of worm burdens may be because of their inability to make high level antibody responses to this candidate antigen.
Literature cited


Figure 1

![Graph showing % Worm Burden for different categories: RPMI, Live, Cuticles, E/S. The RPMI category has the highest % Worm Burden, followed by Cuticles and E/S, with Live having the lowest.](image)
Figure 2

% Worm Burden

Naive  Larvae primed  E/S primed

- 0%

* *
Figure 3

% Worm Burden

Unprimed  L3 larvae  L3 E/S  L4 E/S  Mf E/S  Adult E/S
Figure 4a

The figure shows a bar graph representing the adhesion score of different hybridomas. The x-axis represents the hybridomas, and the y-axis represents the adhesion score. The bars indicate the mean adhesion scores with error bars, showing the variability. The hybridomas are labeled as Primed, Naive, 1B3, 1B9, 1C5, 1D4, 1D9, 1D11, 1E7, 1E9, 1F3, 1F9, 1G5, 1G8, 1G9, 2B1, 2B2, 2C2, 2C7, 2D5, 2D6, 2D10, 2E4, 2G3, and 3G6.
Figure 4b
Figure 5

% Worm Burden

Naive Serum  Primed Serum  1E9

*
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<th>ALT 2</th>
<th>TCTP</th>
<th>E4</th>
<th>Tick Sal. Prot</th>
<th>E/S</th>
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<th>BSA</th>
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Figure 7

![Bar chart showing % Worm Burden for E4, E/S, TCTP, and Alt-2.](chart.png)
Figure 8