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Granuloma Formation around Filarial Larvae Triggered by Host Responses to an Excretory/Secretory Antigen▽

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In previous studies using a murine model of filarial infection, granuloma formation was found to be a most important host-protective mechanism. We have also shown that *in vitro* cytoadherence is a surrogate for the formation of antifilarial granulomas *in vivo* and that it requires "alternatively activated" host cells and a source of antifilarial antibody. We show here 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 (no. 1E9) was positive in this assay. We then screened a novel expression library of filarial antigens displayed on the surface of T7 bacteriophage for reactivity with 1E9. Phage expressing two filarial antigens (TCTP and BmALT-2) reacted with 1E9. Immunization of mice showed that the cohort immunized with BmALT-2 cleared a challenge infection with infective *Brugia pahangi* L3 in an accelerated manner, whereas cohorts immunized with TCTP cleared larvae with the same kinetics as in unimmunized mice. These data confirm that BmALT-2 is the antigenic target of granuloma-mediated killing of *B. pahangi* L3. 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 may provide host protection.

Normal, immunocompetent mice quantitatively eliminate infections with filarial 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$^{\alpha}\beta$ 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 extracellularly 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 that in 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 antifilarial 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 the 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, the immunization of mice with BmALT-2, even in the absence of adjuvants, results in elimination of infective larvae with accelerated kinetics. These observations 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

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serum IgM in randomly selected mice as determined by sandwich enzyme-linked immunosorbent assay (ELISA).

Infectious larvae. *Brugia pahangi* L3 was harvested at either TRS Inc., Athens, GA, the University of Georgia (John McCall), or the University of Louisiana (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 larvae intraperitoneally in 500 μl of RPMI using 1-ml syringes fitted with 5/8-in. 25-gauge needles. For challenge infections, 50 L3 larvae of the same species were injected intraperitoneally into mice previously sensitized with 25 L3 larvae 2 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% fetal bovine serum (FBS) (Gibco BRL Inc.; catalog no. 12571) at 37°C with 5% carbon dioxide and 95% humidity. The medium was supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin, 2 μg/ml ceftazidime, and 2 μg/ml ciprofloxacin. Ascorbic acid was added to a final concentration of 75 μM on day 5 of culture. Duration postculture was calculated from the time that 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 excretory/secretory (E/S) products collected from the culture of 25 worms per mouse. We chose this priming dose because earlier studies showed that priming with 25 L3 larvae is sufficient to engender accelerated clearance of a challenge infection. A group of five mice injected with 500 μl RPMI each served as controls. After a month, these four sets of mice were challenged with 50 live L3 larvae. The mice were euthanized 10 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 larvae, group 2 was a cohort of BALB/cByJ mice injected with 25 L3 larvae, and group 3 consisted of control BALB/cByJ mice that received RPMI. All mice were euthanized on day 12, and serum was collected.

Another three groups of secIgM$^{-/-}$ mice received an injection of 50 *B. pahangi* L3 larvae. Twelve days after injection, the 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 percent worm burdens were determined. For the protection experiment using 1E9, 125 μl of 1E9 monoclonal antibody (MAb) (which contained amounts of immunoglobulin similar to those in 250 μl of serum) was injected into the secIgM$^{-/-}$ mice 12 days after the L3 injection.

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

A BALB/cByJ mouse was injected with 25 L3 larvae. A month later, the mouse was euthanized and the splenocytes were harvested. These splenocytes were mixed with NSO cells at a ratio of 4:1, and they were fused using polyethylene glycol (PEG) 4000 (ATCC, VA). The 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 and used in the cytoadhesion assay. Of the 50 wells tested, cells pooled from 24 wells tested positive. The supernatants from these 24 wells were then individually tested to narrow down the positive clones to six wells. Limiting dilution was performed on these six clones. Among these, one clone, 1E9, was retained for further study.

The Cytometric bead array (BD Biosciences, San Jose, CA) for mouse immunoglobulins was used for typing the antibodies generated in the hybridoma supernatants. Per the manufacturer's instructions, supernatant was diluted 10 fold and 50 μl of diluted supernatant and detector antibodies was incubated with the beads for 2 h at room temperature. Standards were prepared as per the manufacturer's instructions. The beads were washed and analyzed on a flow cytometer 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 the 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, 50 μl of a 2-mg/ml stock of 1E9 was injected intraperitoneally.

Phage display library screening. To identify the cognate antigen of the 1E9 antibody, it was used to screen a cDNA expression library in T7 phage (4). Briefly, a *B. malayi* phage display library was prepared by ligating PCR-amplified cDNAs from a *B. malayi* cDNA library into T7. The T7 bacteriophage DNA was introduced into bacteria, where it was packaged and amplified. The phage particles display different *B. malayi* proteins as a part of their capsid. The phage particles thus obtained were panned using 1E9. High-binding 96-well titer plates were coated with the 1E9 antibody. The phage particles were added to this plate and allowed to bind. The unbound phage particles were washed off. The bound phage was eluted. Successive rounds of amplification and biopanning were performed. The *B. malayi* cDNA was PCR amplified out of the T7 DNA and sequenced. The sequencing results were BLAST searched against the *B. malayi* expressed sequence tag (EST) database to identify the genes encoding the proteins that 1E9 recognizes.

***In vitro* cell adherence assay.** Peritoneal exudate cells (PECs) were recovered from secIgM$^{-/-}$ mice at 14 days postinfection. 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 20×10^6 per ml (12). Sera were collected from naïve or infected wild-type C57BL/6 or secIgM$^{-/-}$ mice and either 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 56°C 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 six-well polystyrene cluster dishes. Five L3 larvae that had been cleaned by three serial passages through RPMI were added per well of a 96-well flat-bottom plate containing 50 μl of complete RPMI. One million PECs were added in a volume of 50 μl. Sera from 20% stocks were added at final concentrations ranging from 2 to 10% before the final volume of the well was adjusted to 200 μl. The plates were incubated at 37°C for 1 h 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 0 to 3: 0 if no cells were attached, 1 if a 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 the recombinant rBmTCTP (rBmTCTP) and rBmALT-2 antigens were host protective, we immunized mice with these two proteins. Three groups of mice were injected intraperitoneally with 1E9 conjugated beads soaked in either 10 μg of rBmALT-2, 10 μg of rBmTCTP, or 10 μg 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 larvae. On day 10 after challenge, mice were euthanized and worm burdens determined.

1E9 binding to live larvae. *B. pahangi* larvae were cultured in a six-well dish with medium containing heat-inactivated serum for at least 24 h. We reasoned that serum proteins would adhere to larvae, blocking nonspecific binding between antibodies and larvae. Viable larvae were transferred to a 24-well dish containing a 1:100 dilution of either 1E9 or Sp6. Sp6 is an IgM-kappa hybridoma against trinitrophenyl (TNP) that we used as an isotype control. After an hour, the larvae were washed by being passed three times through α-MEM. They were then incubated in a 1:3,000 dilution of goat anti-mouse fluorescein isothiocyanate (FITC) (Sigma Aldrich, St. Louis, MO)- or rhodamine-conjugated isotype control. The larvae were washed again, mounted on a slide using Immu-mount (Thermo Scientific, Waltham, MA), and observed under a fluorescence microscope.

Statistical methods. The statistical significance of differences between comparable groups was estimated using appropriate nonparametric tests, with the level of significance set at a P value of <0.05. Unpaired two-tailed t tests were used in comparing worm burdens between groups. All values are reported as means ± standard errors of the means (SEM).

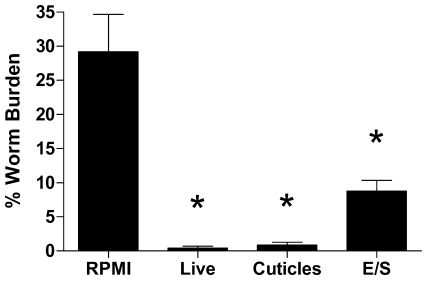

FIG. 1. Percent worm burdens in mice primed with L3 E/S products, L3-L4 cuticles, L3 larvae, and RPMI. Three sets of BALB/cByJ mice (eight mice per group) were primed with various worm fractions on the same day. The first group of mice received 25 live L3 *B. pahangi* larvae per mouse. The second group received 25 cuticles obtained from the L3-L4 molt during *in vitro* culture per mouse. The third group received the supernatants collected from the culture of 25 worms per mouse (L3-L4 E/S products). A group of four mice injected with 0.5 μl RPMI each served as controls. After a month, these four sets of mice were challenged with 50 live L3 larvae. The mice were sacrificed 10 days after the challenge, and the number of worms surviving in the peritoneal cavity were counted. The bars represent the average percent worm burdens (depicted on the *y* axis) in these groups of mice. The results marked with an asterisk are significantly different ($P < 0.05$) from those for the RPMI group. This experiment is representative of four similar experiments.

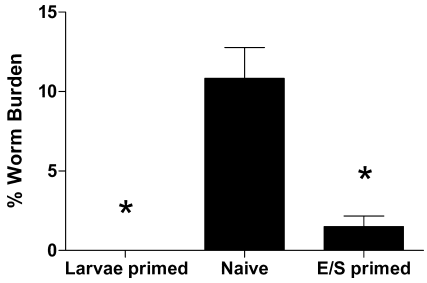

FIG. 2. Percent worm burdens in secIgM$^{-/-}$ mice reconstituted with serum from L3-primed, naïve, and E/S-primed BALB/cByJ mice. A group of BALB/cByJ mice were primed with E/S products from 25 L3 larvae. Two other groups of BALB/cByJ mice were injected with either L3 larvae or RPMI. Each group contained three to five mice. Sera were obtained from each of these groups 2 weeks after infection and pooled groupwise. Three groups of secIgM$^{-/-}$ mice (six to eight mice per group) were infected with 50 *B. pahangi* L3 larvae. Twelve days later, these mice were reconstituted with serum from one of the three groups described above. Six days after the transfer, the mice were sacrificed and their percent worm burdens were calculated. The bars represent the average percent worm burdens (depicted on the *y* axis) in these groups of mice. The results marked with an asterisk are significantly different ($P < 0.05$) from those for the group receiving serum from naïve mice. This experiment is representative of three similar experiments.

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%) (Fig. 1). The percentages of remaining worm burdens in the cuticle-primed mice and in the E/S product-primed mice were comparable to those seen in the mice primed with live L3 (0.42% ± 0.27%).

Serum from E/S-product 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 larvae. Two other groups were injected either with L3 larvae or with RPMI. Three groups of secIgM$^{-/-}$ mice were infected with 50 *B. pahangi* L3 larvae 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 percent 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 (Fig. 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 a protective antigen(s) is 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 larvae, L4 larvae, adults, or microfilariae in serum-free culture medium for 7 days. At the end of the 7 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 larvae. Ten days after challenge, mice were euthanized and worm establishment determined. An additional group of control mice were injected with 0.5 ml RPMI, and another control group was injected with 25 L3 larvae.

We found that only E/S products obtained from the cultures of L3 larvae conferred protection comparable to that seen in the mice which received live larvae for priming (Fig. 3). The other groups that received E/S products from the culture supernatants of L4 larvae, adults, 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 larvae. One month later, their spleens were harvested and the splenocytes were fused with cells from a nonsecreting myeloma cell line (NSO cells) using PEG 4000. 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 pos-

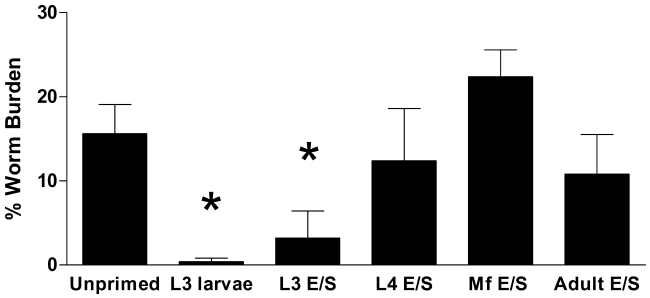

FIG. 3. Percent worm burdens in mice primed with RPMI, L3 larvae, L3 E/S products, L4 E/S products, microfilarial E/S products, and adult E/S products. L4 larvae, adults, or microfilariae were cultured in serum-free culture medium for 7 days. At the end of the 7 days, the culture supernatants were collected, and groups (five mice per group) of BALB/cByJ mice were primed with these E/S products. A month later, these mice were challenged with 50 L3 larvae, and 10 days later, the mice were sacrificed and the number of surviving worms was counted. As controls, a group of five mice were injected with 0.5 ml RPMI and another group were injected with 25 L3 larvae. The bars represent the average percent worm burdens (depicted on the *y* axis) in these groups of mice. The results marked with an asterisk are significantly different ($P < 0.05$) from those for the group primed with RPMI. This experiment is representative of three similar experiments.

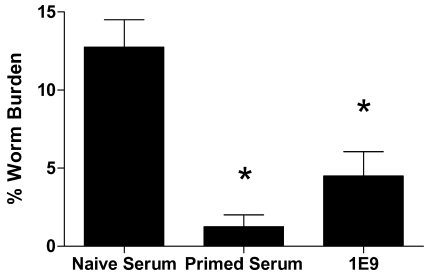

FIG. 5. Percent worm burdens in secIgM$^{-/-}$ mice reconstituted with serum from naïve mice, L3-primed mice, and purified 1E9 antibodies. Three sets of five secIgM$^{-/-}$ mice were challenged with 50 L3 larvae. On day 12, these mice were reconstituted with primed serum, naïve serum, or purified 1E9 antibodies. On day 18, the mice were sacrificed and the worms in the peritoneal cavity were quantitated. The bars represent the average percent worm burdens (depicted on the *y* axis) in these groups of mice. Statistically significant ($P < 0.05$) changes compared to results for naïve serum-reconstituted mice are designated with an asterisk. This experiment is representative of three similar experiments.

itive 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 (Fig. 4). This clone had the best growth and antibody production. 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 larvae. secIgM$^{-/-}$ mice, which do not possess circulating IgM, are unable to clear an infection of L3 larvae. If we reconstituted these mice with primed serum, they were 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 larvae. 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 larvae. 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%) (Fig. 5). The mice reconstituted with 1E9 cleared a significant number of the worms (4.5% ± 3.2%). This was significantly different from what was seen in the group reconstituted with naïve serum ($P < 0.05$). 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

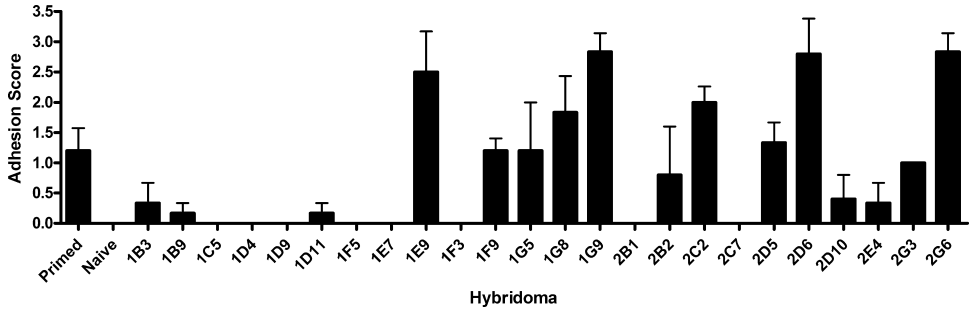

FIG. 4. Identification of 1E9. Hybridomas were prepared as described in the text. Cell-free supernatants of different clones were collected. Worms were plated at five worms per well. Twenty microliters of supernatant or serum from primed wild-type (WT) mice or naïve WT mice were added to each well. PECs were harvested from secIgM$^{-/-}$ mice infected with *B. pahangi* L3 larvae 14 days prior. A total of 10^6 PECs were added per well, and the volume was made up to 200 μl per well. The plate was incubated at 37°C in 5% CO_2 for 1 h. After an hour the plates were removed and adhesion was scored by a blinded observer. Mean adhesion scores (± SEM) for each of the supernatants are shown. The experiment was repeated with a selected cohort of supernatants for reproducibility. Five clones with adhesion scores greater than or equal to that with primed serum were selected. Of these, 1E9 was selected for reproducibility of results and growth characteristics.

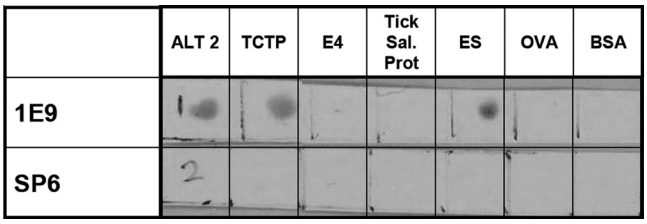

FIG. 6. Dot blot testing the reactivities of 1E9 to ALT-2, TCTP, E4 (a leishmanial protein), a tick salivary protein, whole E/S, OVA, and BSA. Various proteins were blotted onto a strip of nitrocellulose membrane. The strip was blocked using 5% milk in PBS (blocking buffer). The strip was then transferred to a 1:100 dilution of 1E9 or isotype control Sp6 in blocking buffer. It was then washed with PBS–0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO). It was then transferred to a solution containing 3 μg/10 ml horseradish peroxidase (HRP)-conjugated anti-IgM antibody. This experiment is representative of four similar experiments.

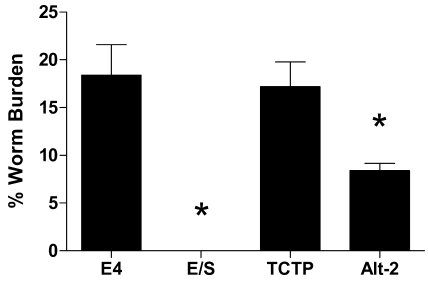

FIG. 7. Percent worm burdens in mice primed with E4, BmTCTP, or BmALT-2. Four groups of mice of five mice each were injected with E4, TCTP, or ALT-2 mixed with 1E9 conjugated beads and were challenged with 50 L3 larvae 3 weeks after the priming injections. The mice were sacrificed on day 10 of the challenge, and the worm burdens were quantitated. An E/S product-primed group served as positive control, while mice primed with E4 were regarded as negative control. The bars represent the average percent worm burdens (depicted on the *y* axis) in these groups of mice. Statistically significant differences ($P < 0.05$) compared to results for E4 are indicated with an asterisk. This experiment is representative of three similar experiments.

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 blotting with purified ALT-2 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 S. K. Wikel, University of Connecticut Health Center, Farmington, CT), OVA, and bovine serum albumin (BSA) (Fig. 6). 1E9 MAbs also recognized E/S products (Fig. 6).

rBmALT-2 immunization protects against a challenge infection with L3 larvae. 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 larvae. Four groups of mice were injected with these beads or with E/S products and challenged with 50 L3 larvae 3 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 gave 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 BmALT-2 by larvae, where production begins on day 2 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%) (Fig. 7). The mice which had received E/S products 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 those in 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 products and that seen in the E4- or TCTP-injected mice.

1E9 specifically adheres to surface of live larvae. *B. pahangi* larvae were cultured for 2 to 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 (Fig. 8), whereas the ones labeled with the control antibodies did not show fluorescence. This shows that 1E9 specifically binds to the surface of L3 larvae.

1E9 recognizes a surface epitope on L3 larvae as well as E/S products. We showed earlier (Fig. 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 (Fig. 9). This shows that 1E9 antibodies recognize antigens present in E/S products and on the surface of L3 larvae.

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

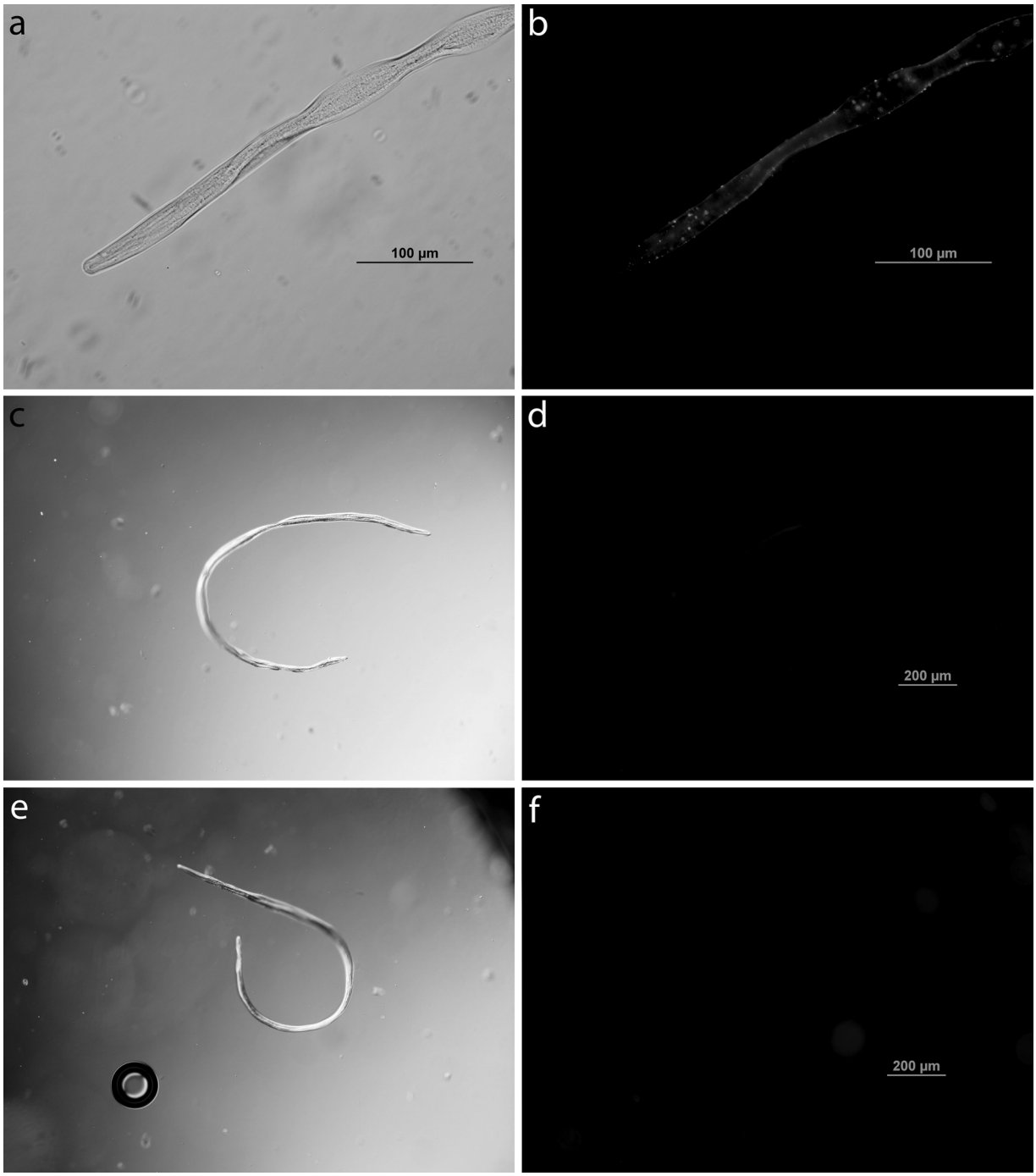

FIG. 8. Surface staining of live L3 larvae with 1E9 antibody. Larvae on which nonspecific binding was mitigated by prolonged culture in serum-containing medium were incubated with either 1E9 (a to d) or Sp6 (e and f) antibodies. (a, b, e, and f) Larvae were incubated with goat anti-mouse IgM–FITC. (c and d) Larvae were incubated with the isotype control goat anti-mouse IgM–FITC. This is a representative of two similar experiments. Each experiment included approximately five larvae per group. All larvae stained uniformly.

indicates that an important host defense mechanism is the formation of multicellular aggregates of leukocytes called granulomas around incoming infectious larvae. Our previous studies indicated an important role 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 antifilarial 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 assay is a good surrogate for *in vivo* granuloma formation. These results, while indicating the ultimate mechanism of host

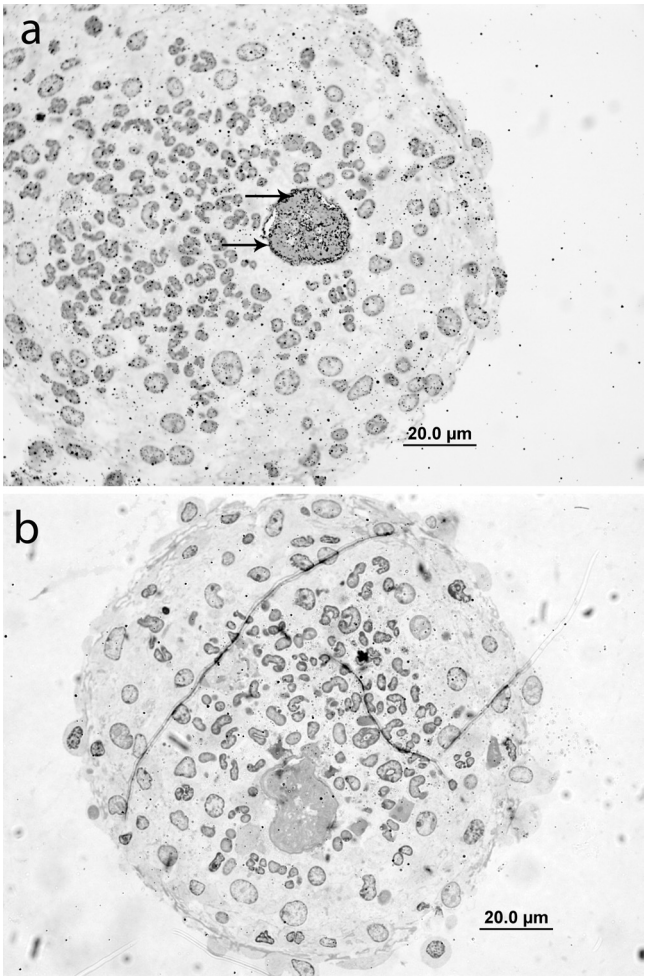

FIG. 9. L3 larvae in granulomas stained with 1E9 antibody. *B. pahangi* L3 larvae were injected into wild-type mice, and the granulomas that were produced were harvested. These granulomas were fixed with 2% glutaraldehyde and embedded in plastic. One-micrometer sections were cut. The sections were blocked with FBS and incubated with 1E9 (a) or isotype matched control antibody Sp6 (b) followed by colloidal gold-conjugated anti-mouse IgM. The gold particles were silver enhanced (arrows) and counterstained with toluidine blue. This is representative of two similar experiments.

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 responses. It is clear that whole, live L3 larvae are an excellent source of antigens to prime mice. However, L3 larvae 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 larvae 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 larvae that could conceivably contain the putative protective antigen(s). Since there is little, if any, L3 death during the first 7 days of culture, the supernatants of our culture system should contain only 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 narrowed the pool of candidate protective antigens to those present either on the surface of the worm or in the E/S products. Sera from mice that have been injected with live L3 larvae show reactivity against E/S products on a dot blot and also against the surface of the cuticle on immunofluorescent labeling (data not shown). Thus, in a normal immune response against L3 larvae, 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 the results were not always statistically significant, we have consistently noted that worm burdens are higher in mice immunized with E/S products than in those immunized with 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 antigen-presenting cells (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 these represent a collection of soluble antigens and are therefore conceivably easier to subfractionate. We approached the problem of antigen identification by making monoclonal antibodies against E/S 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 E/S products and 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, BmALT-2 and BmTCTP. The ALT-1 and ALT-2 antigens are the most highly expressed genes in L3 larvae. 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 *Wuchereria bancrofti*.

Priming mice with BmALT-2 helped mice to eliminate a challenge infection of L3 larvae with accelerated kinetics, whereas priming with BmTCTP did not. However, it is worth noting that the protection induced by BmALT-2, although significant, was less than that with E/S products. This gives us

reason to believe that BmALT-2 may be just one of the protective antigens present in E/S products, 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 products 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. Thus, it is likely that while antibodies are produced in response to the ALT-2 present in E/S products, 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 host-protective antigens of filarial origin 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. Independently, they too have discovered that BmALT-2 is host protective. Sera from immune individuals were noted to react to BmALT-2, and it appeared to be the immunodominant 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 the use of other vaccine candidates or combinations of candidate antigens (17). Our experiments differ from the protein vaccination studies in that, in those studies, 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 among our experiments difficult.

It is interesting to note that Ramachandran et al. (13) have found that normal individuals in an area of endemicity react to purified BmALT-2 more strongly than individuals with asymptomatic microfilaremia. One is tempted to speculate that the reason that individuals with asymptomatic microfilaremia harbor high worm burdens may be because of their inability to produce high-level antibody responses to this candidate antigen.

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