

A NOVEL GENE FROM *BRUGIA* SP. THAT ENCODES A CYTOTOXIC FATTY ACID BINDING PROTEIN ALLERGEN RECOGNIZED BY CANINE MONOCLONAL IgE AND SERUM IgE FROM INFECTED DOGS

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ABSTRACT: *Brugia pahangi* infection of dogs is a well characterized model of human lymphatic filariasis in which sera consistently show IgG or IgE reactivity to a 35-kDa antigen. Using dog lymph node B cells, we previously established a heterohybridoma cell line producing canine monoclonal IgE (cmAb 2.39) that activates and degranulates canine mast cells, and specifically recognizes a 35-kDa *B. pahangi* antigen. By affinity purification and sequencing of the native protein from *B. pahangi* adults, a 19-amino acid sequence was obtained; the derived nucleotide sequence showed homology to a *Brugia malayi* and 2 related *Onchocerca volvulus* expressed sequence tag (EST) clones from the Filarial Genome Project database. Consensus primers amplified a 244-bp product from adult and infective larval stage cDNA libraries of *B. malayi*, *O. volvulus*, and *Wuchereria bancrofti*, but not from those of nonfilarial nematodes. The *B. malayi* EST clone only showed nucleotide sequence homology to *O. volvulus* EST sequences. A 684-bp region from the open reading frame was expressed as a glutathione S-transferase fusion protein designated BmA1-1. CmAb 2.39, as well as serum IgE from dogs infected with *B. pahangi* and canine filarial heartworm, *Dirofilaria immitis*, recognized BmA1-1 on enzyme-linked immunosorbent assay and Western blots. BmA1-1 showed high binding affinity for a fatty acid; however, a search for sequence homology with known fatty acid binding proteins indicated that BmA1-1 is a unique fatty acid binding protein. This 35-kDa protein seems to be highly conserved in different stages and species of filarids, and it represents a previously unknown allergen that is possibly involved in the pathogenesis of filarial disease.

Experimental infection of dogs with the filarial nematode *Brugia pahangi* has proven to be an excellent model system to study disease pathogenesis associated with lymphatic filariasis. We have found that the acute and chronic lesions (Snowden and Hammerberg, 1989), pathogenesis (Snowden and Hammerberg, 1987, 1989), and the immunological responses (Miller et al., 1991) are very similar to those reported for human filariasis (Ottesen, 1982; Nielsen et al., 2002; Semnani and Nutman, 2004). We described an association of elevated release of histamine and tumor necrosis factor (TNF) from lymph node cells in dogs with a genetic predisposition to limb edema formation upon infection with *B. pahangi*, and we noted increased mast cell numbers in the uninfected peripheral lymph nodes of this group of dogs compared with lymph nodes from nonedema-forming dogs (Orton et al., 1998). Mast cell release of preformed histamine and TNF- α is mediated by surface-bound IgE antibodies.

In a previous study, canine popliteal lymph node cells were harvested at the onset of clinical disease from the rear limb of a dog infected with *B. pahangi*, and they were fused with a mouse myeloma cell line to form canine $\times$ mouse heterohybridomas. Of the several canine immunoglobulin-producing clones from this fusion, clone 2.39 was found to produce monoclonal canine IgE (cmAb 2.39) that was specific for a filarial antigen (Gebhard et al., 1995). The IgE antibody recognized a 35-kDa band in Western blots of adult *B. pahangi* extracts run under both reducing and nonreducing conditions, whereas immunohistochemical analyses showed binding to somatic muscle tissue in both male and female adult worms.

Dogs chronically infected with *B. pahangi* and dogs reinfected during chronic infection have consistently shown IgG or IgE antibodies to a 35-kDa antigen, while demonstrating vari-

able to undetectable responses to the nematode polypeptide antigen (NPA) or “ladder antigen” (Snowden and Hammerberg, 1987; Allen et al., 1995; Britton et al., 1995; Kennedy et al., 1995). We were interested in knowing whether the 35-kDa antigen recognized by the canine monoclonal IgE was homologous to the common ladder antigen or the filarial antigens that preferentially induce polyclonal and antigen-specific IgE and IgG4 antibodies (Garraud et al., 1995; Lobos et al., 1996), and whether the recombinant antigen would be able to signal degranulation of canine mast cells activated with cmAb 2.39. Furthermore, we wanted to examine whether canine serum IgE from filarid-infected dogs recognized the recombinant fusion protein. In the present study, we describe the isolation and characterization of a novel 52-kDa *B. malayi* allergen-glutathione S-transferase (GST) recombinant fusion protein, designated BmA1-1, that contains the epitope recognized by monoclonal canine IgE generated from B cells of a *B. pahangi*-infected dog.

MATERIALS AND METHODS

Isolation of native, IgE-specific *Brugia* antigen

The *B. pahangi* antigen (BpA) preparation was a saline-soluble extract of male and female adult worms recovered from the peritoneal cavity of experimentally infected jirds (Snowden and Hammerberg, 1989). BpA-specific canine monoclonal IgE, cmAb 2.39 (available as dog IgE monoclonal from Bethyl Laboratories, Montgomery, Texas), was linked to protein A-agarose beads using the ProFound coimmunoprecipitation kit following manufacturer's instructions (Pierce Chemical, Rockford, Illinois), which leaves the Fab end of cmAb 2.39 free to bind specific antigen. BpA in phosphate-buffered saline (PBS) was applied to the column, and after repeated washing, bound antigens were eluted from the column with 0.1 M glycine-NaOH, pH 11.0. The affinity-purified antigens were concentrated with a 10-kDa molecular weight cut-off Centrprep filtration unit (Millipore Corporation, Billerica, Massachusetts) and assayed for protein content using the Bradford reagent assay (Bio-Rad, Hercules, California). Fifty μ g of affinity-purified parasite antigens were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and nonreducing conditions on a 10% gel, and they were transferred to 0.2- μ m pore size polyvinylidene difluoride (PVDF) membranes (Bio-Rad) as described previously (Towbin et al., 1979). To reveal the band of protein recognized by cmAb 2.39, a single strip of the membrane blots was incubated

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with cmAb 2.39 and detected with phosphatase-labeled affinity-purified goat anti-dog IgG, heavy and light chain-specific antiserum (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland) that cross-reacted with canine IgE. This stained strip was used as a reference for excision of area of the PVDF membrane containing the 35-kDa cmAb 2.39-reactive peptide from the remaining blot run under nonreducing conditions. The excised area of membrane bearing the 35-kDa protein was submitted for amino acid sequencing to the Louisiana State University Medical Center Core Labs (New Orleans, Louisiana).

Antisera production

A 20-amino acid synthetic peptide, corresponding to the N-terminal sequence of the 35-kDa antigen to which an N-terminal cysteine was added to allow linkage to keyhole limpet hemacyanin (KLH), was generated by the Louisiana State University Medical Center Core Labs. One hundred micrograms of the synthetic peptide was mixed with Titermax adjuvant (Sigma-Aldrich, St. Louis, Missouri) and injected subcutaneously into New Zealand White rabbits at 4 sites. The rabbits were boosted with 50 µg of protein in Titermax adjuvant after 4 wk, and sera were harvested 10–14 days postboost. Enzyme-linked immunosorbent assay (ELISA), as described under immunochemical procedures, was used to determine antibody titers. IgG antibodies specific for the KLH carrier were removed by passage over an affinity column of KLH linked to Affi-Gel 10 beads (Bio-Rad), and the remaining IgG antibodies were purified by protein G-agarose bead chromatography (Pierce Chemical).

For production of polyclonal rabbit anti-canine IgE, rabbits were immunized with cmAb 2.39, and immune sera were adsorbed with canine IgG linked to Affi-Gel 10 beads before protein G-agarose bead affinity chromatography was used to purify IgG antibodies specific for canine IgE.

Polymerase chain reaction (PCR) amplification and sequence analysis

The peptide and deduced nucleotide sequence from the N-terminal 19 amino acids of the 35-kDa antigen were compared with public databases (NCBI, NIH, Bethesda, Maryland) using basic local alignment search tool (BLAST) (Altschul et al., 1990). An approximately 1.1-kb PCR product showing homology to a *B. malayi* adult male expressed sequence tag (EST) sequence in the Filarial Genome Project (FGP) database, was obtained from S. A. Williams (Smith College, Northampton, Massachusetts). A 5'-insert-specific primer (primer A: 5'-GA GTGGTTCAGTTAAATT-3') and 3'-oligo(dT) primer were used to amplify the entire clone for sequencing, because only 45% bp of the clone had been sequenced by the FGP. Lasergene software (DNASTAR, Inc., Madison, Wisconsin) was used to generate consensus primers (5'WVEK: 5'-TTGGGTTGAAAAAGATGC-3' and 3'RGEQ: 5'-CTC ACGCCACTGTTACAC-3' [Genosys, Woodlands, Texas]) based upon highly homologous regions found between the *B. malayi* adult male and *O. volvulus* infective larva (L3) and adult female EST sequences in the FGP database. PCR conditions used for cross-species comparisons were 94 C for 45 sec, 55 C for 30 sec, and 72 C for 45 sec for 45 cycles, and a final extension at 72 C for 10 min.

cDNA libraries for *B. malayi* L3, *W. bancrofti* adult male, and *O. volvulus* L3 were obtained from the FGP repository (S. A. Williams). The *Trichinella spiralis* (Arasu et al., 1994) and *Ancylostoma caninum* (P. Arasu, unpubl. obs.) adult stage cDNA libraries were constructed using the λZAP expression vector (Stratagene, La Jolla, California). Protein analysis on the derived sequences was done using the Protean software (DNASTAR, Inc.).

Construction of BmAl-1 expression vector

A 684-bp open reading frame (ORF) from the 1-kb EST clone was amplified with sequence-specific primers designed with 5'BamHI and 3'EcoRI sites (5'-CGGGATCCATGAAACAATTCTTTAATCC-3') and (5'-GCGGAATTCCT-ATTTCATATTCATGGAA-3'), respectively. PCR conditions were 94 C for 60 sec, 50 C for 90 sec, and 72 C for 120 sec for 45 cycles, followed by a final extension at 72 C for 10 min. The Bam HI–EcoRI-digested PCR product was purified and ligated into the pGEX2T vector (GE Healthcare, Piscataway, New Jersey) for subsequent construction of a GST fusion protein.

Expression and purification of BmAl-1 in *Escherichia coli*

For expression of the protein, the recombinant construct was transformed into *E. coli* strain BL-21(DE3)pLysS (Novagen, Madison, Wisconsin) and induced for 2 hr using 1 mM isopropyl β-D-thiogalactoside (IPTG) when the culture optical density (OD)₆₀₀ had reached 0.6. Cells transfected with GST vector alone were used as controls. The bacterial cell pellet was harvested by centrifugation and resuspended in a buffer containing 100 mM sodium chloride, 10 mM Tris, pH 8.0, 1 mM EDTA with 100 µg/ml lysozyme (STE). Cell lysis was completed by addition of dithiothreitol in 10% Sarkosyl/STE (Sigma-Aldrich) immediately before centrifugation to clarify the lysate (Smith and Johnson, 1988; Fragoni and Neel, 1993). Recombinant fusion protein was purified by affinity chromatography using glutathione-Sepharose 4B (Pfizer Inc., New York, New York). After induction, the presence of recombinant protein was confirmed by immunoblot analysis as described above using rabbit anti-GST (Sigma-Aldrich) and cmAb 2.39.

Immunochemical methods

In addition to detection of cmAb 2.39 by cross-reactive rabbit antisera against canine IgG as described above, binding of cmAb 2.39 to BmAl-1 on Western blot was detected with a biotinylated mouse monoclonal anti-canine IgE, mAb 5.91 (Hammerberg et al., 1997), followed by incubation with streptavidin-phosphatase (Zymed Laboratories, South San Francisco, California), and developed with alkaline phosphatase substrate (Bio-Rad). Detection of cmAb 2.39 was also conducted with rabbit anti-canine IgE, followed by phosphatase-labeled goat anti-rabbit IgG (Kirkegaard and Perry Laboratories). ELISA on microtiter plates was used to detect binding to BmAl-1 by cmAb 2.39 and canine serum IgE from filarid-infected dogs. CmAb 2.39 and canine serum IgE antibodies specific for BmAl-1 were detected with either biotinylated mAb 5.91 or biotinylated protein G-purified rabbit anti-canine IgE, followed by streptavidin-peroxidase (Zymed Laboratories) and developed with ammonium 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonate) peroxidase substrate (Kirkegaard and Perry Laboratories). The standard curve consisting of cmAb 2.39 ranging from 0.5 to 100 ng/ml allowed quantitation of BmAl-1-specific IgE in dog sera. Color development was measured by absorbance at 405 nm on a Fisher Microtek microtiter plate reader (Fisher, Raleigh, North Carolina). ELISA was also used to measure canine serum IgG specific for BmAl-1. However, this ELISA was only qualitative in nature. IgG was detected with peroxidase-labeled goat anti-canine IgG γ chain specific (Kirkegaard and Perry Laboratories). Serum from each dog was analyzed at 4 dilutions, each of which was run in triplicate. Sera were serially diluted 1:5 through 1:40 for BmAl-1-specific IgE and 1:50 through 1:400 for BmAl-1-specific IgG levels.

Source of infected and uninfected dogs

Blood from *B. pahangi*-infected dogs was collected 8.5–9 wk after infection with 20 *B. pahangi* L3 by subcutaneous injection at the dorsum of the rear paw as described previously (Orton et al., 1998). Pre-infection serum was also collected from these dogs; however, all of the *B. pahangi*-infected dogs were born to mothers that had patent *B. pahangi* infections. All *B. pahangi*-infected dogs were raised and maintained under closed housing conditions. Blood samples from dogs naturally infected with *D. immitis* were obtained after the animals were killed at a local animal shelter by lethal injection and infections were confirmed at necropsy.

"Outdoor housing" dogs were privately owned dogs, maintained on outdoor runs, and bled with owner consent at the time medical histories were obtained. All dogs were being maintained on monthly anthelmintics to prevent adult *D. immitis* and to minimize ascarid and hookworm infection. "Indoor housing" dogs were colony reared and protected from filarial parasite exposure. Blood was collected from 6 of the dogs at 6 mo of age.

Passive cutaneous anaphylaxis (PCA)

Two colony-reared dogs, raised and maintained in indoor housing without exposure to helminth parasites, were used to test BmAl-1 cross-linking by specific IgE with passive cutaneous anaphylaxis. The right thoracic area of the 2 dogs was shaved, and intradermal injections of 100 µl of purified cmAb 2.39 (at 1 mg/ml) were given at 12 sites.

Another 12 sites were injected with 100 μ l of saline. Forty-eight hours later, duplicate injections of 100 μ l of BmAl-1 at 1.0, 0.5, 0.1, 0.05, and 0.01 mg/ml were injected intradermally into sites previously injected with cmAb 2.39 or saline, respectively. GST at 1.0 mg/ml was also injected as a control. Rabbit polyclonal anti-canine IgE (20 μ g/ml) and saline were injected as positive and negative controls, respectively. Reactive regions at the test sites were evaluated using caliper measurement for wheal diameter, and erythema intensity for flare response by an independent observer at 20 min and 24 hr after injection of the antigen.

Trypan blue dye exclusion

Two different cell types were used for dye exclusion testing, i.e., a canine mastocytoma cell line, C2 (Lazarus et al., 1986); and a feline lymphosarcoma cell line, E (English et al., 1993). Cells were incubated with buffer, GST (100, 50, and 10 μ g/ml), or BmAl-1 (100, 50, and 10 μ g/ml) for 30 min at either 4 C or 37 C. Cells were then incubated with 0.1% trypan blue at room temperature for 5 min. Cell counts and dye uptake assessments were done using a hemocytometer.

Fatty acid binding capacity of BmAl-1

BmAl-1 was tested for its ability to bind fatty acids using dansylated fatty acid 11-((5-dimethyl-aminonaphthalene-1-sulphonyl) amino) undecanoic acid (DAUDA) (Invitrogen, Carlsbad, California) as described previously (Wilkinson and Wilton, 1986; Kennedy et al., 1997; McDermott et al., 1999). A 1 mM stock solution of DAUDA was made in methanol, and serial dilutions of proteins were made in PBS, pH 7.2. Bovine serum albumin (BSA) and *Ascaris suum* major allergen (ABA-1) were used as reference fatty acid binding proteins. Each cuvette contained 0.2 ml of protein (at 0.1 or 0.05 mg/ml) in PBS and 2 μ l of 1 mM DAUDA. After 10 sec, the fluorescence was read on a Versa-Fluor Fluorometer System (Bio-Rad) using excitation and emission filters at 340–364 nm and 510–520 nm, respectively.

Statistics

Significance of differences of the means between groups was evaluated using the Student's *t*-test assuming unequal variances. Any *P*-value less than 0.05 was considered significant. Statistical analysis was done using GraphPad Prism software (GraphPad Software Inc., San Diego, California).

RESULTS

Isolation and sequencing of native *Brugia* protein

Previous studies showed that cmAb 2.39 recognized a 35-kDa protein in Western blots of *B. pahangi* adult worm extracts (Gebhard et al., 1995). The native protein was isolated by passing *B. pahangi* adult worm-soluble extracts over a column to which cmAb 2.39 was covalently bound by the Fc portion to protein A beads. After elution and concentration of the affinity-purified material, SDS-PAGE and transfer to PVDF membrane showed a major band at 35-kDa by protein staining and Western analysis with cmAb 2.39 under both reducing and nonreducing conditions (Fig. 1). A portion of the blot run under nonreducing conditions containing the protein of interest was submitted for sequencing. The resulting 19 amino acid N-terminal sequence, depicted by single-letter amino acids, is as follows: I-H-D-G-N-N-Y-K-D-G-D-T-W-V-E-K-D. Homology searches using the derived 57-nucleotide sequence with the genetic code favored by *Brugia* sp. revealed 100% nucleotide sequence homology with nucleotides 101–157 of a 1-kb *B. malayi* adult stage EST clone (AA680477) from the FGP database. Nucleotide sequencing of the clone revealed a 774-bp ORF with start and stop codons, and short 5'- and 3'-untranslated sequences. The deduced 257-amino acid sequence was in-frame with the 19-amino

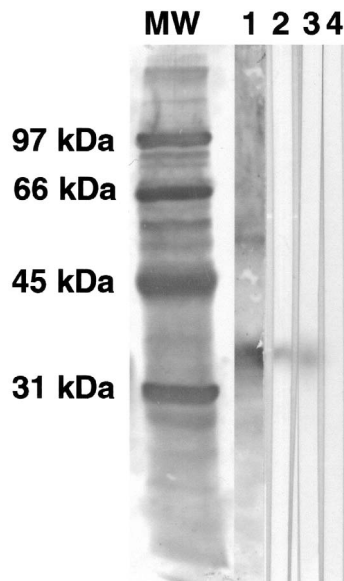


FIGURE 1. Reactivity of cmAb 2.39 on Western blot against affinity-purified *B. pahangi* soluble extracts, fractionated by 10% SDS-PAGE under reducing and nonreducing conditions. Protein stain of affinity-purified *B. pahangi*-soluble extract showing peptide at 35-kDa under reduced conditions (lane 1). Reactivity of cmAb 2.39 against *B. pahangi* extracts separated under nonreducing (lane 2) and reduced (lane 3) conditions. Detecting antibody control (lane 4).

no acid sequence of the N terminus of the native 35-kDa protein (Fig. 2).

Cloning and expression of BmAl-1

From the ORF of the EST clone, a 684-bp region was amplified and directionally cloned into pGEX2T and transformed into *E. coli* BL-21 cells. After IPTG-induced expression, the cell lysates were subjected to affinity column chromatography with glutathione 4B-Sepharose for purification of the 29-kDa-tagged GST-fusion protein. A major band of about 52 kDa was detected on a SDS-PAGE gel run under reducing conditions. SDS-PAGE run under nonreducing conditions also showed a band at 52 kDa, in addition to minor bands at approximately 115 kDa and larger, indicating possible aggregation of the recombinant peptide into dimers and/or trimers (data not shown).

BmAl-1-specific IgE and IgG

Canine mAb 2.39 and sera from *B. pahangi*-infected, *D. immitis*-infected, and uninfected dogs housed indoors without exposure to filarial parasites or housed outdoors were tested for reactivity to the recombinant BmAl-1 and control GST proteins by Western blot, ELISA, or both. Western blot analysis of the affinity-purified recombinant protein showed strong binding of cmAb 2.39 to BmAl-1 as detected by monoclonal and polyclonal anti-canine IgE. A major band was seen at 52 kDa and a minor band at about 115 kDa, again suggesting the occurrence of some dimerization, because GST itself is known to form natural dimers (Fig. 3). CmAb 2.39 did not bind control GST protein.

Purified cmAb 2.39 was used to construct a standard curve for measuring absolute quantities of BmAl-1-specific IgE in

GGCACGAGGTCATAAGAGTGGTTCAGTTAAATTTCAAATTTAAGCAATAACAAA (5'untranslated)

ATGAAATCAATTCTTTTAATCCTATTTTCTCTATTTAATCCACTAAATGGATGCATACA
M K S I L L I L F S L F N P L N G C I H 20

TGATGGTAATAATTACAAGGATGGCGATACTTGGGTTGAAAAAGATGCATTGTGTATGCG
D G N N Y K D G D T W V E K D A F V M R 40

ATGTCGCATGACTGATGATGGGACATCTTGGATGGTTGAGATAACAGGATGTAAGACACC
C R M T D D G T S W M V E I T G C K T P 60

ATCGGGTGCAACAATATCAATTAATTCAAGTATAATTGATGGAGATTATGAATGGAAATG
S G A T I S I N S S I I D G D Y E W K C 80

• *
 T TCAAAAAATAATGATGGACAAATTGTAATGCAAAAAACTTTACATGCAAAATGCAAAATG
S K N N D G Q I V M Q K T L H A N A K C 100

TGATGAACATCAGCGTGGTGAACAGTGGCGTGAGAAATCATTTTTATATGAATGTGGAGC
D E H Q R G E Q W R E K S F L Y E C G A 120

AGGTGGCCAGAAAAAATTGATAGCCTGTTTTGGTCAAGATAATGAACAAATAAATGTTGG
G G Q K K L I A C F G Q D N E Q I N G 140

TGAATCAAAAAGAAATTGGTGGATATATTGTGAAATGCGAAAAATATGATAATGGAACGGT
E S K E I G G Y I V K C E K Y D N G T V 160

*
 T ATAGTACATGGCATAACGCAAAGGAACCGAAAAATGGAACAACATTTCAGGTGGAATGCAT
I V H G I R K G T E N G T T F Q V E C I 180

• *
 AGATTGCAAAAGGTGAACATCATGTTGCAGATTTCATGGTGGATAGATGATCAACGATTCAA
D S K G E H H V A D S W W I D D Q R F N 200

*
 TAAGACATGTTTATCATCT GGCAAAATTGAAGTATTAAATTGCATTTCAAAAGATGGCATT
K T C L S S G K I E V L N C I S K D G I 220

AAAATTCCATTGAATAATGAAATAAGCCGTTGGTGCACGAAATACACATGTGAGAAAACA
K I P L N N E I S R W C T K Y T C E K T 240

AGCGATGGTTTCAGTACGTTTTGCATCT GGTCCAATTGATGCTAATGGTAAATAA
S D G S V R F A S G P I D A N G K STOP 257

TTAATAATGAAAATTAATGGAAGGAAGAGAAAAACA (3'untranslated)

FIGURE 2. Nucleotide and derived amino acid sequence of the 774-bp ORF, BmA1-1. Numbers on the right designate the amino acid residue. The numbering starts at the initiation codon. The predicted signal peptide, residues 1–16 (bold), *N*-glycosylation sites (*), myristilation sites (●), and phosphorylation sites (underlined) are indicated. Nucleotide sequence data reported in this article are available in the GenBank database under the accession AF335566.

dog sera on BmA1-1-coated microtiter plates. Results showed that all sera from dogs infected with *B. pahangi* had significantly higher levels of BmA1-1-specific IgE compared with nondetectable levels in preinfection sera from the same dogs, i.e., 146 ± 72 ng/ml compared with <0.5 ng/ml, respectively ($P < 0.01$) (Table I). Sera from 4 of 11 *D. immitis*-infected dogs had detectable levels of IgE specific for BmA1-1. There was no detectable binding of IgE to the recombinant GST in any of the sera tested.

Measurement of BmA1-1 binding IgG showed results that were different from BmA1-1-specific IgE. All sera tested showed detectable levels of IgG specific for BmA1-1 in all groups of dogs. These groups included dogs housed indoors and outdoors without exposure to filarial parasites, pre- and post-*B. pahangi*-infected dogs born to mothers with patent *B. pahangi*

infections, and *D. immitis*-infected dogs. ELISA results showed detectable amounts of serum IgG binding to both BmA1-1 and GST, albeit at relatively high serum concentrations and low OD signal. Even in the dogs housed indoors without any exposure to filarial parasites, there were detectable levels of BmA1-1 binding IgG, comparable with the other groups of sera. Subtraction of the OD values attributable to GST did not alter the conclusion that OD values due to binding to BmA1-1 in sera collected from dogs after *B. pahangi* infection were not significantly different from sera collected before infection (data not shown).

Biological activity of BmA1-1

PCA studies were carried out in dogs to assess in vivo the IgE cross-linking capacity of BmA1-1. Dogs were injected in-

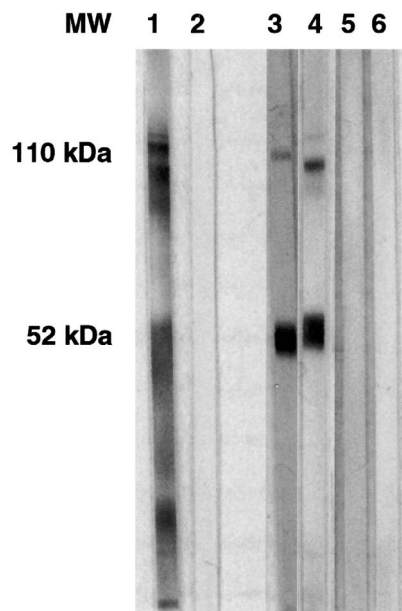


FIGURE 3. Binding of cmAb 2.39 to Western blots of BmAl-1 under nonreducing conditions. Lane 1, polyclonal rabbit anti-GST antibody as a positive control. Lane 2, horseradish peroxidase-labeled goat anti-rabbit IgG antibody control. Lane 3, cmAb 2.39. Lane 4, rabbit polyclonal antisera against the 19-amino acid sequence derived from direct sequencing of the *B. pahangi* peptide. Lanes 5 and 6, secondary antibody controls to detect nonspecific binding. Note binding of cmAb 2.39 and rabbit polyclonal sera against the recombinant peptide bands at 52 and 110 kDa.

tradermally with cmAb 2.39 or PBS, and then they were challenged at the same sites 48 hr later with BmAl-1 or GST only. Sites injected with polyclonal anti-canine IgE were read as positive controls compared with saline only sites that were used as negative controls. At BmAl-1 concentrations of 0.5 and 1.0 mg/ml, wheal and flare reaction sites measured 13–14 mm in diameter, regardless of whether the sites were previously injected with cmAb 2.39. At lower concentrations of BmAl-1, the sites previously injected with cmAb 2.39 had a slightly larger wheal and flare response than sites that did not receive cmAb 2.39 injections given, respectively, as follows: at 0.1 mg/ml, 14.5 versus 12 mm; at 0.05 mg/ml, 14 mm versus 11.5 mm; and at 0.01 mg/ml, 13 mm versus 11 mm. Because only 2 sites for each sample on each of 2 dogs were tested, the statistical significance of these results could not be determined. The sites injected with either GST or saline alone did not show any reactivity. With regard to erythematous (flare) responses, there were no differences at any of the sites for any of the treatments. We did not measure lipopolysaccharide (LPS) levels in the BmAl-1 used for intradermal injection, because LPS alone has been shown not to induce an immediate skin response (Michel et al., 1991). The weak reactivity in PCA may indicate that recombinant BmAl-1 only contains a single epitope that is inadequate for cross-linking IgE on mast cells.

Because an immediate intradermal wheal and flare response to BmAl-1 occurred independently of cmAb 2.39 injection, direct effects on cells, especially mast cells, by BmAl-1 was assessed by trypan blue dye exclusion (Table II). Incubation of 2 different cell types for 30 min at 37 C with BmAl-1 at concentrations of either 100 or 50 µg/ml resulted in 100% of cells

TABLE I. BmAl-1-specific IgE in dog sera.*

Groups of dog sera	BmAl-1-specific IgE (ng/ml)
<i>B. pahangi</i> infected	
n = 15	146 ± 72
<i>D. immitis</i> infected	
n = 7	<0.5
n = 4	1,592 ± 1,548
Uninfected sera	
n = 25	<0.5

* Results are mean values and standard error for triplicates of each sample. Detectable levels of BmAl-1-specific IgE were measured in sera from all 15 *B. pahangi*-infected dogs. However, only 4 of the 11 total *D. immitis*-infected dog sera had measurable levels of BmAl-1-specific IgE, and they were thus divided into n = 4 and n = 7 subgroups.

being unable to exclude the dye, indicating loss of cell membrane integrity. Similar results at 4 C indicated that the effects of BmAl-1 were independent of temperature. The effect was not observed at concentrations of 10 µg/ml or less. Cell counts before and after incubation with BmAl-1 were not different, indicating that the effect of incubations with BmAl-1 was not cytolytic.

Sequence analysis of BmAl-1 and homology to other filarids

Homology searches of databases revealed 85% nucleotide sequence homology of BmAl-1 with *Onchocerca volvulus* EST clones from the infective larvae cDNA library SAW94WL-OvL3, (clones AA611009, AA917241.1, and AI09647.1) and adult female cDNA library SAW98MLW-OvAF (clone AI151561). The derived amino acid sequences of the 2 *O. volvulus* EST clones (based on codon usage favored by *Brugia* sp.) were compared against the BmAl-1 sequence (Fig. 4). The *O. volvulus* adult female clone had 66% homology, whereas the L3 clone showed 71% homology to the BmAl-1 sequence between amino acids 25–137. The protein encoded by BmAl-1 was not homologous with any known protein, including previously described nematode allergens (Lobos et al., 1992; Garraud et al., 1995; Lobos et al., 1996; Kennedy, 2001).

TABLE II. BmAl-1-induced dye uptake.*

Treatment†	% Stained cells (37 C)	
	C2 cells	E cells
Saline	8.3	38
GST		
0.1	19.6	34
0.05	12.1	33.8
0.01	13.5	28.5
BmAl-1		
0.1	100	100
0.05	100	100
0.01	9.1	37.1

* Membrane permeability was assessed by trypan blue dye staining after 30 min. The results represent the average of duplicate counts.

† Units for GST and BmAl-1 are in milligrams per milliliter.

[illegible]

FIGURE 4. Comparative analysis of the derived amino acid sequence of BmAl-1, *O. volvulus* L3 stage (OvL3), and adult female (OvAdfem) EST clones based upon codon use favored by *Brugia* species. Identical nucleotides are in bold.

Based upon the high percentage of homology between *B. malayi* and *O. volvulus* nucleotide sequences, 5' and 3' consensus primers were developed for PCR analysis on filarial and nonfilarial cDNA libraries. As expected, the predicted 244-bp fragment was amplified from BmAl-1, *O. volvulus* L3, and adult female EST clones, as well as from *B. malayi* L3 and *Wuchereria bancrofti* adult male cDNA libraries (Fig. 5). Additionally, other bands were detected on the gel, suggesting nonspecific PCR amplification products or that this sequence is part of a multigene family. Analysis of cDNA libraries from nonfilarial nematode libraries (*A. caninum* adult, *T. spiralis* L1, and *Caenorhabditis elegans* mixed stage) did not result in amplification of the expected 244-bp consensus sequence.

Peptide sequence analysis of BmAl-1

Given that the recombinant BmAl-1 protein is from an uncharacterized sequence with no strong homology to proteins of known function, various analyses were conducted to determine

the nature of the encoded protein. The recombinant protein construct used in these studies may have near tertiary conformation due to its recognition by cmAb 2.39 and IgE from *Brugia*-infected dogs, and the observed functional activity in binding fatty acids (see below). The predicted average molecular weight of the 257-amino acid protein is 28.7 kDa, with an isoelectric point of 5.88. This is less than an expected 35-kDa molecular weight of the native protein, and is likely due to posttranslational modification. The 5'-(N-terminal) region of the deduced amino acid sequence that was absent from the extracted protein, resembles a typical eukaryotic signal peptide in that 9 of the first 16 N-terminal amino acids have hydrophobic and uncharged residues (von Heijne, 1986). The sequence encoded within the open reading frame contains four potential N-linked glycosylation sites (Asn₆₈, Asn₁₅₇, Asn₁₇₁, and Asn₂₀₀), which follow the general rule of Asn-X-Thr/Ser, where X is any residue except perhaps aspartate, glutamic acid, or proline (Mononen and Karjalainen, 1984) (Fig. 2). Highly glycosylated gly-

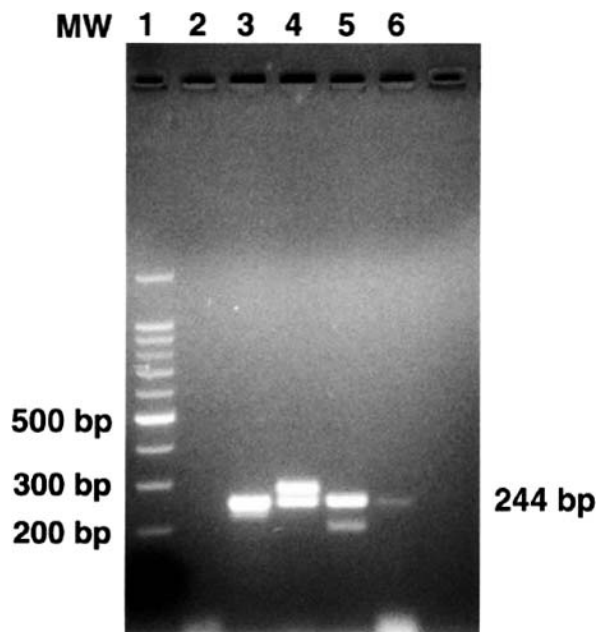


FIGURE 5. PCR analysis using 5'-WVEK and 3'-RGEQ consensus primers to screen filarial cDNA libraries for the predicted 244-bp consensus sequence (found in *B. malayi* adult male and *O. volvulus* L3 and adult female EST clones) by 2% agarose gel electrophoresis. Lane 1 is a 1.5-kb molecular weight marker. Lane 2 is a negative water control. Lane 3 is positive control using cloned BmAl-1. Lanes 4, 5, and 6 are reactions using cDNA from a *B. malayi* L3, *W. bancrofti* adult male, and *O. volvulus* L3 library, respectively. Similar analysis with *T. spiralis*, *A. caninum*, and *C. elegans* cDNA libraries were negative (data not shown).

coproteins are common in nematode parasites (Meghija and Maizels, 1986; Maizels et al., 1989), and extensive posttranslational glycosylation may be present in the native peptide, but it is not present in BmAl-1, because bacterial expression does not provide this posttranslational modification.

Lipid binding capacity of BmAl-1

Sequence analysis revealed several α -helical regions containing hydrophobic residues that are characteristic of nematode fatty acid binding proteins (Kennedy, 2001). BmAl-1 was tested for its ability to bind DAUDA, a dansylated fatty acid that shows a shift in fluorescence when bound by protein. Incubation with 100 μ g/ml BmAl-1 showed fluorescence that was greater than fluorescence for the common fatty acid binding protein BSA and comparable with the strong affinity binding of fatty acids shown by ABA-1 at the same concentrations (Table III).

DISCUSSION

In the present study, we describe the cloning, expression, and characterization of a novel allergen, BmAl-1, from *Brugia* sp. The native allergen was isolated using cmAb 2.39, a canine monoclonal IgE specific for a 35-kDa protein from *B. pahangi*. CmAb 2.39 was produced from a canine $\times$ mouse heterohybridoma using B cells from the lymph node of a *Brugia*-infected dog (Gebhard et al., 1995). In previous studies, we showed that dogs chronically infected with *B. pahangi*, and

TABLE III. Fatty acid binding capacity of BmAl-1.*

Treatment†	Relative fluorescence units (mean $\pm$ SD)
BSA	
0.1	363 $\pm$ 4.6
0.05	229 $\pm$ 2.0
ABA-1	
0.1	954 $\pm$ 8.5
0.05	671 $\pm$ 3.6
BmAl-1	
0.1	1,217 $\pm$ 38.5
0.05	970 $\pm$ 24.1

* Each sample was run in triplicate. Results for relative fluorescence units are reported as the mean $\pm$ SD. The values are the average of samples run in triplicate.

† Units for BSA, ABA-1, and BmAl-1 are in milligrams per milliliter.

rechallenged with the nematode, had IgG antibodies, IgE antibodies, or both to a 35-kDa antigen from adult worm extracts (Snowden and Hammerberg, 1987), even when there was no detectable response to the NPA or ladder antigen. The ladder antigen belongs to a family of conserved nematode antigens that includes the ABA-1 allergen of *A. suum* and *A. lumbricooides* (Spence et al., 1993; Tweedie et al., 1993), proteins from *D. immitis* (Poole et al., 1992), and *Dictyocaulus viviparus* (Britton et al., 1995). IgE antibodies specific for a 37-kDa allergen distinct from NPA, designated BmR1, has been reported in humans infected with *B. malayi* (Rahmah et al., 1998); however, there is no sequence similarity between BmAl-1 and BmR-1.

CmAb 2.39, as well as serum IgE from *Brugia*-infected dogs and 4 of 11 of *D. immitis*-infected dogs, bound the BmAl-1 recombinant protein; there was no IgE reactivity in sera from uninfected dogs. The mean amount of BmAl-1-specific IgE in *B. pahangi*-infected dogs was remarkably high at 146 $\pm$ 72 ng/ml. However, total serum IgE values for dogs are highly variable (Hill et al., 1995; Felsburg, 1998), and, in our own previous investigations using purified canine IgE as a reference standard, total serum IgE values ranged from 0.5 ng/ml to 2 μ g/ml (Hammerberg et al., 1997). Yet, there is no significant difference in total serum IgE levels between helminth-infected and normal nonparasitized or nonatopic dogs (Hill et al., 1995). Within this context, the relatively high levels of IgE specific for BmAl-1 are difficult to interpret. The detection of BmAl-1-specific IgE in the sera of all 10 *Brugia*-infected dogs and a number of *D. immitis*-infected dogs suggests that this allergen or its close homologue is common to filarial nematodes. Variability in immune response among individuals in the random-bred population of *D. immitis*-infected dogs compared with the line-bred colony population of *B. pahangi*-infected dogs is similar to the variability seen in humans infected with filarial parasites (Nielson and Simonsen, 2002).

In contrast to IgE anti-BmAl-1, IgG binding to BmAl-1 was detectable in all samples tested. The presence of low levels or low affinity BmAl-1 binding IgG in sera from normal, uninfected, and unexposed dogs could not be explained. However, this may be due to prior exposure to antigens with epitopes similar to those present on BmAl-1, or due to BmAl-1 binding through interactions with constant region sites on canine IgG.

The source of exposure to potential epitopes in the BmA1-1 sequence is unknown for uninfected dogs, because BLAST searches showed homology exclusively with filarial nematode sequences. Furthermore, with regard to the lack of difference in IgG between outdoor-housed dogs and dogs born and raised indoors, there may have been exposure to filarial antigens from mothers potentially exposed to filarial infection before being moved inside and subsequently giving birth to puppies that were born and raised indoors.

The in vivo PCA response showed that skin sites previously injected with cmAb 2.39 had a slightly stronger wheal and flare response compared with sites that were not previously sensitized. The weak in vivo response compared with strong in vitro reactivity shown by Western immunoblot analysis and ELISA suggests that the recombinant protein may only bear a single epitope recognized by IgE, thereby making it a weak cross-linking agent for IgE antibodies on skin mast cells. The strong wheal and flare responses at high concentrations of BmA1-1, i.e., 100 and 50 $\mu\text{g/ml}$, regardless of whether the skin sites had previously been injected with IgE, led us to investigate its direct effect on cells, in particular, mast cells in culture. BmA1-1, at concentrations above 10 $\mu\text{g/ml}$, has a permeabilizing effect on cell membrane integrity, as shown by the cell's inability to exclude trypan blue dye. This functional property will be investigated in further characterization of the recombinant allergen. Trypan blue is an indicator of loss of cellular membrane integrity (Lorenz et al., 1988). BmA1-1 is not cytolytic in that the cell counts before and after incubations with the recombinant fusion protein were not different.

Based on consensus regions within the BmA1-1 nucleotide sequence, a 244-bp PCR product was amplified from cDNA libraries of *O. volvulus* and *W. bancrofti*; this has not yet been tested with *D. immitis* cDNA. Amplification was not observed with cDNA of distantly related nonfilarial nematodes, but it was observed with different developmental stages and sex of various filarial species. These data and the absence of sequence homology on both nucleotide and amino acid searches would suggest that, based on our testing, this sequence is filarial specific. As shown in Figure 4, the 5' amino terminus of the derived amino acid sequences of the *B. pahangi* adult male and *O. volvulus* L3 clones shared greater homology compared with the *O. volvulus* adult female sequence. Furthermore, multiple bands were detected with the *B. malayi* L3 and *W. bancrofti* adult male cDNA templates in the PCR analyses shown in Figure 5. Together, the results suggest that these BmA1-1-related sequences may be part of a multigene family or that there may be differential splicing of the transcripts. These sequences are also not sex or stage restricted, in that the consensus sequence was found in adult male and female libraries, as well as L3 libraries. This is consistent with the polyprotein fatty acid binding NPA from *A. suum*, ABA-1 that is abundant in the pseudocoelomic fluid of the adult worms, but is also found in the secretions of all parasitic stages (Kennedy and Qureshi, 1986; McGibbon et al., 1990). There are 2 classes of fatty acid and retinol binding proteins that are both rich in α helix structures, i.e., the polyprotein allergens and the fatty acid and retinol binding (FAR) proteins secreted by many nematodes. To date, the in vivo function of these proteins remains to be determined. The large polyprotein allergen/antigens are synthesized as large polypeptides that are posttranslationally cleaved, resulting in

multiple copies of similar lipid binding proteins (ladder antigens), whereas the FAR proteins are synthesized as individual proteins of approximately 20 kDa (Garofolo et al., 2002, 2003; Solovyova et al., 2003). FAR proteins have been described in multiple nematode species, including *C. elegans*, *O. volvulus*, *B. malayi*, and *A. suum*.

Sequence analysis showed several α helical regions containing hydrophobic residues, characteristic of fatty acid binding proteins. The capacity to bind fatty acids is part of a list of common characteristics shared by nematode allergens (Wilkinson and Wilton, 1986; Kennedy et al., 1997; McDermott et al., 1999). NPAs and FARs of filarial nematodes have shown high-affinity binding of fatty acids using the dansylated fatty acid DAUDA (Kennedy et al., 1995; McDermott et al., 1999). Many mammalian allergens are lipocalins that represent a diverse group of lipid binding proteins (Virtanen et al., 1999). We found that BmA1-1 bound DAUDA to the same degree as ABA-1, one of the strongest allergens known. The fatty acid binding capacity of BmA1-1 should provide clues to the biological function of the native protein, possibly similar to lipid transfer properties described for ABA-1 and Ov-FAR-1 (McDermott et al., 2002). Sequence analysis and comparisons with various nematode FARs did not show any significant homology with BmA1-1 (Blast alignment tools; <http://www.ncbi.nlm.nih.gov/BLAST/>) (Altschul et al., 1997).

The results reported here describe a novel fatty acid binding protein that seems to be highly conserved in different stages and species of filarial nematodes and that may represent a major allergen involved in the pathogenesis of filarial disease. The recombinant protein form of this allergen and the canine monoclonal IgE specific for it will facilitate further studies of the possible role of this protein in filarial fatty acid transport and in allergen-related mechanisms of lymphatic pathologies seen in lymphatic filariasis, suggested by studies in *Brugia*-infected dogs (Snowden and Hammerberg, 1989; Orton et al., 1998).

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