Cross-reactive IgE antibody responses to tropomyosins from Ascaris lumbricoides and cockroach

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Background: Evidence indicates that infection with Ascaris lumbricoides may promote development of allergy and asthma. Objective: To study the role of tropomyosin, a pan-allergen in invertebrates, in IgE responses to A lumbricoides. Methods: Recombinant A lumbricoides and Periplaneta americana tropomyosins were expressed in Pichia pastoris. Levels of IgE to tropomyosins from A lumbricoides and P americana were determined by chimeric ELISA in sera from 119 children living in a parasite-endemic area and 112 patients with cockroach allergy. Presence of tropomyosin in A lumbricoides larvae at L3 stage was evaluated by immunofluorescence using mAb 1A6, directed against mite tropomyosin. Molecular modeling of P americana and A lumbricoides tropomyosins was performed by using the MODELLER program. Results: A lumbricoides tropomyosin showed 69% to 98% sequence identity to tropomyosins from other invertebrates. The predicted structure of A lumbricoides tropomyosin was similar to that of P americana tropomyosin and showed the characteristic coiled-coil structure. Strong correlation was found for IgE antibodies to tropomyosins from A lumbricoides and P americana in sera from children living in a parasite-endemic area and from patients with cockroach allergy. Larvae of A lumbricoides reacted strongly with mAb 1A6. Conclusion: Tropomyosin induces IgE responses in A lumbricoides–infected children and in patients allergic to cockroach. (J Allergy Clin Immunol 2008;121:1040-6.)

Key words: Tropomyosin, Ascaris lumbricoides, Periplaneta americana, IgE cross-reactivity, allergy, parasites, asthma

It is estimated that 2 billion individuals worldwide are currently infected with the intestinal parasites Ascaris lumbricoides, Trichuris trichiura, hookworm, and Strongyloides stercoralis.1 Similar to patients with allergy, individuals infected with helminths often develop increased secretion of Th2-type cytokines, eosinophilia, and high levels of IgE.2 In addition, certain parasite infections, particularly those with Schistosoma mansoni, have been shown to trigger regulatory responses with increased IL-10 production, which may underlie the observation of inhibition of atopy and development of allergic symptoms in infected individuals.3,5 Studies performed in an endemic area of schistosomiasis in Brazil revealed reduced skin prick test reactivity to inhalant allergens, and an attenuated course of asthma in individuals harboring S mansoni eggs in their feces.6,7 However, the protective role of parasite infection in allergy and asthma may not be generalized to all species. Previous reports and a recent meta-analysis revealed that current infection with A lumbricoides was associated with a significant increase in the risk of asthma.8-10 whereas infection with hookworm was associated with a reduction in risk.10

Intervention studies aimed at clarifying the role of parasite infections in allergy and asthma have provided conflicting results.4 An open-label randomized controlled trial performed in Gabon showed that antihelmintic treatment of children with a high prevalence of infection, using praziquantel and mebendazole every 3 months, resulted in increased skin test reactivity to house dust mites.11 In Venezuela, an open-label randomized study demonstrated that monthly treatment with albendazole over a period of 1 year reduced symptoms of wheeze and the need for asthma medications.12 More recently, a cluster-randomized study in Ecuador revealed that treatment of a large group of children currently infected by A lumbricoides, T trichiura, Ancylostoma duodenalis, or S stercoralis with albendazole every 2 months for 12 months was highly effective in reducing helminth infection, but it was not associated with increase in the prevalence of atopy or allergy symptoms.13

We have focused our studies on the role of tropomyosin in IgE responses to A lumbricoides. Tropomyosins are highly conserved proteins that have been shown to be pan-allergens in invertebrates including shrimp and other crustaceans and mollusks, mites, and cockroach.14 In addition, tropomyosin has been identified in Anisakis simplex, a fish parasite that can cause allergic reactions
in human beings. The high degree of amino acid sequence identity among invertebrate tropomyosins provides support for immunological cross-reactivity, and some studies have suggested that this cross-reactivity may be clinically relevant. Sensitization and allergic symptoms after ingestion of snails and shrimp have been reported after specific immunotherapy with house dust mite, and increased severity of allergic reactions to snails has been observed in children receiving specific immunotherapy with dust mite extracts. IgE antibody reactivity to shrimp among Orthodox Jews, who are prohibited from eating shellfish, is thought to be a result of tropomyosin.

Preliminary data from our laboratory revealed that a mAb directed against mite tropomyosin showed strong binding to A lumbricoides striated muscle tissue from adult worms by means of immunofluorescence. In the current study, we have determined the amino acid sequence of A lumbricoides tropomyosin, expressed recombinant tropomyosins from A lumbricoides and from the cockroach species Pamericana, and compared IgE antibody responses to both proteins in patients with asthma and/or rhinitis allergic to cockroach, and in children living in an endemic area for A lumbricoides.

METHODS

Subjects

A panel of sera from 112 patients with asthma, wheezing, and/or rhinitis, age 2 to 52 years, who participated in previous studies from our group was analyzed for the presence of IgE antibodies to P americana and A lumbricoides tropomyosin. All patients presented allergy to cockroach, defined by presence of a positive skin test to extracts of Pamericana and/or Blattella germanica and/or the presence of specific IgE to B germanica in serum (ImmunoCap class ≥2). Sera from 119 children age 5 to 6 years who attended a day care center in Natal, Northeast Brazil, an endemic area for A lumbricoides, were also analyzed. Twenty-nine of these children participated in a previous study on the role of parasitic infection in allergy and asthma. Four subjects without allergy served as controls for assays for IgE antibodies to tropomyosin. The study was approved by the Ethics Committee of the School of Medicine of Ribeirão Preto.

Identification of cDNA coding for tropomyosin in A lumbricoides

Total RNA was extracted from 100 mg of an adult A lumbricoides worm by using Trizol LS (Invitrogen, Carlsbad, Calif.). DNA amplification was performed by RT-PCR, using primers synthesized on the basis of the sequences of Pamericana tropomyosin (Genbank AF106961) and Anisakis simplex tropomyosin (Genbank Y19221). Previous sequencing of partial cDNA coding for A lumbricoides tropomyosin, performed in our laboratory, using primers based on the DNA coding for Pamericana tropomyosin conserved sequences (MDA1KKB and LK6AEATRAE) revealed that tropomyosins from A lumbricoides presented 98% identity to A simplex tropomyosin. Primer sequences were as follows: forward primer 5’ ATGGAGCGCCATCAAGAA 3’, where M represents A or C; reverse primer 5’ ATATCCCAGGAAATGT GTG 3’. RT-PCR was performed as previously described. Briefly, 10 μl A lumbricoides RNA was mixed with deoxynucleotides 20 μM/L, reverse primer 20 μM/L, dithiothreitol 0.1 μM/L, reverse transcriptase 0.5 μL (Invitrogen, 200 U/L), and recombinant RNase Out Ribonuclease Inhibitor (Amersham Pharmacia, Uppsala, Sweden, 20,000 U/mL) 0.5 μL for 10 minutes at room temperature, and incubated at 37°C for 1 hour and at 95°C for 10 minutes. The forward primer was added, followed by 3 minutes incubation at 95°C. Reactions were performed in 50 μL volume, with denaturation at 95°C for 1.5 minute, annealing at 55°C for 1.5 minute, extension at 72°C for 1 minute for 34 cycles, and a final 10-minute step at 72°C using Taq polymerase 0.25 μL (Invitrogen, 5 U/μL). Amplified DNA was ligated into pCR2.1 vector (TA cloning kit; Invitrogen) and prepared for sequencing (QLAGEN Plasmid Purification system).

Production of recombinant tropomyosins from A lumbricoides and P americana

A lumbricoides RNA was used as a template to generate an 879-bp product for cloning into the pPICO Pichia pastoris vector (Invitrogen), and DNA amplification was performed by RT-PCR as described. Primers for PCR were synthesized as follows: 5’ GGGCTCTAATGAGCGCCTAGAA AA 3’ (sense), containing a Sma I restriction site, and 5’ ATAAAGAATGGCCGCATCCGGAAATGCTTT 3’ (antisense), containing a Not I restriction site. Digested PCR products were ligated into Sma I and Not I sites of pPIC9, and expression of recombinant proteins in the P pastoris system (Invitrogen) was performed following the manufacturer’s instructions. Plasmid DNA encoding Pamericana tropomyosin identified from a UNI-Zap expression library was used as a template to generate a 864-bp PCR product for cloning into the pPIC9. Primers for PCR were synthesized as follows: 5’ GCCG TACGTAATGGGCAGCGCATCAAGAA 3’ (sense), and 5’ ATAAAGAATGGCCGCATCCGGAAATGCTTT 3’ (antisense), containing Sma I and Not I restriction sites, respectively. After an initial denaturation step of 5 minutes at 95°C, PCR incubations were performed for 1 minute at 55°C and 3 minutes at 72°C for 30 cycles followed by a final extension at 72°C for 15 minutes.

Cultures were grown at 30°C with shaking, with addition of a 0.5% final concentration of methanol every 24 hours, and samples were collected at 24, 48, 72, and 96-hour time points for analysis. Pichia-expressed tropomyosins from A lumbricoides and P americana were purified from culture supernatants separate over mAb affinity columns, using anti-tropomyosin mAb 1A6. This mAb was originally raised against Dermatophagoides pteronyssinus tropomyosin; however, it recognizes tropomyosins from cockroach, mites, and shrimp. Twenty-five milligrams of mAb 1A6 were coupled to 1 g cyanogen bromide–activated Sepharose (Amersham Pharmacia), and culture supernatants were applied to the columns at 4°C. Proteins were eluted with 0.005 mol/L glycine pH 2.8 and analyzed by silver-stained SDS-PAGE using 8% to 25% gradient gels (Pharmacia Phast System).

Molecular modeling of tropomyosins from A lumbricoides and P americana

The MODELLER program was used for modeling of tropomyosins from A lumbricoides and P americana with the α-carbon atomic coordinates from porcine tropomyosin (Protein Data Bank code 1C1G). Spatial restraints and CHARMM energy terms were combined into an objective function. A total of 250 models were generated for each protein, and optimized final models were selected on the basis of stereochemical quality, assessed by the program PROCHECK. The cutoff for hydrogen bonds and salt bridges was 3.4 Å.

Immunofluorescence analysis of A lumbricoides larvae at stage L3

Eggs were obtained from adult worms of A lumbricoides by dissection and cultured for 4 weeks. Eggs containing larvae at the L3 stage were concentrated by centrifugation at 1500 rpm for 10 minutes, immersed in tissue-freezing medium (Tissue Tek, Electron Microscopy Sciences, Hatfield, Pa.), and frozen with dry ice and acetone. Ten-micrometer sections were placed on glass slides, rinsed 5 times with PBS, and blocked with 2% BSA-PBS for 15 minutes at room temperature. Sections were incubated for 1 hour with mAb 1A6 (5 μg/mL), rinsed 5 times with PBS, and blocked with 2% BSA-PBS for 15 minutes at room temperature. Sections were incubated for 1 hour with mAb 1A6 (5 μg/mL), rinsed 5 times with PBS, and incubated for 30 minutes with goat anti-mouse IgG conjugated to AlexaFluor 594 (25 μg/mL; Molecular Probes; Invitrogen). Slides were rinsed 8 times with PBS, mounted with Fluormount-G (Electron Microscopy Sciences), and examined by using a Nikon Ultraphot FX microscope (Nikon Instruments, Melville, NY). Slides incubated without primary antibody or with unrelated primary antibody, mAb 1D8, directed to mite allergen Der p 2 (2.5 μg/mL), served as controls.
Results of specific IgE to A lumbroicoides and P americana tropomyosin

Levels of IgE to A lumbroicoides and P americana tropomyosin were measured by chimeric ELISA. Briefly, microtiter plates were coated with 1 μg/well mAb 1A6 overnight at 4°C in carbonate-bicarbonate buffer, pH 9.6. After washing, plates were incubated with recombinant tropomyosin from A lumbroicoides and P americana (rPer a 7) at 0.5 μg/mL. Sera were added at 1:10 dilution, followed by incubation with biotinylated goat antihuman IgE (1:4000) and streptavidin-peroxidase (1:1000). The reaction was de-quanti ted by using a gas-phase sequencer PPSQ-21 A (Shimadzu, Kyoto, Japan) and similarity searches compared with those not sensitized to tropomyosin. Likewise, Ascaris-infected children with detectable IgE to tropomyosin showed no differences in the frequency of wheezing or lung problems compared with those not sensitized to tropomyosin. However, this region of the molecule has not previously been identified as containing IgE epitopes. 

Molecular models derived from the sequences of A lumbroicoides and P americana tropomyosins showed the characteristic coiled-coil structure common to tropomyosins (Fig 3).

Recombinant P americana tropomyosin (Per a 7 allergen) was expressed in P pastoris culture supernatants at 24 hours, with maximum production in 72 to 96 hours (data not shown). After purification, SDS-PAGE analysis revealed a 68-kd band under nonreducing conditions and a 34-kd band, corresponding to monomeric protein, under reducing conditions. These results suggested that the 68-kd protein corresponded to a homodimer of tropomyosin (Fig 4, A). The yield was 7 mg purified protein/L culture. A lumbroicoides tropomyosin was expressed as a recombinant protein by using a similar method, with a yield of 0.22 mg purified protein/L culture. Analysis of recombinant tropomyosin revealed major bands of estimated molecular weights of 30 kd and 8 kd (Fig 4, B). Amino acid sequencing of a peptide comprising residues 11 to 32, identified in both higher-molecular-weight bands, and of a peptide comprising residues 224 to 233, obtained from the lower-molecular-weight band, confirmed the identity of the A lumbroicoides recombinant protein to tropomyosin and suggested that the protein had been cleaved into several large peptides during expression. Addition of protease inhibitors in expression cultures has not modified this apparent protease activity (data not shown).

No differences of severity of asthma or rhinitis symptoms compared with those negative for IgE to this protein, measured by chimeric ELISA. Briefly, microtiter plates were coated with recombinant protein to tropomyosin and suggested that the protein had been cleaved into several large peptides during expression. Addition of protease inhibitors in expression cultures has not modified this apparent protease activity (data not shown). N-terminal sequencing showed that both recombinant proteins lacked the first 10 amino acid residues; however, this region of the molecule has not previously been identified as containing IgE epitopes.16

40 Forty-seven of the 112 patients from the allergy clinic (42%) had IgE to Per a 7 and to A lumbroicoides tropomyosin. Levels of IgE antibodies to Per a 7 varied from 0.4 to 300 IU/mL (geometric mean [GM], 1.0 IU/mL) and to A lumbroicoides tropomyosin ranged from 0.4 to 325 IU/mL (GM, 2.4 IU/mL). Among the 119 children living in Northeast Brazil, 90 (75.6%) and 93 (78.1%) subjects presented IgE to Per a 7 and to A lumbroicoides tropomyosin, respectively. Levels of IgE antibodies to Per a 7 varied from 0.4 to 175 IU/mL (GM, 2.1 IU/mL) and to A lumbroicoides tropomyosin ranged from 0.4 to 325 IU/mL (GM, 2.4 IU/mL). There was a significant correlation of levels of IgE to A lumbroicoides and P americana tropomyosin in sera of patients from Ribeirão Preto (r = 0.97; P < .0001) and of children from Natal (r = 0.95; P < .0001; Fig 5, A and B). Patients seen in the allergy clinics with positive IgE to tropomyosin presented no differences of severity of asthma or rhinitis symptoms compared with those not sensitized to tropomyosin.
invertebrates, including cockroach, mites, and shrimp. Invertebrate tropomyosins share greater than 70% sequence identity, whereas comparisons of amino acid sequences of invertebrate tropomyosins and vertebrate tropomyosins, which are nonallergenic, reveal a degree of 51% to 58% identity. Molecular modeling revealed that *A. lumbricoides* tropomyosin shares the typical α-helical, coiled-coil structure with tropomyosins from other sources.

![FIG 2. Sequence alignment of *A. lumbricoides* tropomyosin to tropomyosins from nematode, mite, and cockroach. The deduced amino acid sequence from cDNA showed 98%, 73%, and 69% identity to tropomyosins from *A. simplex* (GenBank Y19221.1), mite (GenBank Y14906), and cockroach (GenBank AF106961.1), respectively. The conserved N-terminal sequence DAIIKK is underlined, and the tropomyosin signature sequence L-K-E-A-E-x-R-A-E is indicated in red.](image)

Tropomyosin is expressed in high levels in L3 stage *A. lumbricoides* larvae, the stage of pulmonary passage of the parasite. Human IgE antibody binding to *A. lumbricoides* tropomyosin and to cockroach tropomyosin from *P. americana* showed a strong correlation.

Although both *A. lumbricoides* and cockroach recombinant tropomyosins produced in the current study lacked the first 10 N-terminal amino acid residues because of cleavage during expression, we believe that the IgE binding activity of the dominant large peptides of up to 38 kd has not been decreased.
Analysis of the *A lumbricoides* tropomyosin sequence at regions of IgE binding epitopes previously identified in shrimp tropomyosin (Pen a 1),\(^3\) which do not encompass the N-terminal portion of the molecule, showed 80% identity or greater to 5 of the 8 epitopes (see this article’s Table E1 in the Online Repository at www.jacionline.org). In the study by Ayuso et al.,\(^3\) IgE recognition of some of the cockroach tropomyosin (Per a 7) epitopes, particularly epitopes 2, 3a, 3b, 4, and 5a, by patients with shrimp allergy was similar to that of Pen a 1 epitopes. Our data suggest cross-reactivity caused by shared sequences within IgE binding epitopes. In the current study, none of the *A lumbricoides*-infected children presented positive skin test results to

**FIG 5.** Levels of IgE to *A lumbricoides* and *P americana* tropomyosins. There was a significant correlation of levels of IgE to *A lumbricoides* and *P americana* tropomyosins in sera of patients with asthma and/or rhinitis from Ribeirão Preto (A) and in sera of children living in Natal (B), areas with low and high prevalence of *A lumbricoides* infection, respectively.

**FIG 6.** Reactivity of antitropomyosin mAb 1A6 with *A lumbricoides* larvae. A, Strong reactivity of mAb 1A6 to L3 stage *A lumbricoides* larvae was observed by immunofluorescence. B, Control sections stained with an unrelated antibody revealed nonspecific fluorescence of the egg outer membrane, despite presence of larvae. C and D, Differential interference contrast images of A and B.
cockroach; likewise, none of the patients with cockroach allergy with asthma and/or rhinitis were currently infected with *A lumbricoides*. However, sera from these individuals presented IgE to tropomyosin from both *P americana* and *A lumbricoides*, and a strong correlation of levels of IgE to both proteins was observed.

Infection with *A lumbricoides* has been associated with lack of protection or even increased risk for allergen sensitization and asthma symptoms in some studies, whereas infections with *Schistosoma* and hookworm were shown to promote protection. Among preschool children living in Brazil, current infection with *A lumbricoides* was strongly and independently associated with wheezing. A study in China revealed that infection with *A lumbricoides* was associated with increased risk of childhood asthma and sensitization with inhalant allergens. In a community in South Africa, children with elevated *A lumbricoides* IgE had a higher frequency of allergic symptoms, atopic diseases, and positive skin prick test results to aerosol allergens than those without elevated IgE to the parasite. The increased risk of atopic symptoms was present among those with negative tuberculin test results. In young adults with asthma or rhinitis, no effect of *A lumbricoides* infection on skin test reactivity to allergens was observed; in addition, IL-10 production by PBMCs stimulated with *D pteronyssinus* antigens showed no difference in patients infected with *A lumbricoides* compared with noninfected patients. On the other hand, studies in Ecuador and Vietnam have pointed to an inverse association of geohelminth infections, including those with *A lumbricoides*, and skin test reactivity to allergens, suggesting a protective effect against sensitization among children in endemic areas.

The pulmonary phase of larval migration that occurs in the life cycle of some parasites including *A lumbricoides* and hookworms has been associated with wheezing, bronchial hyperresponsiveness, pneumonitis, pronounced pulmonary eosinophilia, elevated serum IgE, and production of cation and macrophage inflammatory protein 1α, in both human and experimental studies. A *lumbricoides* adult and larval-stage antigens induced increased proliferative responses in PBMCs of infected subjects compared with uninfected individuals, which were accompanied by increased expression of IL-4 and IL-5, with no differences in parasite-specific IL-10 production in the 2 groups. It is thought that this highly polarized Th2 immune response in the lung mucosa could cause symptoms and even enhance allergic reactivity to environmental allergens. We speculate that presence of tropomyosin in *A lumbricoides* L3 larvae in the respiratory tissue early in life could enhance Th2 polarized responses. In endemic areas, children often get infected with *Ascaris* through ingestion of parasite eggs before the first year of life. Most of the larvae passing through the lung tissue die locally, allowing release of antibodies including tropomyosin to be taken up by antigen-presenting cells, which in turn undergo migration to regional lymph nodes to stimulate Th2 responses and IgE production. Progressively these children also get exposed through the inhalation route to allergens derived from mites and cockroach, which share the highly homologous tropomyosin. It is possible that IgE responses to tropomyosin derived from inhalant allergens could be amplified or develop more promptly as a result of previous sensitization to *Ascaris* tropomyosin, triggering persistent lung inflammation.

The protective effect of hookworms could not be explained on the basis of direct effect on lung tissue. Infection with hookworms occurs later when children are able to walk independently and become susceptible to infection by larvae penetrating the skin. Therefore, it has been suggested that the age at which a parasitic infection is acquired may be an additional determining factor for subsequent modulation of allergic responses to environmental allergens by parasites.

Schistosomes, the worms implicated in suppression of allergy, on the other hand, do not present a pulmonary passage of larvae during their life cycle, and strongly induce IL-10 production. Schistosome-specific lysophosphatidylserine, a lipid structure derived from eggs and adult worms, has been shown to activate Toll-like receptor 2, leading to development of mature dendritic cells with the capacity of inducing IL-10–producing regulatory T cells. In addition, *Schistosoma* tropomyosin shows only 58% sequence identity to *A lumbricoides* tropomyosin.

In conclusion, the results of the current study show that tropomyosin is an important protein that induces IgE response in *A lumbricoides*–infected populations and in patients allergic to cockroach. Our results provide evidence to support immunologic cross-reactivity of allergens derived from *A lumbricoides*, inhalants (arthropods), and foods (crustaceans and mollusks), and suggest that *A lumbricoides* tropomyosin may have a role in development of allergy and asthma. The clinical relevance of IgE cross-reactive responses to tropomyosin would be best investigated in the setting of a birth cohort study, following infants from birth to school age, designed to detect the initial infections with *Ascaris* or other parasites; the development of IgE antibody responses, including those directed to tropomyosin; and the onset of allergic symptoms, in the context of other risk factors for development of allergy and asthma, including respiratory viral infections, allergen and endotoxin exposure, and breast-feeding.

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**Clinical implications:** Structural similarities of invertebrate tropomyosins could account for cross-reactive IgE antibody responses. Presence of tropomyosin in *A lumbricoides* larvae at the stage of pulmonary passage could enhance sensitization to tropomyosins from inhalant sources and contribute to allergic lung inflammation.

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**TABLE E1.** Sequence identity of *A lumbricoides* tropomyosin to IgE epitopes previously identified in shrimp tropomyosin (allergen Pen a 1)

<table>
<thead>
<tr>
<th>Epitope no.</th>
<th>Amino acid residues in Pen a 1 sequence</th>
<th>Sequence</th>
<th>Identity (%)</th>
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<tr>
<td>1</td>
<td>43-55</td>
<td>VHNLOKRMQQLEN</td>
<td>46</td>
</tr>
<tr>
<td>2</td>
<td>87-101</td>
<td>ALNRRQQLLEELER</td>
<td>80</td>
</tr>
<tr>
<td>3a</td>
<td>137-141</td>
<td>DEERM</td>
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<tr>
<td>3b</td>
<td>144-151</td>
<td>LENQLKEA</td>
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<tr>
<td>4</td>
<td>187-197</td>
<td>ESKIVELEEEL</td>
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</tr>
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Eight IgE-binding epitopes within Pen a 1 allergen have been identified by Ayuso et al.\(^1\) with sera from patients allergic to Pen a 1 who presented severe allergic reactions within 1 hour after ingestion of shrimp. Alignment of Pen a 1 sequences to homologous epitopes in *A lumbricoides* revealed sequence identities of 80% or greater to 5 of the 8 epitopes.

**REFERENCE**