Reduction of Worm Fecundity and Canine Host Blood Loss Mediates Protection against Hookworm Infection Elicited by Vaccination with Recombinant Ac-16

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Hookworm infection is one of most important parasitic infection of humans, occurring in 740 million people. Here we report the protective vaccination of dogs with Ac-16, an immunodominant surface antigen from the hookworm Ancylostoma caninum. We show that immunization with Ac-16 formulated with AS03 elicited specific humoral and cellular immune responses and provided partial protection against hookworm infection and morbidity as evidenced by a significant reduction of hookworm egg counts (64% reduction; P = 0.0078) and worm-induced blood loss (P < 0.05). Moreover, specific anti-Ac-16 antibodies recognized the native protein on the surface of third-stage larvae and blocked their migration through tissue in vitro. Our data support the use of Ac-16 as a potential candidate for vaccination against hookworm infection.

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MATERIALS AND METHODS

Cloning of Ac-16. Partial cDNA sequence information for Ac-16 was obtained by searching the Parasite Genomes Database through Wu-Blast2 (http://www.ebi.ac.uk/blast2/parasites.html) using the DNA sequence of Ost1, a gene encoding an immunodominant hypodermal antigen of Onchocerca volvulus (4). In order to clone the 5′ and 3′ cDNA regions of Ac-16, gene-specific primers (Ac-16-F1, ATGCTTGAAAGTCTGACTGAT; Ac-16-R1, CTCCTGAGTTATCCTCTCAT TGCACTCC; and Ac-16-R2, TGCTCCATTGTATCCCTGAC) were designed based on partial expressed sequence tag sequence information obtained from the Parasite Genomes Database. The 5′ and 3′ regions of Ac-16 cDNA were isolated from first-strand cDNA of an L3 A. caninum by a modified RNA ligase-mediated rapid amplification technique (GeneRacer; Invitrogen) using Ac-16 gene-specific primers as described previously (34). The full-length cDNA sequence of Ac-16 was obtained by aligning 5′ and 3′ regions of Ac-16 sequences.

Sequence analysis. DNA and predicted protein sequences were analyzed using ESEE version 3.1. Sequences were aligned using CLUSTAL W (http://clustalw.sourceforge.net) and prepared for display using BOXSHADE (http://bioweb.pasteur.fr/seqanal/interfaces/boxshade-simple.html).

Expression and purification of Ac-16. The entire coding sequence minus the N-terminal signal peptide of Ac-16 was cloned in frame into pET28a using BamHI and XhoI restriction sites, and BL21 Escherichia coli cells were trans-
formed with the recombinant construct using standard methods. Expression of the recombinant protein containing a His tag at the N terminus was induced with IPTG (isopropyl-β-D-thiogalactopyranoside) at a final concentration of 1 mM for 3 h at 37°C. The cells were centrifuged at 8,000 x g for 10 min, and the supernatant was removed. The pelleted cells were lysed by resuspending in TE (10 mM Tris, 1 mM EDTA) buffer (pH 8.0) containing 100 μg/ml lysozyme and 0.1% Triton X-100. After incubation at 30°C for 20 min, samples were sonicated on ice using an Ultra Processor XL 2020 sonicator (Misonix Incorporated) until no longer viscous. Soluble and insoluble fractions were separated by centrifugation and analyzed in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels under reducing conditions. Proteins were visualized by staining with Coomassie brilliant blue. For purification of the recombinant Ac-16, the pellet from 2 liters of culture medium was resuspended in 60 ml of 1.0% SDS and 0.5% 2-mercaptoethanol, boiled for 5 min, cooled to room temperature (−16), the pellet from 2 liters of culture medium was resuspended in 60 ml of 1.0% SDS and 0.5% 2-mercaptoethanol, boiled for 5 min, cooled to room temperature, and shaken overnight. The extract was dialyzed against 2 liters of 0.1% SDS in phosphate-buffered saline (PBS) for 24 h with two changes of buffer and applied to a 10-ml HisBind resin column (Novagen) until no longer viscous. Soluble and insoluble fractions were separated by centrifugation and analyzed in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels under reducing conditions. Proteins were visualized by staining with Coomassie brilliant blue. For purification of the recombinant Ac-16, the pellet from 2 liters of culture medium was resuspended in 60 ml of 1.0% SDS and 0.5% 2-mercaptoethanol, boiled for 5 min, cooled to room temperature, and shaken overnight. The extract was dialyzed against 2 liters of 0.1% SDS in phosphate-buffered saline (PBS) for 24 h with two changes of buffer and applied to a 10-ml HisBind resin column (Novagen). Chromatography was conducted according to the manufacturer’s instruction except that all buffers included 0.1% SDS.

Study dogs and animal husbandry. Purpose-bred, parasite-naive, male beagles aged 8 ± 1 weeks were purchased (Harlan Lab), identified by ear tattoos, and maintained in The George Washington University Animal Research Facility (16). The experiments were conducted according to a protocol approved by the University Animal Care and Use Committee.

Vaccine study design and immunizations. The vaccine trial was designed to test Ac-16 formulated with the adjuvant AS03 obtained from GlaxoSmithKline (16, 33). To prepare six doses of Ac-16 formulated with AS03, 600 μg of recombinant protein was mixed with 1.2 ml of 20 mM Tris-HCl, 0.5 M NaCl, pH 7.9, and 1.5 ml of AS03 (15). Dogs were immunized with 100 μg of formulated antigen in a final volume of 0.5 ml AS03 control was prepared as described above, with PBS instead of recombinant antigen.

Vaccinated dogs received three doses of the formulated hookworm recombinant antigen by intramuscular injection. The vaccine was administered on days 0, 21, and 42, beginning when the dogs were 62 ± 4 days of age. Control animals were also injected intramuscularly with an equivalent amount of AS03 using the same schedule. Two weeks after the final vaccination, dogs were challenged with 500 A. caninum L3 organisms by the footpad infection method (16). Each experimental group consisted of five animals.

Serological analysis. Blood was collected at least once every 21 days, with sera separated and stored at −80°C. Antigen-specific canine immunoglobulin G1 (IgG1), IgG2, and IgE were measured by an indirect enzyme-linked immunosorbent assay (ELISA) performed as described elsewhere (10). Briefly, 96-well microplates (Maxisorp; Nalgene Nunc Int.) were coated with 5 μg/ml of Ac-16 diluted with PBS (pH 7.4). Sera were added at dilutions of 1:100 and 1:500, followed by 3x serial dilutions thereafter. Plates were probed with peroxidase-conjugated anti-canine IgG1, IgG2, or IgE (Bethyl Laboratories Inc., Montgomery, TX) at a dilution of 1:1,000, developed with 100 μl/well of o-phenylenediamine (Sigma-Aldrich Co.), and then read at 490 nm after 30 min on an automated ELISA microplate reader (SpectraMax 240 PC reader; Molecular Devices, Sunnyvale, CA) with SOFTmax Pro software (Molecular Devices). The mean optical density of control canine (nonimmunized animals) sera was used as a baseline. The last serum dilution greater than three times above baseline was considered the titration endpoint. The geometric means of these endpoints were calculated for the five dogs from each group.

Western blot assay. Soluble and insoluble fractions of larval (L3) and adult (AE) crude extract antigens were boiled in SDS sample buffer, separated on a 4 to 20% gradient SDS-PAGE gel (InnovoTec), and transferred onto a nitrocellulose membrane (Schleicher & Schuell Biotechnology Inc.). The blot was probed with pooled sera (5 μl each) from vaccinated and control dogs at a dilution of 1:1,000. Horseradish peroxidase-conjugated anti-dog IgG (Bethyl) was used as a secondary antibody at a 1:5,000 dilution. Development was performed with ECL reagent (Amersham Biosciences, England) according to the manufacturer’s instructions.

Lymphoproliferation assay. Lymphoproliferation assays were performed using whole blood as previously described (10, 26). All tests were performed in triplicate with proliferation responses expressed as the stimulation index (SI = mean proliferation of stimulated cultures/mean proliferation of unstimulated cultures).

Interleukin 4 (IL-4), IL-10, and gamma interferon (IFN-γ) production by cultured whole blood. The whole blood was diluted 1:8 in RPMI supplemented with 3% antibiotic/antimycotic solution ( Gibco, Grand Island, NY) in a 48-well, flat-bottomed culture plate with a final volume of 1.0 ml per well. Cells were stimulated by the addition of 25 μg/ml of recombinant antigens. After 48 h of incubation at 37°C, 700 μl of supernatant was removed from each well and stored at −20°C until required for the cytokine assay.

IL-4, IL-10, and IFN-γ were measured using a capture ELISA assay for dogs (R & D Systems, Minneapolis, MN) following the manufacturer’s instructions. Biotin-labeled anti-canine detection antibodies were used and revealed with streptavidin-horseradish peroxidase (Amersham Biosciences) and an ortho-phenylenediamine substrate system (Sigma-Aldrich Co.). Results are expressed as the net production of cytokines (production of cytokine in stimulated wells – production of cytokine in control wells).

Cellular profile of peripheral blood. The phenotypic expression of peripheral blood cell populations was performed using whole-blood samples collected at the indicated days as described previously (10). Cells were stained with anti-canine CD3- fluorescein isothiocyanate (FITC), anti-canine CD4-FITC, anti-canine CD8-FITC, anti-canine B cells-phycocerythrin, anti-canine major histocompatibility complex class II-FITC, and anti-human CD14-FITC (all from Serotec Inc.). Eosinophils were analyzed by light scatter and by autofluorescence (7, 29, 31). Ig-FITC and phycocerythrin controls (Serotec Inc.) were used in all experiments. The data on fluorescently labeled cells were acquired in a FACSCalibur flow

FIG. 1. Alignment of the deduced amino acid sequence of Ac-16 with putative homologues from Ascaris suum (Ac14; GenBank accession no. BAB67769), Acanthocheilonema viteae (Av-RAL-2; AAB53809), Setaria digitata (Sd-SXP-1; AAX18863), Brugia malayi (Bm-SXP-1; AAA67319), and Onchocerca volvulus (Ov17; P36901). Sequences were aligned using CLUSTAL W and prepared for display using BOXSHADE. Identical amino acids are shaded in black, and similar amino acids are in gray. The percentage of sequence identity to Ac-16 is shown at the end of each sequence.

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cytometer (Becton Dickinson, San Jose, CA), selecting the lymphocyte population. Thirty thousand events were counted.

**Hemoglobin measurements.** To determine the hemoglobin concentrations of experimental dogs, 1 ml of blood was collected in EDTA and analyzed using a QBC VetAutoread hematology system and VETtest software (IDEXX Laboratories Inc., Westbrook, MA).

**Parasitological evaluation.** Quantitative hookworm egg counts (using the McMaster technique) (15) were performed three times per week beginning two weeks after challenge infection. Four weeks after the challenge infection, the dogs were euthanized by intravenous barbiturate injection, with adult hookworms recovered from the small and large intestines at necropsy (12). Approximately 1 to 2 cm of small intestine was separated and placed into formalin for future histopathological analysis.

**Immunolocalization.** Exsheathed *A. caninum* L3 organisms were fixed for 60 min at room temperature in 0.25% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, containing 1% sucrose and processed for immunoelectron microscopy, as described previously (18). Thin sections of embedded worms were probed overnight at 4°C with a 1:100 dilution of pooled dog sera from *Ac*-16-vaccinated animals (after vaccination but before hookworm challenge), washed, and incubated with rabbit anti-dog IgG (50 μg/ml) followed by protein A coupled with 10-nm gold particles (Amersham Biosciences). Preimmune serum was used as control. After staining with uranyl acetate, sections were observed with a Philips 410 electron microscope (Philips, Holland).

**In vitro L3 tissue migration assays.** Tissue migration assays were performed as described elsewhere (3, 32). Experiments were conducted in triplicate. The baseline was determined by counting the number of L3 organisms remaining on the skin in the presence of control dog serum. The number of L3 organisms that did not migrate in the presence of anti-*Ac*-16 sera was calculated by subtracting the skin in the presence of control dog serum. The number of L3 organisms that did not migrate in the presence of anti-*Ac*-16 sera was calculated by subtracting this baseline and then determining the percent reduction in L3 migration.

**Statistical analysis.** The small sample size (*n* = 10) did not provide enough data to discriminate between Gaussian and non-Gaussian distributions; so we used a nonparametric test (Mann-Whitney) to determine if there were statistical differences between the medians of two independent groups (vaccinated and control groups). When more than 20 values were available, the one-sample Kolmogorov-Smirnoff test was used to determine if the variable was normally distributed. To determine statistical differences among three or more normally distributed sets of data, we used analysis of variance. Statistical significance was set at a *P* value of ≤0.05 for all tests.

**Nucleotide sequence accession number.** The full-length nucleotide sequence of *Ac*-16 has been submitted to GenBank with accession number DQ445995.

**RESULTS**

**Ac*-16 cDNA cloning.** The 5′ and 3′ regions of *Ac*-16 were isolated from *A. caninum* L3 first-strand cDNA based on the partial expressed sequence tag sequence (AW735476) obtained from the Parasite Genomes Database. A full-length, 551-bp cDNA consensus sequence of *Ac*-16 was obtained by linking the 5′ and 3′ regions generated by 5′ and 3′ GeneRacer. The full-length *Ac*-16 cDNA encodes an open reading frame (ORF) of 147 deduced amino acids. There is a predicted 16-amino-acid hydrophobic signal peptide identified at the N terminus. The ORF ends with a TAA stop codon and is followed by a putative polyadenylation signal (AATAAA) and a poly(A) tail. The predicted *Ac*-16 protein has a calculated molecular mass of 15.3 kDa and a theoretical pI of 5.19.

Database searches revealed that the predicted amino acid sequence of *Ac*-16 belongs to a member of the SXP/RAL-2 superfamily of proteins that exist in a broad range of nematodes (14, 23, 27, 28, 30). An alignment of the putative *Ac*-16 ORF with SXP/RAL2 family proteins from other nematodes is shown in Fig. 1, and it demonstrates that *Ac*-16 exhibits 39 to 41% amino acid sequence identity with these homologues.

**Specific antibody production elicited by immunization with *Ac*-16.** Immunization with recombinant *Ac*-16 formulated with AS03 induced high titers of IgG1, IgG2, and IgE against *Ac*-16 (Fig. 2). Anti-*Ac*-16 antibody titers were observed in immunized animals only after the second dose of vaccination. IgE titers were low throughout the trial (Fig. 2C). Dogs immunized with adjuvant alone did not have any IgG1 and IgE response against *Ac*-16 at any time.

*Ac*-16 was detected by immunolocalization on the L3 surface.
(Fig. 3); the dog antibody labeled primarily the epicuticle as well as the basal layer of the cuticle.

Detection of Ac-16 in larval and adult A. caninum crude extracts. Western blot analysis showed that antibodies from Ac-16-vaccinated dogs recognized antigens from both L3 and AE antigen extracts (Fig. 4A). Only soluble antigens were detected in the L3 and AE extracts. Sera from control animals (immunized only with AS03) did not recognize any proteins in either extract (data not shown). Differences in the sizes of recombinant and native Ac-16 (Fig. 4) were expected due the presence of a His tag at the N-terminal portion of the recombinant protein.

Inhibition of larval invasion through tissue in vitro by anti-Ac-16 antibodies. Three replicates from three different experiments were used to determine the effect of anti-Ac-16 sera on migration of L3 organisms through tissue in vitro. Only 17.78 ± 25.87 of 300 L3 organisms that had been incubated in 50 μl of

<table>
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<tr>
<th>Group (n = 9)</th>
<th>No. of L3 organisms that failed to migrate</th>
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<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>213.3</td>
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<tr>
<td>Control</td>
<td>17.78</td>
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*Approximately 300 L3 organisms were applied to the skin, and the number of L3 organisms that did not successfully penetrate was counted. The percent reduction in migration after incubation of L3 organisms with serum from vaccinated dogs (Ac-16) versus control dogs (AS03) was 65.2% (P < 0.0001).
control sera remained in the skin, i.e., 5.9% did not migrate through tissue. In contrast, 213.3/1006.48 out of 300 L3 organisms that had been incubated in 50 l of anti-Ac-16 sera remained on the skin, i.e., 71.1% did not migrate through tissue. Therefore a 65.2% reduction (P < 0.0001) in migration of L3 organisms through tissue occurred in the presence of anti-Ac-16 sera (Table 1).

Cell proliferation, cytokine production, and cellular profile. Vaccination with Ac-16 induced a high level of specific lymphocyte proliferation (P < 0.05; Fig. 5A). No significant cell proliferation against Ac-16 was observed before the immunization (data not shown). Immunization with Ac-16 induced a significantly higher IFN-γ production in vaccinated animals after antigen stimulation (P = 0.0241; Fig. 5B). No significant difference in the IFN-γ production before and after vaccination was observed in the control group. No significant production of IL-4 or IL-10 after stimulation with Ac-16 was detected in either vaccinated or control groups (data not shown). In addition, no statistical differences were found in the cellular profiles of peripheral blood from animals immunized with Ac-16 or AS03 alone. Ac-16-vaccinated animals presented average values for T CD4+, T CD8+, B cells, major histocompatibility complex class II, CD11/18, monocytes, and eosinophils of 47.7 ± 6.3, 16.2 ± 2.2, 18.5 ± 2.9, 47.7 ± 1.4, 2.5 ± 3.3, 8.9 ± 2.1, and 9.9 ± 3.9, respectively.

Parasitological evaluation. Female hookworms recovered from the intestines of vaccinated dogs were less fecund than worms from control dogs, with a decrease of 63.4% in egg production (P = 0.0078; Fig. 6A). The egg counts were calculated from the mean of the daily egg counts from the last two days of the experiment. A reduction in adult hookworm burden in the small intestine was observed in animals immunized with Ac-16 (25.3%; 25.3% for female worms and 25.7% for male worms); however, these differences were not statistically significant.

Vaccination with Ac-16 protects against anemia. After challenge infection, hemoglobin levels of animals vaccinated with Ac-16 were significantly (P < 0.05) elevated compared with levels for control dogs (Fig. 6B). The mean hemoglobin concentration of vaccinated dogs immediately prior to necropsy was 12.12 ± 1.25 g/dl. Conversely, control dogs showed a decrease in hemoglobin levels (10.00 ± 1.09 g/dl) corresponding to established times of patency (adult worms arriving in the gut).

DISCUSSION

Here we report the cloning, anatomic location, and protective role of Ac-16, a member of the SXP/RAL-2 family. We show that vaccination with Ac-16 protects against hookworm...
Ac-induced blood loss. The similarity of disease by reducing hookworm egg counts and hookworm-induced blood loss. The similarity of Ac-16 to other proteins from the SXP/RAL-2 family, such as from *Ascaris suum* (*As14*), *Acanthocheilonema viteae* (*Av*-RAL-2), *Setaria digitata* (*Sd*-SXP-1), *Brugia malayi* (*Bm*-SXP-1), and *Onchocerca volvulus* (Ov17 and P36991), suggests that Ac-16 might share some biological properties with these (apparently) nematode-specific proteins (23). While little is known of the functions of these proteins, some of them were identified as targets for nematode vaccines due to the protection they conferred upon immunization. Vaccination of gerbils with recombinant *B. malayi* SXP protein showed significant reductions in worm burden and microfilaria (hatched L1) levels following challenge (30). The *O. volvulus* homologue, Ov17/Ov-RAL-2, induced protective immunity of 51 to 60% in mice against an L3 challenge (19). Moreover, the *Ascaris* homologue (*As14*) has been shown to confer protective immunity against *A. suum* infection in mice (27). Our results show that dogs immunized with Ac-16 were protected against challenge infection, as demonstrated by a decrease in egg counts and a marked reduction in adult worm burden. It should be emphasized that the protective immunity elicited by Ac-16 differs from the protection elicited by Ac-16 homologues (*As14*, *Av*-RAL-2, *Sd*-SXP-1, *Bm*-SXP-1, Ov17, and P36991), which were tested in animal laboratory models that were not the natural-host parasite systems (e.g., mice). Since the current data reflect the impact of vaccination on a natural-host parasite system (i.e., *A. caninum* in canines), they are much more likely to reflect the efficacy of this vaccine for human hookworm infection.

Immunolocalization using specific antibodies against Ac-16 demonstrates its expression on the L3 surface, mostly in the epicuticle and the basal layer of the cuticle. Detection by immunoblotting of native Ac-16 in soluble extracts from both L3 and adult worms demonstrates its presence in both larval and adult stages of hookworms. Other proteins of the SXP/RAL-2 family, such as *Wb*-SXP-1 (from *Wuchereria bancrofti*), have also been localized to the hypodermis of the adult worm, concentrating in the sheath proteins, which suggests secretion of the protein (25). Other studies showed SXP proteins localized in the filarial hypodermis (4) or as secreted proteins (14, 28).

Vaccination with Ac-16 elicited protective immunity against parasite challenge as observed by decreased egg production, (nonsignificant) reduction of adult worms, and protection against anemia caused by the parasite’s blood feeding. Vaccination also induced antibodies that impaired L3 migration through animal tissue (skin), probably also impairing migration of larvae in the host (10). Conversely, while an impairment of L3 migration was demonstrated in vitro, the difference in the reduction of adult worms in the gut was modest. Therefore, although most L3 organisms matured and migrated to the gut, the physical attenuation of L3 organisms by anti-Ac-16 antibodies probably attenuated their reproductive development, accounting for the diminished fecundity of adult female worms. We saw a similar phenomenon when dogs were vaccinated with recombinant ASP-2 from *A. caninum* (11). On the other hand, the reduced egg output could result from the lower number of female adult worms established in the intestine as a direct effect of vaccination. However, since the egg count reduction was more pronounced than the adult worm reduction, a lower number of eggs per female was observed, and it suggests the impairment of the fecundity of those worms.

Proliferation of peripheral blood leukocytes was observed in vaccinated dogs after antigen stimulation, indicating that the immunization process induces a memory response in previously primed cells (10). Interestingly, although the presence of IgG1 and IgG2 antibodies to Ac-16 suggests the presence of a mixed Th1/Th2 response in dogs, only IFN-γ production was detected in whole-blood cultures from vaccinated animals in response to Ac-16 stimulation, suggesting that Ac-16 induces a Th1 response. Similar results were obtained when dogs were vaccinated with recombinant Ac-APR-1 hemoglobinase, which also induced a mixed IgG1/IgG2 serologic response but only an IFN-γ cytokine response after antigen stimulation in vaccinated animals (15). Since both Ac-APR-1 and Ac-16 demonstrate protection against parasite infection and also against anemia, we cannot exclude that a Th1 immune response might have an important role in vaccine-mediated protection against hookworm infection. In fact, a mixed Th1/Th2 response is observed in dogs immunized with irradiated hookworm larvae (the gold standard for a hookworm vaccine) (10), indicating that a Th1 immune response induced by a recombinant vaccine is not detrimental to protection.

Protection against anemia was also described in the experimental vaccination of hamsters with *A*-ASP-2 (cloned from *Ancylostoma ceylanicum*) prior to challenge with *A. ceylanicum* (11, 20) and vaccination of dogs with Ac-APR-1 prior to challenge with *A. caninum* (15). Ac-APR-1 degrades hemoglobin in the gut lumen of the worm; therefore, it is not surprising that interruption of the function of APR-1 via neutralizing antibodies reduces blood loss and has a deleterious effect on the establishment of worms, particularly female worms and their subsequent egg production (15). It is less clear how anti-Ac-16 antibodies protect against blood loss, given that adult worm burdens were not significantly reduced. However, the developmental attenuation of L3 organisms by anti-Ac-16 antibodies might affect their ability to blood-feed, just as it appears to compromise reproductive capacity in adult worms.

Our strategy to vaccine against human hookworm infection is based on employing antigens that are uniquely expressed in a single developmental stage of the parasite within the host, by targeting either the larval stage (to reduce the numbers of L3 larvae entering the gastrointestinal tract) or the adult blood-feeding stage (to block its establishment, survival, and/or fecundity). Several hookworm antigens, such as Ac-ASP-2 (from larvae), Ac-APR-1, and Ac-GST-1 (both from adults), have been demonstrated as potential candidates for a human hookworm vaccine. Ac-16 is unique in that it is (to date) the only efficacious antigen that is present in both adult and larval stages, and as such this supports its use as a monovalent vaccine against human hookworm infection in the future.

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