INFECTION AND IMMUNITY, Apr. 2004, p. 2214–2221 Vol. 72, No. 4
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.4.2214–2221.2004

Molecular Characterization of *Ancylostoma ceylanicum* Kunitz-Type Serine Protease Inhibitor: Evidence for a Role in Hookworm-Associated Growth Delay

Daniel Chu,[1] Richard D. Bungiro, Jr.,[1] Maureen Ibanez,[1] Lisa M. Harrison,[1] Eva Campodonico,[1] Brian F. Jones,[1] Juliusz Mieszczanek,[1,2] Petr Kuzmic,[3] and Michael Cappello[1]*

Child Health Research Center, Departments of Pediatrics and Epidemiology & Public Health, Yale School of Medicine, New Haven, Connecticut[1]; Department of Parasitology, Warsaw Agricultural University, Warsaw, Poland[2]; and BioKin Ltd., Pullman, Washington[3]

Received 26 November 2003/Returned for modification 15 December 2003/Accepted 24 December 2003

Hookworm infection is a major cause of iron deficiency anemia and malnutrition in developing countries. The *Ancylostoma ceylanicum* Kunitz-type inhibitor (AceKI) is a 7.9-kDa broad-spectrum inhibitor of trypsin, chymotrypsin, and pancreatic elastase that has previously been isolated from adult hookworms. Site-directed mutagenesis of the predicted P1 inhibitory reactive site amino acid confirmed the role of Met^{26} in mediating inhibition of the three target serine proteases. By using reverse transcription-PCR, it was demonstrated that the level of AceKI gene expression increased following activation of third-stage larvae with serum and that the highest level of expression was reached in the adult stage of the parasite. Immunohistochemistry studies performed with polyclonal immunoglobulin G raised against recombinant AceKI showed that the inhibitor localized to the subcuticle of the adult hookworm, suggesting that it has a potential in vivo role in neutralizing intestinal proteases at the surface of the parasite. Immunization with recombinant AceKI was shown to confer partial protection against hookworm-associated growth delay without a measurable effect on anemia. Taken together, the data suggest that AceKI plays a role in the pathogenesis of hookworm-associated malnutrition and growth delay, perhaps through inhibition of nutrient absorption in infected hosts.

Hookworm infection remains a major global health problem, and over one billion people are reportedly infected in developing countries (9, 14). Hookworms, which are blood-feeding intestinal nematodes, are a major cause of iron deficiency anemia and malnutrition (15, 20, 59–61, 64). While the anemia is presumably due to the cumulative effect of chronic intestinal blood loss, the molecular mechanisms underlying the pathogenesis of hookworm malnutrition remain unknown. Although it has been suggested that hookworm malnutrition and growth delay occur secondary to chronic iron deficiency, particularly in children, evidence from prior clinical studies suggests that hookworm infection is also associated with various degrees of intestinal malabsorption (18, 35, 54, 57, 62). It has been hypothesized that this hookworm malabsorption syndrome might occur secondary to mucosal inflammation triggered by the adult worm attached to the intestinal epithelium or might be a result of secretion of parasite inhibitors of host digestive enzymes (18).

As part of a series of ongoing studies aimed at characterizing adult hookworm secretory proteins, a cDNA corresponding to the gene encoding a putative Kunitz-type serine protease inhibitor was previously identified from adult *Ancylostoma ceylanicum* RNA by using a PCR-based approach (48). The *A. ceylanicum* Kunitz-type inhibitor (AceKI) cDNA was expressed in *Escherichia coli*, and the recombinant protein was found to inhibit the pancreatic enzymes chymotrypsin, pancreatic elastase, and trypsin in vitro, with equilibrium inhibitory dissociation constant (K_i) values ranging from picomolar levels to low nanomolar levels. The native AceKI protein was also purified from adult hookworm excretory-secretory (ES) products, which strongly suggests that it has a role in the biology of the adult hookworm.

We report here studies aimed at characterizing the molecular mechanism of action of AceKI, as well as the role of AceKI in the pathogenesis of hookworm-associated malnutrition and growth delay. Our data confirm that AceKI inhibits its target serine proteases by a mechanism that involves interaction with the amino acid residue Met^{26}, the predicted P1 inhibitory reactive site amino acid. In addition, immunohistochemistry studies demonstrated that the native AceKI protein localizes to the subcuticle of the adult worm, suggesting that it has a possible in vivo role in inhibiting proteolytic damage by host digestive enzymes. Finally, immunization with recombinant AceKI (rAceKI) was found to confer partial protection against hookworm-associated growth delay, strongly suggesting that the protease inhibitor has a role in disease pathogenesis.

MATERIALS AND METHODS

Site-directed mutagenesis. The cDNA encoding the mature AceKI protein was cloned into the pET32a expression vector (Invitrogen) as described previously (48). Orientation of the inserted gene sequence was confirmed by sequencing plasmid DNA purified from *E. coli* transformed with the AceKI-pET32a construct. Point mutations were incorporated into the proposed reactive site of AceKI (Met^{26}) by using amplification primers (Fig. 1) that made the desired sequence changes; these mutagenesis primers were designed to anneal to opposite strands of the AceKI-pET32a plasmid. This was followed by PCR amplification of plasmids containing each of the mutations.

Briefly, a large amount of template (750 ng) was combined with primers,

* Corresponding author. Mailing address: Yale Child Health Research Center, P.O. Box 208081, New Haven, CT 06520-8081. Phone: (203) 737-4320. Fax: (203) 737-5972. E-mail: michael.cappello@yale.edu.

AceKI: Translated amino acid sequence of mature protein cDNA
AEEAGKKLTDEERCNAPTHLDGPQC*M*AFFKRYTYNKEKKQC
EEFVYGGCRPSPNNFETMEECKKTCVK

Primers for Met26 → Lysine Mutagenesis
5'- CCACAATGCAAAGCGTTCTTCAAGAGGTACACC - 3'
5'- TCCATCTAGGTGAGTCGGAGCATTACATCTCTC - 3'

Primers for Met26 → Alanine Mutagenesis
5' - CCACAATGCGCGGCGTTCTTCAAGAGGTACACC - 3'
5' - TCCATCTAGGTGAGTCGGAGCATTACATCTCTC - 3'

Primers for Met26 → Glycine Mutagenesis
5' - CCACAATGCGGGGCGTTCTTCAAGAGGTACACC - 3'
5' - TCCATCTAGGTGAGTCGGAGCATTACATCTCTC - 3'

FIG. 1. Translated amino acid sequence of AceKI and oligonucleotide primer sequences used to generate P1 reactive site mutants. The predicted P1 inhibitory reactive site (Met^{26}) of the mature AceKI protein (48) is indicated by boldface italics. The oligonucleotide primers were utilized to amplify AceKI coding sequences containing the specified site-directed mutations. The underlined nucleotide sequences correspond to the amino acid residue altered for each mutant.

deoxynucleoside triphosphates, and *Taq* polymerase (2.5 U of Amplitaq; Applied Biosystems) and placed in a thermal cycler under the following conditions: one cycle of 94°C for 2 min, 50°C for 1 min, and 72°C for 2 min, followed by eight cycles of 94°C for 30 s, 50°C for 1 min, and 72°C for 1 min and a final extension for 5 min at 72°C. The methylated template DNA was then digested with the endonuclease DpnI, and the double-stranded amplification product was treated with *Pfu* DNA polymerase to create blunt ends. The resulting cDNA was then ligated by using T4 DNA ligase and used to transform ultracompetent DH5α cells. Following sequence confirmation, the plasmid construct was transformed into *E. coli* ORIGAMI (Novagen) competent cells.

Purification and expression of rAceKI mutants. The three rAceKI mutants were purified from lysates of cells that had been transformed with the appropriate pET32 plasmids and induced with isopropyl-β-D-thiogalactopyranoside (IPTG). Each insoluble fraction was removed by centrifugation (13,000 × *g*), and the supernatant was applied to a HiTrap chelating column (Amersham Pharmacia Biotech). Bound protein was eluted from the column with imidazole, and the fractions containing recombinant protein were pooled. Following affinity chromatography, the partially purified recombinant protein was subjected to reverse-phase high-pressure liquid chromatography by using a C_{18} column (Vydac, Hesperia, Calif.) (10, 19, 48). The relative purity and molar concentration of the recombinant protein were determined by electrospray ionization mass spectrometry and quantitative amino acid analysis as previously described (12, 26, 48).

Protease inhibition assays. Single-stage chromogenic assays (48) were used to characterize the inhibitory activities of the various purified AceKI preparations against three serine proteases: trypsin, chymotrypsin, and pancreatic elastase. The recombinant proteins were preincubated with each of the purified enzymes for 15 min at 25°C, and this was followed by addition of the appropriate chromogenic substrate. In 200-μl (total volume) mixtures in individual wells of a 96-well microtiter plate, the final concentrations of enzyme and substrate were as follows: 1.9 nM pancreatic elastase (Sigma Aldrich, St. Louis, Mo.) and 250 μM Suc-Ala-Ala-Pro-Ala-paranitroanilide (pNA) (Bachem, Torrence, Calif.); 4.6 nM α-chymotrypsin (Sigma) and 200 μM Suc-Ala-Ala-Pro-Phe-pNA (Bachem); and 10 nM trypsin (Sigma) and 250 μM H-D-Pro-Phe-Arg-pNA (DiaPharma, West Chester, Ohio). The kinetic rate of substrate hydrolysis (optical density at 405 nm per minute) was measured for 5 min by using a kinetic microplate reader (MRX HD; Dynex Laboratories, Chantilly, Va.).

For each of the recombinant protein-serine protease combinations, inhibitory data were generated by incubating increasing concentrations of rAceKI with a fixed concentration of enzyme for 15 min at 25°C. After addition of the chromogenic substrate, the initial rate (optical density at 405 nm per minute) of substrate hydrolysis over 5 min was measured as described above. The rate of hydrolysis in the presence of increasing amounts of inhibitor was then plotted against the corresponding inhibitor concentration (10–12, 48). The initial velocities were analyzed by using the software DYNAFIT (42) by assuming the competitive inhibition mechanism under the rapid-equilibrium approximation (56). Asymmetrical confidence intervals at the 95% confidence levels were computed by using the t-profile method of Bates and Watts (1).

Antibody production and purification. Polyclonal antiserum directed against native rAceKI was produced by immunizing a 3-month-old male New Zealand White rabbit with 100 μg of reverse-phase high-pressure liquid chromatography-purified protein emulsified in Freund's adjuvant. After 3 weeks, the rabbit was given a booster immunization containing 50 μg of recombinant protein in Freund's incomplete adjuvant. All animal immunizations were carried out by certified personnel from the Yale Animal Resources Center by using protocols approved by the Yale Animal Care and Use Committee. Serum was collected following the second boost, and the immunoglobulin G (IgG) fraction was purified by protein G affinity chromatography as previously described (19, 25). Protein concentrations in the fractions containing the purified IgG were determined by using reagents from a bicinchoninic acid kit (Pierce, Rockford, Ill.).

Stage- and tissue-specific expression of AceKI. The stage specificity of AceKI RNA expression was evaluated by reverse transcription (RT)-PCR. Briefly, total RNA was isolated from 5,000 *A. ceylanicum* third-stage larvae (L3) or 30 adult parasites by homogenizing whole worms in Trizol (Life Technologies) (5, 19, 26). RNA was also isolated from 5,000 L3 that had been activated by incubating them in 50% fetal bovine serum for 2 h at 37°C. This process has been shown to induce feeding of hookworm L3 and upregulate the expression of selected genes (27–32). The primers used for the RT-PCR corresponded to a 200-bp fragment of the AceKI cDNA. As a positive control, an identical aliquot of RNA from each set of hookworms was used as a template for amplification of a 200-bp fragment of the previously identified nematode 5.8S ribosomal DNA (13). Synthesis of the first cDNA strand was carried out by using Superscript reverse transcriptase (Life Technologies) with either a poly(T) primer or the reverse 5.8S ribosomal DNA primer (ACAACCCTGAACCAGACGTG). Equal amounts of cDNA were then used as templates for a PCR performed with either the AceKI primers (forward primer GTCTTCTGGTGGTGCTGCTT and reverse primer TCGCATTGCTT CTTTTCCT) or the 5.8S ribosomal DNA primers (forward primer CGTATCG ATGAAAAACGCAG and reverse primer ACAACCCTGAACCAGACGTG). The conditions for amplification were as follows: 30 cycles of 94°C for 10 s, 55°C for 10 s, and 72°C for 30 s. The PCR products were subjected to agarose gel electrophoresis, and the PCR products were visualized by ethidium bromide staining.

The purified anti-AceKI IgG was used to localize the native protein in whole adult *A. ceylanicum* hookworms, as well as pooled ES products collected from adult worms. Immunoblots were prepared by subjecting rAceKI or pooled *A. ceylanicum* ES products to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to nitrocellulose. The blots were incubated with purified anti-rAceKI IgG, and the bound antibody was detected by using horseradish peroxidase-labeled anti-rabbit IgG and chemiluminescence as previously described (5, 6, 25). For immunohistochemistry studies, live *A. ceylanicum* adults were fixed in 10% formalin. Individual worms were embedded in paraffin, and contiguous sagittal sections and cross sections were fixed on microscope slides. The slides were deparaffinized and rehydrated by incubating them in solutions containing decreasing concentrations of ethanol. The rehydrated slides were then incubated with anti-AceKI IgG for 4 h at 25°C. After washing, the bound antibody was detected by incubating the slides in Texas red-labeled anti-rabbit IgG (Sigma) and was visualized by fluorescent microscopy. Control slides were prepared by using preimmune rabbit IgG as the primary antibody.

Vaccine study with rAceKI. Twenty-one-day-old male Syrian hamsters of the outbred strain Lak:LVG (SYR)BR (Charles River Laboratories) were immunized with purified rAceKI adsorbed to Rehsorptar 2% aluminum hydroxide gel (alum) by using a previously reported protocol (7). The individuals in groups of five hamsters were each immunized with 0.2 ml of an antigen solution subcutaneously in the scruff of the neck. The initial immunization, consisting of 100 μg of rAceKI, was followed by two booster immunizations (50 μg each). The control animals received only the alum solution without recombinant protein. One week after the second booster immunization the animals were each infected by oral gavage with 100 infectious L3. Weights and hemoglobin concentrations were determined throughout the course of the study. For hemoglobin studies, blood samples were collected from the orbital plexus in heparinized capillary tubes (Fisher Scientific) and were assayed by using reagents from a hemoglobin assay kit (Sigma Diagnostics) as previously described (6, 7). Data are presented below as means ± standard errors. Significance testing was conducted by using the StatView 4.51 statistical analysis software package (Abacus Concepts, Inc., Berkeley, Calif.). For multiple-group comparisons, analysis of variance was performed, followed by Fisher's protected least-significant-difference test as a post-test. *P* values of <0.05 were considered significant.

RESULTS

Confirmation of the P1 inhibitory reactive site of AceKI. Previous work has demonstrated that rAceKI is a tight-binding

TABLE 1. Competitive inhibition constants (K_i) and corresponding 95% confidence intervals derived from kinetic analysis of AceKI mutants

Inhibitor	Chymotrypsin		Trypsin		Elastase	
	K_i (nM)	CI_{95} (nM)[a]	K_i (nM)	CI_{95} (nM)	K_i (nM)	CI_{95} (nM)
Wild type	0.10	0.07–0.14	23	14–37	42	33–56
M26G	1.22	0.95–1.58	960	670–1,620	1.9	0.9–4.1
M26A	0.16	0.10–0.29	135	50–880	10.3	6.5–17.5
M26K	0.21	0.15–0.29	2.2	1.2–4.1	1,530	710[b]

[a] CI_{95}, 95% confidence interval.
[b] The upper limit of the 95% confidence interval could not be calculated, as the M26K mutant has no significant inhibitory activity against pancreatic elastase.

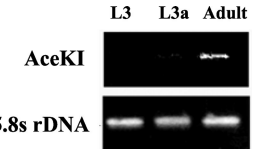

FIG. 2. Stage-specific expression of the AceKI mRNA. The expression of AceKI was monitored by RT-PCR amplification by using nondegenerate oligonucleotide primers based on the AceKI cDNA. (Top panel) A clear band at the expected size (200 bp) was amplified from adult *A. ceylanicum* RNA but was not detected in L3. A faint signal was detected in the lane containing RNA isolated from L3 that had been activated with host serum (L3a). (Bottom panel) Control RT-PCR amplification mixtures included the same template RNA, but oligonucleotide primers corresponding to the nematode 5.8S ribosomal DNA (rDNA) (13) were used.

inhibitor of trypsin, chymotrypsin, and pancreatic elastase (48). Tight-binding inhibitors act via a mechanism that involves interaction of the enzyme's reactive site with the P1 amino acid residue of the inhibitor (43, 44, 51). Although this interaction is reversible, the enzyme-inhibitor complex is sufficiently stable that considerable inhibition can be detected at nearly equimolar concentrations. Based on an alignment of AceKI with other members of the Kunitz-type family, we identified Met^{26} as the likely P1 inhibitory reactive site amino acid (8, 43, 49, 66). Therefore, in order to more clearly elucidate the role of Met^{26} in defining the spectrum of inhibition of AceKI, a series of site-directed mutants were produced that had various substitutions incorporated at the P1 site (23, 33, 41, 46, 51, 65). These recombinant proteins were expressed and purified, and their inhibitory activities against the three serine protease targets of AceKI were measured. Each site-directed mutant exhibited a distinct inhibitory pattern against trypsin, chymotrypsin, and pancreatic elastase when a single-stage chromogenic assay was used. Analysis of the inhibitory equilibrium dissociation constants (K_i values) for each of the rAceKI proteins confirmed the effect of replacement of Met^{26} on the affinity for each target protease. As shown in Table 1, in the case of chymotrypsin, the only significant change in the inhibitory activity was observed with the glycine mutant (M26G), which was approximately 10 times less potent based on the inhibition constant (K_i). However, in the case of trypsin, all three mutations (glycine, alanine, and lysine in place of Met^{26}) resulted in significant changes in inhibitory activity. The glycine mutant (M26G) was almost 2 orders of magnitude less potent than the wild-type inhibitor against trypsin. The opposite effect was observed for the lysine mutant (M26K), which exhibited binding to trypsin that was 10 times stronger than the binding of the wild type. In the case of pancreatic elastase, the glycine and lysine mutations had the inverse effects, at least compared to their effects on the inhibition of trypsin. The glycine mutant was found to be an approximately 20-fold-stronger inhibitor than the wild-type rAceKI, whereas the lysine mutant bound so weakly that the upper limit of the 95% confidence interval for the K_i could not even be determined. Statistically speaking, the inhibitory effect of the M26K mutant on pancreatic elastase was negligible.

Stage- and tissue-specific expression of native AceKI. An activity consistent with AceKI has previously been identified in adult *A. ceylanicum* ES products, and the native protein has successfully been purified from soluble protein extracts of adult hookworms (48). The stage specificity of AceKI gene expression was further characterized by RT-PCR. Total RNA was isolated from *A. ceylanicum* L3 cultured from the feces of infected hamsters (21, 22), as well as from adult worms. Additional RNA was isolated from *A. ceylanicum* L3 that had been activated by incubation in 50% fetal calf serum, which has been shown to stimulate feeding of hookworm larvae and upregulate expression of certain genes (27–32). As shown in Fig. 2, the 200-bp product consistent with AceKI gene expression was not detected in L3, although there was a faint band in the lane containing RNA from activated L3. In contrast, the adult hookworm RNA produced a prominent signal, confirming that the greatest levels of AceKI gene expression occur in the adult, intestinal stage of the parasite.

In order to confirm that the native AceKI is secreted by the adult worm, immunoblots of ES proteins separated by SDS-PAGE were probed with the anti-rAceKI IgG. As shown in Fig. 3, the IgG raised against the recombinant protein recognized the pET32-rAceKI fusion protein, which had a predicted molecular mass of 25.9 kDa. In addition, the IgG also recognized a band in *A. ceylanicum* ES products at an estimated molecular mass of 6 to 8 kDa, which is similar to the predicted molecular mass of the native AceKI protein. No immunoreactivity was observed when normal rabbit IgG was used as the primary antibody (data not shown).

In order to localize the exact region where AceKI is produced, sections of adult *A. ceylanicum* were probed with a polyclonal IgG raised against the recombinant protein (19). As

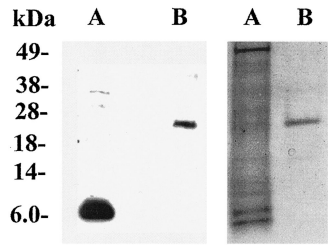

FIG. 3. AceKI is secreted by the adult hookworm. (Left panel) Pooled adult *A. ceylanicum* ES products (7.5 μg) (lane A) and purified rAceKI fusion protein (lane B) were subjected to SDS-PAGE followed by transfer to nitrocellulose and probing with anti-AceKI IgG and detection by chemiluminescence. (Right panel) Adult hookworm ES products and rAceKI were subjected to SDS-PAGE and stained with Coomassie blue.

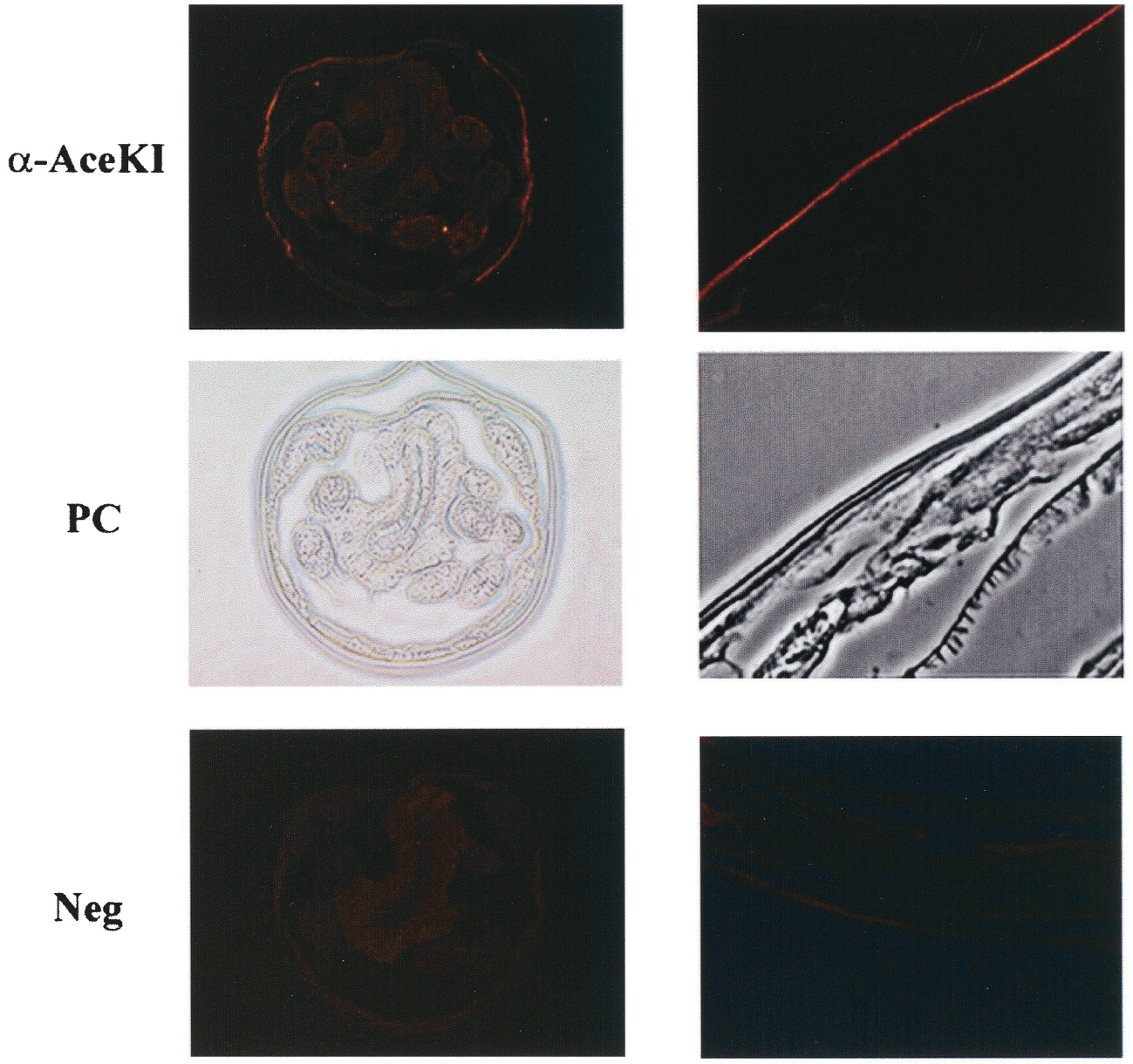

FIG. 4. Immunolocalization of AceKI. (Top panels) Paraffin-embedded cross section (left panel) and sagittal section (right panel) of *A. ceylanicum* adults were probed with anti-rAceKI IgG (α-AceKI), and this was followed by detection with rhodamine-labeled secondary antibody and fluorescent microscopy. The IgG localizes to the subcuticle of the adult worm, which is located just beneath the outer tegument. (Middle panels) Sections viewed by phase-contrast microscopy (PC). (Bottom panels) Contiguous sections probed with preimmune rabbit IgG as a negative control (Neg).

shown in Fig. 4, the protease inhibitor localized primarily to the subcuticle, near the outermost surface of the worm's tegument. No significant staining was detected in other structures, including the esophagus, intestinal tract, and major secretory glands. This pattern of staining suggests that AceKI, by localizing to the outer surface of the adult hookworm, may function as an in vivo inhibitor of the digestive enzymes most likely to be encountered at the site of attachment.

Effect of rAceKI vaccination on hookworm-associated growth delay. Based on the observation that AceKI is a potent inhibitor of intestinal digestive enzymes that localizes to the outer surface of the adult hookworm, we hypothesized that the presence of AceKI in the small intestine might protect the worm but also impair host absorption of essential nutrients, thereby contributing to hookworm-associated growth delay. In order to test this hypothesis, we vaccinated naïve hamsters with wild-type rAceKI in alum, and this was followed by a challenge infection with 100 *A. ceylanicum* L3. The animals were monitored for 60 days postinfection in order to characterize the long-term effect of immunization on disease progression. In order to account for the loss of one animal in the AceKI immunization group that died on day 32 postinfection, one animal in each of the remaining groups (uninfected, alum immunized, soluble hookworm extract [HEX] immunized) was

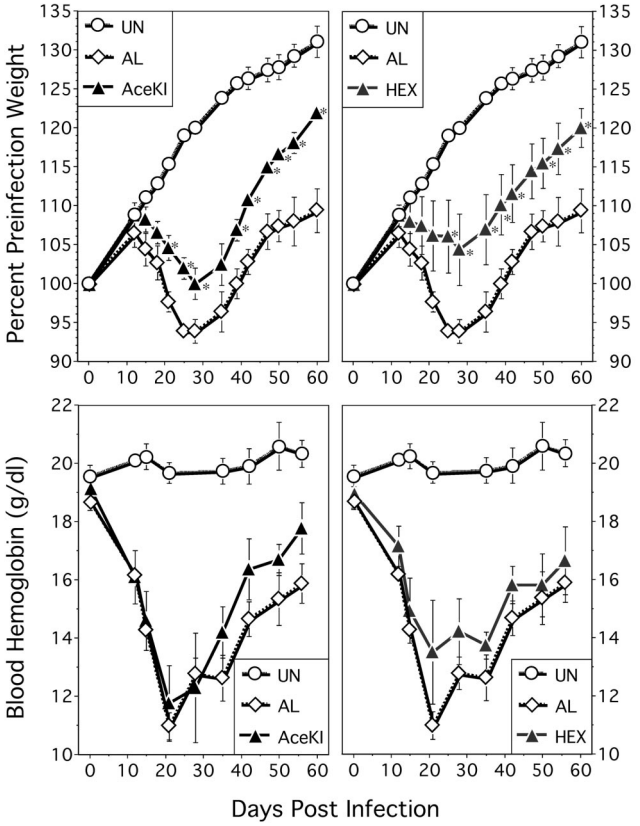

FIG. 5. Effect of rAceKI immunization on hookworm-associated growth delay. Hamsters were immunized subcutaneously three times with rAceKI in alum (AceKI), HEX in alum (HEX), or alum alone (AL). Following infection with 100 *A. ceylanicum* L3, the growth (top panels) and anemia (bottom panels) of the animals were monitored. An asterisk indicates that the *P* value is <0.05 for a comparison with the alum controls. Growth and hemoglobin levels were also determined for age-matched control hamsters that were not immunized and not infected (UN).

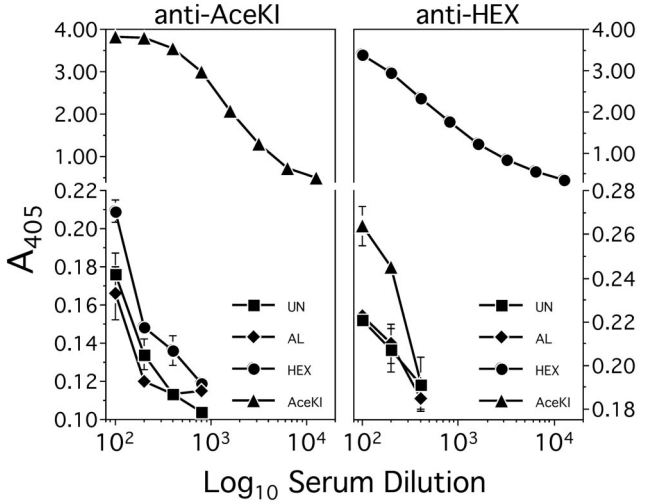

FIG. 6. Cross-reacting antibody responses in hamsters immunized with rAceKI or HEX. Pooled sera from hamsters immunized with rAceKI or HEX were tested for cross-reacting antibodies by an enzyme-linked immunosorbent assay (6, 7). (Left panel) Anti-rAceKI IgG responses. (Right panel) Anti-HEX IgG responses. Data were collected by using pooled sera from animals immunized with rAceKI (AceKI), HEX (HEX), or alum (AL) or from uninfected age-matched controls (UN).

removed from the analysis. In order to minimize any potential bias toward a beneficial effect of immunization in the subsequent analysis, the animal excluded from each group was the animal which exhibited the greatest degree of growth delay at day 32.

As shown in Fig. 5, the animals immunized with rAceKI experienced less dramatic growth delays following challenge infection than the animals that were immunized with alum adjuvant alone experienced. The differences in mean weights between the rAceKI-immunized animals and the alum-immunized animals were found to be statistically significant at 9 of the 12 times measured between days 10 and 60 postinfection. The degree of protection from growth delay in the AceKI immunization group was comparable to the degree of protection exhibited in response to vaccination with HEX, which is a complex mixture of native *A. ceylanicum* proteins previously demonstrated to confer partial protection from hookworm-associated growth delay (7). Of note, immunization with rAceKI did not have a protective effect on the depth or duration of anemia following challenge infection, as measured by serial evaluation of blood hemoglobin levels.

The antibody responses to immunization with rAceKI and HEX were also evaluated. As shown in Fig. 6, each vaccine group reacted strongly against its immunizing antigen. Pooled sera from hamsters immunized with rAceKI contained a higher level of anti-HEX IgG, as measured by an enzyme-linked immunosorbent assay, than sera from unimmunized animals or sera from animals immunized with alum alone contained. By comparison, pooled sera from animals immunized with HEX contained more IgG directed against rAceKI than either type of control sera contained.

DISCUSSION

Although malnutrition, which is often manifested as a growth delay, is a well-recognized consequence of hookworm infection, the molecular mechanisms underlying this clinical effect remain to be elucidated (58, 59, 64). There are a number of potential mechanisms by which hookworms may cause malnutrition. First, it is well established that hookworms cause substantial losses of iron and serum proteins from intestinal bleeding, as each adult *Ancylostoma* worm causes the loss of up to 0.2 ml of blood per day (52, 53). Over time, the resulting chronic iron deficiency, even in the absence of profound anemia, can suppress growth in young children (2, 15, 39, 47). Second, hookworm infection may cause decreased appetite and anorexia, either directly through intestinal irritation or secondarily through mediation of systemic expression of inflammatory cytokines like tumor necrosis factor alpha (15, 16, 59). Damage to the intestinal epithelium might impair absorption of nutrients, as suggested by previous studies which reported abnormal histology for intestinal biopsies from hookworm-infected individuals (24, 57), although a definitive causal role for hookworm infection in these histological changes has not been proven.

Hookworm infection may also be associated with a specific malabsorption syndrome. Work by Darke in Uganda showed that malabsorption was correlated with worm burdens and that the problem improved following anthelminthic treatment. Subsequent studies demonstrated that increased fecal fat excretion and abnormal absorption were associated with hookworm infection (18, 55). Unfortunately, it is difficult to identify the specific effects of hookworm infection in people who are at risk for malnutrition from multiple other causes, which makes these human studies difficult to interpret. However, taken together, these studies suggest that the etiology of hookworm malnutrition is multifactorial and may be a result of the cumulative effects of both local and systemic factors mediated by the adult worm as it resides in the small intestine. Due to the relative paucity of data from nutritional studies of permissive animal host systems, the molecular mechanisms of hookworm malnutrition remain poorly characterized.

Interestingly, Darke hypothesized that the etiology of hookworm malnutrition might be due in part to the action of "antiproteolytic substances" secreted by the adult worm (18). As a broad-spectrum inhibitor of the digestive enzymes trypsin, chymotrypsin, and pancreatic elastase, AceKI represents an obvious molecular candidate for mediating hookworm malnutrition via this mechanism. Previously, the cDNA corresponding to AceKI was amplified from adult *A. ceylanicum* RNA by using a PCR-based approach (48). The translated cDNA suggested that AceKI represents a novel member of the Kunitz family of serine protease inhibitors, which are characterized by six cysteine residues that form three intramolecular disulfide bonds (8, 43, 44, 49). Based on alignment of the AceKI amino acid sequence with the sequences of previously identified members of the Kunitz family, we hypothesized that Met^{26} was the likely P1 inhibitory reactive site of the protein. In order to test this hypothesis, by using site-directed mutagenesis we produced rAceKI mutants contained various substitutions at Met^{26} in order to characterize the effect of this amino acid residue on the protein's inhibitory spectrum. As summarized in Table 1, data from chromogenic assays confirmed that modifying the Met^{26} residue substantially alters the relative activity of AceKI against the three proteases tested.

To a great extent, the pattern of inhibition exhibited by each mutant could be predicted based on what is known about the biochemical properties of the serine proteases tested (4, 23, 34, 41). For instance, the change of residue 26 from Met to Lys was expected to increase the activity against trypsin, which has a much greater affinity for basic amino acids in the P1 amino acid position. The same M26K mutant, as expected, exhibited substantially weaker inhibitory activity against pancreatic elastase, which is known to have poor affinity for basic amino acids like lysine at the P1 site. Chymotrypsin, which prefers bulky hydrophobic residues in the P1 position, was substantially less inhibited by the M42G mutant, as glycine is a small, hydrophilic amino acid. Thus, the patterns of inhibition exhibited by the Met^{26} mutants essentially confirm that AceKI inhibits its target enzymes primarily through the canonical enzyme-substrate interaction that is characteristic of the other members of the Kunitz inhibitor family (43, 44, 51). Whether additional, non-active-site-mediated interactions between AceKI and its target proteases are possible remains to be determined.

Based on the spectrum of inhibitory activity of AceKI, we hypothesized that the role of this broad-spectrum inhibitor of pancreatic digestive enzymes might be to protect the worm in the proteolytic environment of the mammalian intestine. Furthermore, we hypothesized that AceKI is therefore expressed primarily by the adult stage of the parasite, which resides buried in the crypts of the small intestine. Using RT-PCR, we demonstrated that AceKI gene expression is upregulated following activation of *A. ceylanicum* L3 but that the highest level is reached in the adult worm (Fig. 2). Immunoblot data further demonstrated that AceKI is secreted by the adult hookworm (Fig. 3), while immunohistochemistry data revealed that the protein localizes to the subcuticicle (Fig. 4). This pattern of staining suggests that the worm might coat its outermost surface with AceKI, thereby neutralizing the effects of trypsin, chymotrypsin, and elastase in its immediate local environment. Such a strategy potentially allows the worm to avoid substantial proteolytic damage while feeding on the submucosa. However, a secondary effect of AceKI might be impairment of host nutrient absorption through the inhibition of pancreatic enzymes, thus lending further credence to Darke's original yet somewhat controversial hypothesis (45, 64). In light of the accumulating evidence that malnutrition is associated with increased susceptibility to infection by a variety of pathogens, the presence of a chronic malnourished state could potentially favor repeated infection with soil-transmitted helminths, including hookworms (3, 17, 38, 40, 50, 59, 63).

In order to confirm the role of AceKI in the pathogenesis of hookworm-associated growth delay, we immunized hamsters with recombinant protein in the adjuvant alum. As shown in Fig. 5, animals that were immunized with rAceKI in alum exhibited improved growth following infection with 100 L3 compared with the growth of animals that received alum alone. Although these animals did not grow as well as uninfected animals, the beneficial effect of rAceKI immunization on growth was similar to the effect obtained with soluble extracts of *A. ceylanicum* adults, which have previously been shown to protect animals in this vaccine model (6, 9). Consistent with our hypothesis, AceKI immunization had no effect on hookworm-associated anemia, as measured by blood hemoglobin levels following infection. As a means of defining the nature of this protective response, we measured IgG antibody levels in pooled serum from immunized animals. As shown in Fig. 6, serum from animals immunized with HEX contained measurable amounts of anti-rAceKI IgG, in contrast to serum from controls immunized with alum alone. Moreover, serum from animals immunized with rAceKI recognized HEX, in contrast to serum from alum-immunized controls. These data suggest that the native AceKI protein is an immunogenic component of HEX and that anti-AceKI antibodies may mediate, at least in part, the protection conferred by HEX immunization.

The observation that rAceKI immunization protects against growth delay but not anemia provides evidence that these two clinical consequences of hookworm infection appear to be distinct physiologic phenomena. This is consistent with data from a previous vaccine study (7), in which HEX immunization was shown to confer a pattern of protection against growth delay that was distinct from the pattern demonstrated for anemia. Thus, these studies provide the first evidence that the molecular basis of hookworm-induced malnutrition and growth delay involves additional mechanisms beyond chronic

iron deficiency. Importantly, these studies further establish the value of utilizing fully permissive animal models to characterize the pathogenesis of hookworm disease under defined experimental conditions.

The vaccine data presented here demonstrate that a single recombinant antigen is capable of conferring partial protection against a major clinical sequela of hookworm infection. This finding is significant because the potential development of a recombinant vaccine against human hookworm disease requires that specific targets be identified by using animal models like the one described here (9, 36, 37). Since it has been demonstrated that AceKI plays a role in the pathogenesis of growth delay and that vaccination with rAceKI confers partial protection in a fully permissive animal model, this novel hookworm virulence factor may show promise as a potential target for human vaccine development.

ACKNOWLEDGMENTS

This work was supported by a clinical research grant from the March of Dimes Birth Defects Foundation (to M.C.) and by a Hellman Family Fellowship from the Office of the President of Yale University (to M.C.).

We thank Aaron Milstone for careful review of the manuscript and the members of the Yale Child Health Research Center for their advice and support during the course of this work.

REFERENCES

1. **Bates, D. M., and D. G. Watts.** 1988. Nonlinear regression analysis and its applications. John Wiley, New York, N.Y.
2. **Beard, J.** 2003. Iron deficiency alters brain development and functioning. J. Nutr. **133:**1468S–1472S.
3. **Bhaskaram, P.** 2002. Micronutrient malnutrition, infection, and immunity: an overview. Nutr. Rev. **60:**S40–S45.
4. **Bode, W., and R. Huber.** 2000. Structural basis of the endoproteinase-protein inhibitor interaction. Biochim. Biophys. Acta **1477:**241–252.
5. **Bungiro, R. D., L. M. Harrison, and M. Cappello.** 2002. *Ancylostoma ceylanicum* excretory/secretory protein 1: purification and molecular cloning of a major secretory protein from adult hookworms. Mol. Biochem. Parasitol. **119:**147–151.
6. **Bungiro, R. D., Jr., B. R. Anderson, and M. Cappello.** 2003. Oral transfer of adult *Ancylostoma ceylanicum* hookworms into permissive and nonpermissive host species. Infect. Immun. **71:**1880–1886.
7. **Bungiro, R. D., Jr., J. Greene, E. Kruglov, and M. Cappello.** 2001. Mitigation of hookworm disease by immunization with soluble extracts of *Ancylostoma ceylanicum*. J. Infect. Dis. **183:**1380–1387.
8. **Capasso, C., M. Rizzi, E. Menegatti, P. Ascenzi, and M. Bolognesi.** 1997. Crystal structure of the bovine alpha-chymotrypsin:Kunitz inhibitor complex. An example of multiple protein:protein recognition sites. J. Mol. Recog. **10:**26–35.
9. **Cappello, M., L. M. Harrison, J. Bungiro, R. D., C. R. Chadderdon, A. Del Valle, A. Milstone, D. Chu, J. Mieszczanek, A. Nerlinger, and P. Kuzmic.** 2003. The molecular pathogenesis of hookworm anemia: prospects for a disease based vaccine. J. Parasitol. **89:**S158–S164.
10. **Cappello, M., J. M. Hawdon, B. F. Jones, W. P. Kennedy, and P. J. Hotez.** 1996. *Ancylostoma caninum* anticoagulant peptide: cloning by PCR and expression of soluble, active protein in *E. coli*. Mol. Biochem. Parasitol. **80:**113–117.
11. **Cappello, M., S. Li, X. Chen, C. B. Li, L. Harrison, S. Narashimhan, C. B. Beard, and S. Aksoy.** 1998. Tsetse thrombin inhibitor: bloodmeal-induced expression of an anticoagulant in salivary glands and gut tissue of *Glossina morsitans morsitans*. Proc. Natl. Acad. Sci. **95:**14290–14295.
12. **Cappello, M., G. P. Vlasuk, P. W. Bergum, S. Huang, and P. J. Hotez.** 1995. *Ancylostoma caninum* anticoagulant peptide: a hookworm-derived inhibitor of human coagulation factor Xa. Proc. Natl. Acad. Sci. **92:**6152–6156.
13. **Chilton, N. B., H. Hoste, G. C. Hung, I. Beveridge, and R. B. Gasser.** 1997. The 5.8S rDNA sequences of 18 species of bursate nematodes (order Strongylida): comparison with rhabditid and tylenchid nematodes. Int. J. Parasitol. **27:**119–124.
14. **Crompton, D. W.** 2000. The public health importance of hookworm disease. Parasitology **121**(Suppl.)**:**S39–S50.
15. **Crompton, D. W., and M. C. Nesheim.** 2002. Nutritional impact of intestinal helminthiasis during the human life cycle. Annu. Rev. Nutr. **22:**35–59.
16. **Crompton, D. W. T.** 2000. The public health importance of hookworm. J. Parasitol. **121:**S39–S50.
17. **Cunha, A. L.** 2000. Relationship between acute respiratory infection and malnutrition in children under 5 years of age. Acta Paediatr. **89:**608–609.
18. **Darke, S. J.** 1959. Malnutrition in African adults. 5. Effects of hookworm infestation on absorption of foodstuffs. Br. J. Nutr. **13:**278–282.
19. **Del Valle, A., B. F. Jones, L. M. Harrison, R. C. Chadderdon, and M. Cappello.** 2003. Isolation and molecular cloning of a secreted hookworm platelet inhibitor from adult *Ancylostoma caninum*. Mol. Biochem. Parasitol. **129:**167–177.
20. **Dreyfuss, M. L., R. J. Stoltzfus, J. B. Shrestha, E. K. Pradhan, S. C. LeClerq, S. K. Khatry, S. R. Shrestha, J. Katz, M. Albonico, and K. P. West, Jr.** 2000. Hookworms, malaria and vitamin A deficiency contribute to anemia and iron deficiency among pregnant women in the plains of Nepal. J. Nutr. **130:**2527–2536.
21. **Garside, P., and J. M. Behnke.** 1989. *Ancylostoma ceylanicum* in the hamster: observations on the host-parasite relationship during primary infection. Parasitology **98:**283–289.
22. **Garside, P., J. M. Behnke, and R. A. Rose.** 1990. Acquired immunity to *Ancylostoma ceylanicum* in hamsters. Parasite Immunol. **12:**247–258.
23. **Grzesiak, A., R. Helland, A. O. Smalas, D. Krowarsch, M. Dadlez, and J. Otlewski.** 2000. Substitutions at the P(1) position in BPTI strongly affect the association energy with serine proteinases. J. Mol. Biol. **301:**205–217.
24. **Guha, D. K., B. N. Walia, B. N. Tandon, and O. P. Ghai.** 1968. Functional and structural changes in the small intestine in children with hookworm infection. Arch. Dis. Child. **43:**235–238.
25. **Harrison, L. M., J. L. Cordova, and M. Cappello.** 2001. *Ancylostoma caninum* anticoagulant peptide-5: immunolocalization and in vitro neutralization of a major hookworm anti-thrombotic. Mol. Biochem. Parasitol. **115:**101–107.
26. **Harrison, L. M., A. Nerlinger, R. D. Bungiro, J. L. Cordova, P. Kuzmic, and M. Cappello.** 2002. Molecular characterization of *Ancylostoma* inhibitors of coagulation factor Xa. Hookworm anticoagulant activity in vitro predicts parasite bloodfeeding in vivo. J. Biol. Chem. **277:**6223–6229.
27. **Hawdon, J. M., and B. Datu.** 2003. The second messenger cyclic GMP mediates activation in *Ancylostoma caninum* infective larvae. Int. J. Parasitol. **33:**787–793.
28. **Hawdon, J. M., and P. J. Hotez.** 1996. Hookworm: developmental biology of the infectious process. Curr. Opin. Genet. Dev. **6:**618–623.
29. **Hawdon, J. M., B. F. Jones, M. A. Perregaux, and P. J. Hotez.** 1995. *Ancylostoma caninum*: metalloprotease release coincides with activation of infective larvae in vitro. Exp. Parasitol. **80:**205–211.
30. **Hawdon, J. M., and G. A. Schad.** 1990. Serum-stimulated feeding in vitro by third-stage infective larvae of the canine hookworm *Ancylostoma caninum*. J. Parasitol. **76:**394–398.
31. **Hawdon, J. M., S. W. Volk, D. I. Pritchard, and G. A. Schad.** 1992. Resumption of feeding in vitro by hookworm third-stage larvae: a comparative study. J. Parasitol. **78:**1036–1040.
32. **Hawdon, J. M., S. W. Volk, R. Rose, D. I. Pritchard, J. M. Behnke, and G. A. Schad.** 1993. Observations on the feeding behaviour of parasitic third-stage hookworm larvae. Parasitology **106:**163–169.
33. **Helland, R., H. Czapinska, I. Leiros, M. Olufsen, J. Otlewski, and A. O. Smalas.** 2003. Structural consequences of accommodation of four non-cognate amino acid residues in the S1 pocket of bovine trypsin and chymotrypsin. J. Mol. Biol. **333:**845–861.
34. **Helland, R., J. Otlewski, O. Sundheim, M. Dadlez, and A. O. Smalas.** 1999. The crystal structures of the complexes between bovine beta-trypsin and ten P1 variants of BPTI. J. Mol. Biol. **287:**923–942.
35. **Holmes, E. G., and S. J. Darke.** 1959. Malnutrition in African adults. 4. Intestinal absorption. Br. J. Nutr. **13:**266–277.
36. **Hotez, P. J., K. Ghosh, J. M. Hawdon, S. Narasimhan, B. Jones, X. Shuhua, L. Sen, Z. Bin, X. Haechou, R. Hainan, W. Heng, and R. A. Koski.** 1999. Experimental approaches to the development of a recombinant hookworm vaccine. Immunol. Rev. **171:**163–171.
37. **Hotez, P. J., B. Zhan, J. M. Bethony, A. Loukas, A. Williamson, G. N. Goud, J. M. Hawdon, A. Dobardzic, R. Dobardzic, K. Ghosh, M. E. Bottazzi, S. Mendez, B. Zook, Y. Wang, S. Liu, I. Essiet-Gibson, S. Chung-Debose, S. Xiao, D. Knox, M. Meagher, M. Inan, R. Correa-Oliveira, P. Vilk, H. R. Shepherd, W. Brandt, and P. K. Russell.** 2003. Progress in the development of a recombinant vaccine for human hookworm disease: the Human Hookworm Vaccine Initiative. Int. J. Parasitol. **33:**1245–1258.
38. **Ighogboja, I. S., and E. I. Ikeh.** 1997. Parasitic agents in childhood diarrhoea and malnutrition. West Afr. J. Med. **16:**36–39.
39. **Kazal, L. A., Jr.** 2002. Prevention of iron deficiency in infants and toddlers. Am. Fam. Physician **66:**1217–1224.
40. **Keusch, G. T.** 2003. The history of nutrition: malnutrition, infection and immunity. J. Nutr. **133:**336S–340S.
41. **Krowarsch, D., and J. Otlewski.** 2001. Amino-acid substitutions at the fully exposed P1 site of bovine pancreatic trypsin inhibitor affect its stability. Protein Sci. **10:**715–724.
42. **Kuzmic, P.** 1996. Program DYNAFIT for the analysis of enzyme kinetic data: application to HIV proteinase. Anal. Biochem. **237:**260–273.
43. **Laskowski, M., Jr., and I. Kato.** 1980. Protein inhibitors of proteinases. Annu. Rev. Biochem. **49:**593–626.

44. **Laskowski, M., and M. A. Qasim.** 2000. What can the structures of enzyme-inhibitor complexes tell us about the structures of enzyme substrate complexes? Biochim. Biophys. Acta **1477:**324–337.
45. **Layrisse, M., N. Blumenfeld, L. Carbonell, J. Desenne, and M. Roche.** 1964. Intestinal absorption tests and biopsy of the jejunum in subjects with heavy hookworm infection. Am. J. Trop. Med. Hyg. **13:**297–305.
46. **Lu, W., I. Apostol, M. A. Qasim, N. Warne, R. Wynn, W. L. Zhang, S. Anderson, Y. W. Chiang, E. Ogin, I. Rothberg, K. Ryan, and M. Laskowski, Jr.** 1997. Binding of amino acid side-chains to S1 cavities of serine proteinases. J. Mol. Biol. **266:**441–461.
47. **MacPhail, A. P.** 2001. Iron deficiency and the developing world. Arch. Latinoam Nutr. **51:**2–6.
48. **Milstone, A. M., L. M. Harrison, R. D. Bungiro, P. Kuzmic, and M. Cappello.** 2000. A broad spectrum Kunitz type serine protease inhibitor secreted by the hookworm *Ancylostoma ceylanicum*. J. Biol. Chem. **275:**29391–29399.
49. **Otlewski, J., M. Jaskolski, O. Buczek, T. Cierpicki, H. Czapinska, D. Krowarsch, A. O. Smalas, D. Stachowiak, A. Szpineta, and M. Dadlez.** 2001. Structure-function relationship of serine protease-protein inhibitor interaction. Acta Biochim. Pol. **48:**419–428.
50. **Pedersen, S., I. Saeed, K. F. Michaelsen, H. Friis, and K. D. Murrell.** 2002. Impact of protein energy malnutrition on *Trichuris suis* infection in pigs concomitantly infected with *Ascaris suum*. Parasitology **124:**561–568.
51. **Qasim, M. A., S. M. Lu, J. Ding, K. S. Bateman, M. N. James, S. Anderson, J. Song, J. L. Markley, P. J. Ganz, C. W. Saunders, and M. Laskowski, Jr.** 1999. Thermodynamic criterion for the conformation of P1 residues of substrates and of inhibitors in complexes with serine proteinases. Biochemistry **38:**7142–7150.
52. **Roche, M., and M. Layrisse.** 1966. The nature and causes of "hookworm anemia." Am. J. Trop. Med. Hyg. **15:**1029–1102.
53. **Roche, M., M. E. Perez-Gimenez, Layrisse, and E. Di Prisco.** 1957. Gastrointestinal bleeding in hookworm infection; studies with radioactive chromium (Cr51); report of five cases. Am. J. Dig. Dis. **2:**265–277.
54. **Salem, S. N., and S. C. Truelove.** 1964. Hookworm disease in immigrants. Br. Med. J. **5390:**1074–1077.
55. **Saraya, A. K., and B. N. Tandon.** 1975. Hookworm anaemia and intestinal malabsorption associated with hookworm infestation. Prog. Drug Res. **19:** 108–118.
56. **Segel, I.** 1975. Enzyme kinetics. John Wiley, New York, N.Y.
57. **Sheehy, T. W., W. H. Meroney, R. S. Cox, Jr., and J. E. Soler.** 1962. Hookworm disease and malabsorption. Gastroenterology **42:**148–156.
58. **Stephenson, L. S., M. C. Latham, E. J. Adams, S. N. Kinoti, and A. Pertet.** 1993. Weight gain of Kenyan school children infected with hookworm, *Trichuris trichiura* and *Ascaris lumbricoides* is improved following once- or twice-yearly treatment with albendazole. J. Nutr. **123:**656–665.
59. **Stephenson, L. S., M. C. Latham, and E. A. Ottesen.** 2000. Malnutrition and parasitic helminth infections. Parasitology **121**(Suppl.)**:**S23–S38.
60. **Stoltzfus, R. J., H. M. Chwaya, J. M. Tielsch, K. J. Schulze, M. Albonico, and L. Savioli.** 1997. Epidemiology of iron deficiency anemia in Zanzibari schoolchildren: the importance of hookworms. Am. J. Clin. Nutr. **65:**153–159.
61. **Stoltzfus, R. J., M. L. Dreyfuss, H. M. Chwaya, and M. Albonico.** 1997. Hookworm control as a strategy to prevent iron deficiency. Nutr. Rev. **55:** 223–232.
62. **Tandon, B. N., B. C. Das, A. K. Saraya, and M. G. Deo.** 1966. Functional and structural studies of small bowel in ankylostomiasis. Br. Med. J. **5489:**714–716.
63. **Tshikuka, J. G., K. Gray-Donald, M. Scott, and K. N. Olela.** 1997. Relationship of childhood protein-energy malnutrition and parasite infections in an urban African setting. Trop. Med. Int. Health **2:**374–382.
64. **Variyam, E. P., and J. G. Banwell.** 1982. Hookworm disease: nutritional implications. Rev. Infect. Dis. **4:**830–835.
65. **Yao, P. L., M. J. Hwang, Y. M. Chen, and K. W. Yeh.** 2001. Site-directed mutagenesis evidence for a negatively charged trypsin inhibitory loop in sweet potato sporamin. FEBS Lett. **496:**134–138.
66. **Zupunski, V., D. Kordis, and F. Gubensek.** 2003. Adaptive evolution in the snake venom Kunitz/BPTI protein family. FEBS Lett. **547:**131–136.

Editor: W. A. Petri, Jr.