MAJOR ARTICLE

Vaccination of Dogs with a Recombinant Cysteine Protease from the Intestine of Canine Hookworms Diminishes the Fecundity and Growth of Worms

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We expressed a catalytically active cysteine protease, *Ac*-CP-2, from the blood-feeding stage of the canine hookworm *Ancylostoma caninum* and vaccinated dogs with the purified protease. Dogs acquired high-titer, antigen-specific antibody responses, and adult hookworms recovered from the intestines of vaccinated dogs were significantly smaller than hookworms from control dogs. There was also a marked decrease in fecal egg counts and the number of female hookworms in vaccinated dogs. *Ac*-CP-2 is expressed by the parasite in the brush-border membrane of its alimentary canal, and anti–*Ac*-CP-2 antibodies were bound to the gut of hookworms from vaccinated dogs, which suggests that these antibodies were ingested by the parasites with their blood meal. IgG from vaccinated dogs decreased proteolytic activity against a peptide substrate by 73%, which implies that neutralizing antibodies were induced by vaccination. These results indicate that cysteine proteases involved in parasite nutrition are promising candidates as vaccines against hookworm disease.

Hookworms infect >700 million people in tropical and subtropical regions of the world. Unlike other human helminthiases, there is no evidence of age- or exposure-related immunity [1]. In fact, recent findings have revealed that the heaviest burdens of worms are found in elderly persons [2]. Although chemotherapy is effective at eliminating existing adult parasites, reinfection occurs rapidly after treatment [3], making a vaccine against hookworm disease a desirable goal [4].

Dogs can be successfully vaccinated against infection with the dog hookworm, *Ancylostoma caninum*, by vaccination with third-stage larvae (L3) that have been attenuated with ionizing radiation [5]. Subsequently, varying levels of vaccine efficacy have been reported for the major antigens secreted by hookworm L3 in murine hosts [6, 7] and dogs [8]. Despite encouraging levels of protection with larval antigens, only partial reductions in fecal egg counts and adult worm burdens have been reported, which suggests that an ideal hookworm vaccine might require a mixture of recombinant proteins that targets both the larval and blood-feeding adult stages of the parasite.

Of the different families of proteins expressed by blood-feeding parasitic helminths, proteolytic enzymes have shown promise as intervention targets for the development of vaccines [9, 10]. Proteases are pivotal for a parasitic existence—they mediate fundamental physiologic processes, such as molting, tissue invasion, feeding, embryogenesis, and the evasion of host immune responses [9, 11].

Parasitic helminths, both trematodes and nematodes, have provided proof of principle that proteases are attractive targets for the development of antiparasite vaccines. Cattle and sheep can be protected against infection with the liver fluke *Fasciola hepatica* by vaccination with the major secreted cysteine proteases [12]. Like

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wise, vaccination with calpain, as both recombinant protein [13] and naked DNA [14], confers partial protection of mice against *Schistosoma mansoni* infection. Protease-enriched extracts are also effective vaccines against blood-feeding nematodes. vaccination with the protease-rich gut membrane extract of *Haemonchus contortus,* H-gal-GP [15, 16], confers >90% protection against adult worms in lambs. H-gal-GP contains proteases of at least 3 major mechanistic classes, including cysteine proteases [17]; moreover, the vaccination of lambs [18] with an extract enriched for membrane-associated cysteine proteases resulted in decreased numbers of adult worms and eggs in vaccinated animals. Although the vaccination of calves with an extract enriched for the cysteine proteases of another trichostrongyle, *Ostertagia ostertagi,* did not significantly protect against adult worms, a 60% reduction in egg counts was seen [19]. Although protection against nematodes has been achieved using native parasite extracts enriched for cysteine proteases, significant protective efficacy has not been shown with a purified, catalytically active recombinant protease from a nematode.

Hookworms digest blood-derived hemoglobin using a range of mechanistically distinct proteases, and preliminary data have suggested that *Ac*-CP-2, a cathepsin B cysteine protease [20] from *A. caninum,* might be involved in this pathway [9]. With the aim of eventually vaccinating people against human hookworm disease, we decided to vaccinate dogs against the canine hookworm *A. caninum* with catalytically active recombinant *Ac*-CP-2, to determine whether vaccinated animals were protected against hookworm disease. We show that the cathepsin B–like protease *Ac*-CP-2 is secreted as a proteolytically active enzyme by the yeast *Pichia pastoris* and that the enzyme is expressed in the intestinal lumen of blood-feeding adult hookworm parasites. The vaccination of dogs with *Ac*-CP-2, formulated with several discrete adjuvants, resulted in reduced fecal egg counts and a decreased size of female and male worms. Moreover, the number of female hookworms present in the intestines of vaccinated dogs was significantly lower than that in control dogs. Antibodies generated by vaccinated dogs bound to the intestinal lumen and to intestinal contents of hookworms recovered from those dogs, and they interfered with the proteolytic function of the recombinant *Ac*--CP-2 enzyme in vitro.

MATERIALS AND METHODS

***Expression of recombinant* Ac-*CP-2 in* P. pastoris.** The entire open-reading frame encoding the proenzyme of *Ac*-CP-2 (spanning Ala-12 to the C terminal Val-340; GenBank accession number U18912), excluding the predicted signal peptide, was cloned into the expression vector pPIC-Zα using the *Xba*I and *Xho*I sites. Colonies were selected from transformed cells, and suspension cultures were grown in flasks then transferred to a Bioflo 3000 fermentor (New Brunswick Scientific) in a 5-L vessel, as described elsewhere [6]. The recombinant protein was secreted into culture medium and affinity purified on nickel-agarose, as described elsewhere [6].

Assessment of catalytic activity and glycosylation of recombinant* Ac-*CP-2. Purified, recombinant *Ac*-CP-2 was assessed for proteolytic activity by use of the fluorogenic peptidyl substrate Z-Phe-Arg-aminomethyl coumarin (AMC; Bachem) [21]. The optimum pH of *Ac*-CP-2 was assessed by use of Z-Phe-Arg-AMC at different pH values, according to protocols published elsewhere [22]. The cysteine protease inhibitor E64 was included in some assays, at a final concentration of 5 μmol/L. To determine whether recombinant *Ac*-CP-2 was glycosylated, recombinant protease was treated with P*N*Gase F and *O*-glycosidase, according to the manufacturer's instructions (Enzymatic CarboRelease kit; QA-Bio), under denaturing conditions, to remove any *N*- and *O*-linked oligosaccharides.

Animal husbandry and vaccination. Purpose-bred, parasite-naive, male beagles aged 8 $\pm$ 1 weeks were purchased from Marshall farms, identified by ear tattoo, and maintained in the George Washington University Animal Research Facility, as described elsewhere [23]. The experiments were conducted according to a protocol approved by the University Animal Care and Use Committee. Before the first vaccination and after each subsequent one, a serum sample was obtained from each dog.

Vaccine study design and antigen-adjuvant formulation. The vaccine trial was designed to test *Ac*-CP-2 formulated with 4 different adjuvants. AS03 and AS02A [24] were obtained from GlaxoSmithKline (a gift from Joe Cohen and Sylvie Cayphas), and ISA 70 was obtained from SEPPIC. Alum was prepared as described elsewhere [7]. To make 6 doses of *Ac*-CP-2 formulated with AS03, 600 μg of recombinant protein (0.3 mL of *Ac*-CP-2 at a concentration of 2 mg/mL) was mixed with 1.2 mL of 20 mmol Tris-HCl/L, 0.5 mol NaCl/L (pH 7.9), and 1.5 mL of AS03; the contents of the tube were vortex mixed for 30 s and then shaken at low speed for 10 min. Dogs were vaccinated with 100 μg of formulated antigen in a final volume of 0.5 mL. To make 6 doses of *Ac*-CP-2 formulated with AS02A, 600 μg of recombinant protein (0.3 mL of *Ac*-CP-2 at a concentration of 2 mg/mL) was mixed with 0.9 mL of 20 mmol Tris-HCl/L, 0.5 mol NaCl/L (pH 7.9), and 1.8 mL of AS02A; the contents of the tube were vortex mixed for 30 s and then shaken at low speed for 10 min. Dogs were vaccinated with 100 μg of formulated antigen in a final volume of 0.5 mL. To make 6 doses of *Ac*-CP-2 formulated with ISA 70, 600 μg of recombinant protein (0.3 mL of *Ac*-CP-2 at a concentration of 2 mg/mL) was mixed with 1.66 mL of ISA 70; the contents of the tube were vortex mixed for 30 s and then shaken at low speed for 10 min. Dogs were vaccinated with 100 μg of formulated antigen in a final volume of 0.327 mL. To make 6 doses of *Ac*-CP-2 formulated with alum, 600 μg of recombinant protein (0.3 mL of *Ac*-CP-2 at a concentration of 2 mg/mL)

mixed with 0.135 mL of 1 mol $NaHCO_3$/L; 0.3 mL of 0.2 mol $AlK(SO_4)_2 12H_2O$/L [7] was added to initiate precipitation. The precipitate was collected by centrifugation at 13,000 *g* for 10 min. The supernatant was collected, and the precipitation reaction was repeated; the supernatant was collected and assayed for nonprecipitated protein by use of a bicinchoninic acid protein assay (Pearce). The 2 precipitates were pooled, washed with PBS, and resuspended in 3 mL of the supernatant; dogs were then vaccinated with 100 μg of formulated antigen in a final volume of 0.5 mL. The alum-only control was prepared as described above, with PBS included instead of *Ac*-CP-2.

Dog vaccinations and antibody measurements. Beagles were vaccinated with formulated *Ac*-CP-2, as described elsewhere [23]. The vaccines were administered intramuscularly 3 times, beginning at age 62 $\pm$ 4 days. Booster vaccinations were administered to the dogs at intervals of 21 days. Blood was drawn at least once every 21 days, and serum was separated from cells by centrifugation. ELISAs were performed as described elsewhere [23]. Recombinant *Ac*-CP-2 was coated onto microtiter plates at a concentration of 5 μg/mL. Serum samples were titrated between 1:100 and $1:2 \times 10^6$, to determine endpoint titers. Anti–dog IgG1, IgG2, and IgE antibodies conjugated to horseradish peroxidase (Bethyl Laboratories) were used at a dilution of 1:1000.

Hookworm infections and parasite recovery. Fourteen to 16 days after the final vaccination, dogs were anesthetized with a combination of ketamine and xylazine (20 and 10 mg/kg, respectively), and 500 *A. caninum* L3 in a final volume of 50 μL were applied to the footpad. After the application of L3, the foot was wrapped in parafilm, gauze padding, and packaging tape, to ensure that L3 did not escape from the site of application. Dogs were monitored for 3 h, after which the parafilm, gauze, and tape were removed. The site of L3 application was rinsed with saline, and any remaining L3 that had not penetrated were counted. Quantitative hookworm egg counts (counted using the McMaster technique) were obtained for each dog 3 days/week, beginning on day 13–15 after infection. Four weeks after infection, the dogs were killed by an intravenous injection of barbiturate, and adult hookworms were recovered and counted from the small and large intestines at necropsy [23]. The sex of each adult worm was determined, and worm lengths were measured as described elsewhere [6]. Approximately 1–2-cm lengths of the small intestine were removed and stored in formalin for future histopathological analysis.

Statistical methods. The percentage reduction or increase in adult hookworm burden in the vaccinated groups was expressed relative to the control group, as described elsewhere [23]. The statistical significance of differences in adult hookworm burdens was determined by use of nonparametric tests: the Kruskal-Wallis test with Dunn procedures and the Mann-Whitney *U* test. Differences between groups in quantitative hookworm egg counts and worm lengths were assessed by analysis of variance. Once determined, the differences among the means of groups were determined, and a Dunnett post hoc multiple-comparison *t* test was used to compare the vaccine treatment groups against the control group. The sex differences of the adult hookworms recovered were statistically compared by use of the Wilcoxon signed rank test for 2 dependent groups. Differences were considered to be statistically significant if the calculated *P* value was $\leq$.10 (2-sided) or $\leq$.05 (1-sided).

Immunohistochemistry. Adult hookworms recovered from vaccinated dogs were fixed, sectioned, and probed with various serum samples and Cy3-conjugated secondary antibodies (BD Biosciences), as described elsewhere [25]. Serum samples from vaccinated dogs and Cy3-conjugated anti–dog IgG were diluted 1:500. Some sections were probed with rabbit anti–*Ac*-CP-1 serum [20], followed by Cy3-conjugated anti–rabbit IgG; both antibodies were diluted 1:500.

Effect of anti–Ac-CP-2 IgG on proteolytic activity. Dog IgG was purified from serum samples of vaccinated dogs by use of protein A agarose (Amersham Biosciences), as described elsewhere [26]. Purified IgG (10–500 ng) was incubated with 1.0 μg of recombinant *Ac*-CP-2 for 45 min before the proteolytic activity was assessed as described above.

RESULTS

Secretion of catalytically active, glycosylated Ac-CP-2 by P. pastoris. *Ac-cp-2* cDNA (GenBank accession number U18912) has been cloned and reported by Harrop et al. [20]. We expressed recombinant *Ac*-CP-2 as a secreted fusion protein in *P. pastoris,* with a yield of 35 mg/L. Secretion was mediated by the α–mating factor signal peptide derived from the pPIC-Zα vector. The protein was purified from *P. pastoris* culture supernatant using nickel-agarose [6]. The purified protein migrated with an apparent molecular size of 48 kDa (figure 1*A*). This was larger than the predicted size of the proenzyme (41.8 kDa) and processed, mature enzyme (32.1 kDa), factoring in the C-terminal *myc* and His tags and the N-terminal EAEAEF motifs (which were introduced by the choice of restriction sites used in the cloning of the construct). *N*-linked glycosylation of the 5 predicted sites in *Ac*-CP-2 probably accounted for some of the discrepancy between the predicted and observed molecular sizes. Deglycosylation with P*N*GaseF reduced the apparent molecular mass of recombinant *Ac*-CP-2 by 5–10 kDa, although numerous bands within this size range were apparent (not shown)—these probably corresponded to partially deglycosylated proteins. N-terminal amino acid sequencing of the major secreted protein, by use of Edman degradation, showed the N-terminal residue to be Glu-13, which suggests that some posttranslational processing of the proregion had occurred. However, this did not correspond with the predicted cleavage

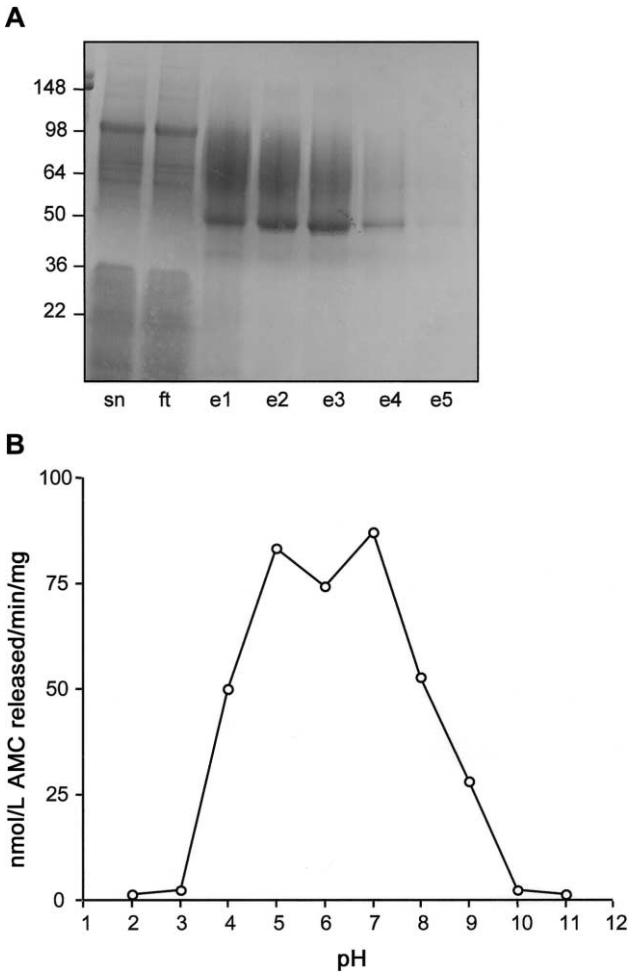

Figure 1. A, Purification of recombinant *Ac*-CP-2 from the culture supernatant of transformed *Pichia pastoris. A,* Molecular size markers (in kDa) are listed at the side. *B,* pH profile of the catalytic activity of recombinant *Ac*-CP-2 against the substrate Z-Phe-Arg–aminomethyl coumarin (AMC). e1-e5, elution fractions after the application of 1 mol imidazoleft/L flowing through from a nickel-agarose column; ft, column flow through; sn, supernatant.

site of the proregion from the mature enzyme (Asp-81–Asp-82, using the numbering scheme of the fusion protein presented here). Although this is only a predicted cleavage site based on the known cleavage site of the proregion of other related enzymes [11], it is unlikely that Glu-13 is the N-terminal residue of the native, secreted protease. Difficulty in obtaining sufficient quantities of native, hookworm-derived *Ac*-CP-2 precluded N-terminal sequence information for comparison. Nonetheless, numerous faint bands with molecular sizes of 30–40 kDa appeared when purified, recombinant *Ac*-CP-2 was stained with silver (not shown), which suggests that a small quantity of the recombinant protein was correctly processed to yield the mature form of the enzyme. This was further confirmed by the catalytic activity seen when recombinant *Ac*-CP-2 was incubated with Z-Phe-Arg-AMC (figure 1*B*). A broad pH range was observed, with activity detected between pH 4 and 8 and optimal catalysis between pH 5 and 7. The addition of the cysteine protease inhibitor E64, to a final concentration of 5 μmol/L, completely ablated cleavage of the peptide substrate (not shown). Moreover, other recombinant proteins (nonproteolytic) expressed and purified in an identical fashion in our laboratory did not cleave Z-Phe-Arg-AMC (not shown). Preliminary attempts at completely activating recombinant *Ac*-CP-2, including autocatalysis at low pH and activation by exogenous proteases, including pepsin and whole-worm extracts, were mostly unsuccessful, and these studies are ongoing in our laboratories. These data and a more detailed biochemical analysis of the recombinant enzyme will be published elsewhere.

Immunogenic recombinant* Ac-*CP-2 in dogs. Dogs vaccinated with recombinant *Ac*-CP-2 formulated with different adjuvants produced IgG1 and IgG2 antibody responses, as measured by ELISA using the recombinant protein (figure 2). IgE

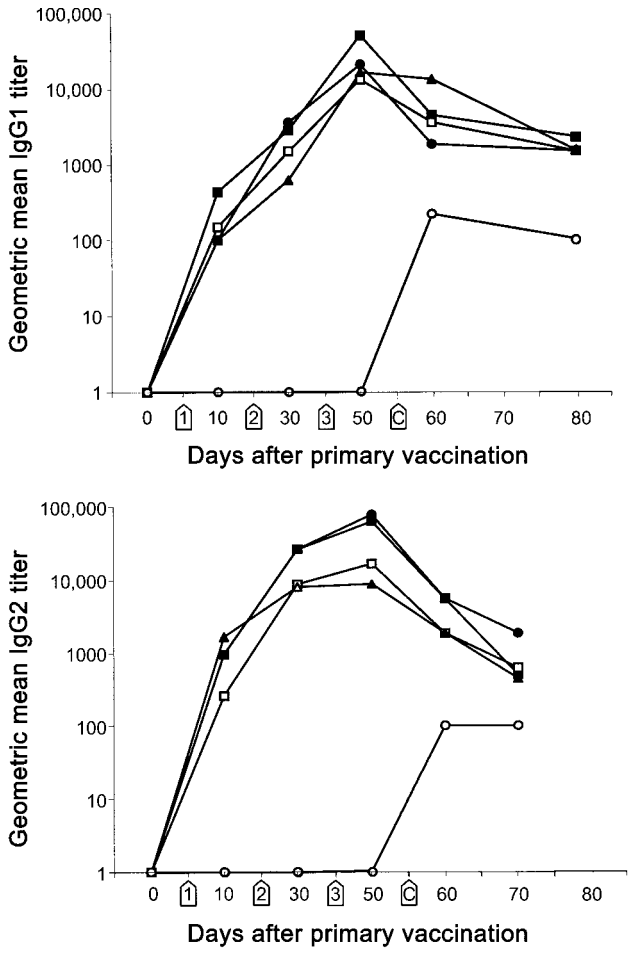

Figure 2. Geometric mean titers of the IgG1 *(A)* and IgG2 *(B)* antibody responses of vaccinated dogs against recombinant *Ac*-CP-2 formulated with AS03 *(black squares),* AS02A *(black circles),* ISA70 *(white squares),* alum *(black triangles),* or alum alone without CP-2 *(white circles).*

titers were low (<1500). We did not adsorb IgG from serum before measuring IgE; however, in previous trials, IgG was removed, and we did not see a difference in antigen-specific IgE titers. The maximum IgG1 titers (geometric mean, 50,452) were induced by formulating *Ac*-CP-2 with AS03. The maximum IgG2 titers (geometric mean, 78,294) were induced by formulating *Ac*-CP-2 with AS02A. Dogs vaccinated with adjuvant alone did not generate detectable immune responses until larval challenge, which suggests that antibodies to *Ac*-CP-2 (or a similar protease) are induced during natural infection with the parasite. *Ac-cp-2* mRNA was not identified from >9000 expressed sequence tags generated from serum-stimulated (induced to feed) *A. caninum* L3 (see http://www.nematode.net; A.L., unpublished data), which implies that the mRNA and protein are only expressed during the adult blood-feeding stages. The increase in anti–*Ac*-CP-2 antibody titers in control dogs after L3 challenge (but before worms would have matured to adulthood) is likely caused by the secretion of antigenically related cysteine proteases by L3; the closest homolog of *Ac*-CP-2 from *A. caninum* L3 cDNAs (EST pb58a11.y1) shares 64% identity at the amino acid level. AS02A and AS03 adjuvants induced the greatest antibody responses, especially of the IgG2 subclass. CP-2 formulated with ISA 70 or alum induced weaker peak responses after the final vaccination (day 50 after the initial vaccination), but, 10 days later (day 60 after the initial vaccination), dogs vaccinated with alum-formulated antigen generated the highest IgG1 titers, which implies that antibody responses to alum-formulated CP-2 were of longer duration (figure 2).

Decreased fecundity of female hookworms after vaccination with* Ac-*CP-2. Dogs rapidly develop age- and exposure-related immunity to *A. caninum* [27]. We therefore observed egg counts from vaccinated animals up to 3 weeks after challenge. At 3 weeks after larval challenge, a significant decrease in egg counts was observed in dogs that had been vaccinated with *Ac*-CP-2 formulated with AS02A, AS03, or alum, compared with dogs that had been vaccinated with alum alone ($P \leq .05$; figure 3).

No significant reduction in adult hookworm burdens after vaccination with* Ac-*CP-2. Statistically significant differences between mean burdens of adult male worms in dogs vaccinated with *Ac*-CP-2 and adjuvant alone were not seen (table 1). The greatest number of female worms was recovered from dogs vaccinated with alum alone (mean, 140); the smallest number of female worms was recovered from dogs vaccinated with *Ac*-CP-2/AS03 (mean, 115). Although the decrease in worm burdens in the latter group was noteworthy, the differences were not statistically significant.

Lower proportion of female worms after vaccination with* Ac-*CP-2/AS02A. Comparison of the proportions of male to female worms revealed that worms recovered from dogs vaccinated with *Ac*-CP-2/alum ($P = .05$) and *Ac*-CP-2/AS02A ($P = .074$) had more male than female worms, compared with worms recovered from dogs vaccinated with adjuvant alone (figure 4).

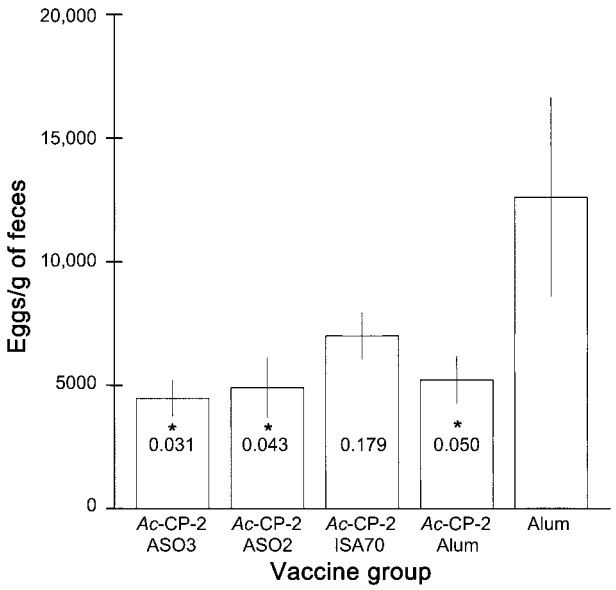

Figure 3. Geometric mean egg counts from dogs vaccinated with *Ac*-CP-2 formulated with different adjuvants or with alum alone (control). The error bars indicate SEs. The nos. within the bars refer to the *P* value of a Dunnett (post hoc) test, a pairwise multiple-comparison *t* test that compares a set of treatments against a single control mean.*$P \leq .05$.

Stunted growth of hookworms after vaccination with* Ac-*CP-2 protease. At necropsy, all worms recovered from the vaccinated dogs were fixed in formalin. The lengths of 100 undamaged worms from each group were measured, and the mean lengths were compared statistically. The mean lengths of female worms recovered from dogs vaccinated with *Ac*-CP-2/AS02A ($P = .003$) and from those vaccinated with *Ac*-CP-2/AS03 ($P = .033$) were shorter than those of worms recovered from dogs vaccinated with adjuvant alone (table 2). Statistically significant differences in lengths of male worms were obtained when male worms from dogs vaccinated with *Ac*-CP-2/AS03 were compared with those recovered from dogs vaccinated with alum alone ($P = .035$).

Anti–*Ac-*CP-2 antibodies in the intestine of feeding hookworms. The site of the anatomical expression of *Ac*-CP-2 within adult hookworms has not been previously reported. We therefore used serum samples from dogs vaccinated with *Ac*-CP-2/AS03 to localize expression to the brush-border membrane of the intestine of adult worms (figure 5*A*). *Ac*-CP-1, on the other hand, was shown by Harrop et al. [20], and confirmed by us (figure 5*E*), to be expressed in the cephalic and excretory glands of the parasite, which accounts for its presence in the excretory and secretory (ES) products of adult *A. caninum* [20].

To determine whether the vaccination of dogs induced circulating antibodies that bound to the intestinal lumen during infection, parasites were removed from vaccinated dogs, fixed,

Table 1. Mean nos. of adult hookworms recovered from the small and large intestines of dogs vaccinated with *Ac*-CP-2 formulated with different adjuvants or with adjuvant alone.

	Small intestine		Large intestine	
Vaccine	Male	Female	Male	Female
Ac-CP-2/AS03	107	111	8	9
Ac-CP-2/AS02A	109	104	7	11
Ac-CP-2/ISA70	113	116	7	8
Ac-CP-2/alum	125	120	4	6
Alum	105	131	6	9

sectioned, and probed with secondary antibody (anti–dog IgG conjugated to Cy3) only. Worms recovered from dogs vaccinated with *Ac*-CP-2 (figure 5*B* and 5*C*), but not from dogs vaccinated with adjuvant alone (figure 5*D*), contained antibodies that were ingested with the blood meal of the worm and subsequently bound specifically to the intestine and intestinal contents.

Neutralized proteolytic activity in vitro by IgG from dogs vaccinated with* Ac*-CP-2. Purified IgG from dogs that were vaccinated with *Ac*-CP-2 was effective at neutralizing the catalytic activity of *Ac*-CP-2. The incubation of 50 ng of pooled IgG from dogs vaccinated with *Ac*-CP-2/AS03 resulted in a 73% reduction in the cleavage of Z-Phe-Arg-AMC by 1.0 μg of *Ac*-CP-2 (table 3). Fifty nanograms of IgG from dogs vaccinated with adjuvant alone resulted in a 3% reduction in proteolytic activity, which implies that vaccination with *Ac*-CP-2 results in the production of antibodies that neutralize the function of the enzyme in vivo.

DISCUSSION

We have described the vaccination of dogs with a recombinant cysteine protease that resulted in partial protection from hookworm infection, as measured by reduced fecal egg counts, the stunting of adult worms, a decreased proportion of female to male worms, and the generation of protease-neutralizing antibodies that bind to the hookworm intestine in vivo. In the 1930s, the late Asa Chandler hypothesized that antibodies directed against critical parasite enzymes mediated a successful antihelminthic immune response by preventing worms from digesting host proteins [28]. To our knowledge, ours is the first report of protective efficacy with a recombinant protease from a parasitic nematode, and it provides support for Chandler's antienzyme theory.

Although it is secreted by *P. pastoris,* the complete processing of recombinant *Ac*-CP-2 to yield a mature enzyme did not occur; nonetheless, proteolytic activity was detected in the purified protein. *P. pastoris,* transformed with a cDNA encoding *F. hepatica* cathepsin L, secreted a partially activated protease that also exhibits catalytic activity; however, unlike *Ac*-CP-2, this enzyme completely autoactivated after 2 h at pH 5.5 [12]. *Ac*-CP-2 displayed a broad pH range, with optimal activity at pH 5–7, which supports earlier work that described an optimal

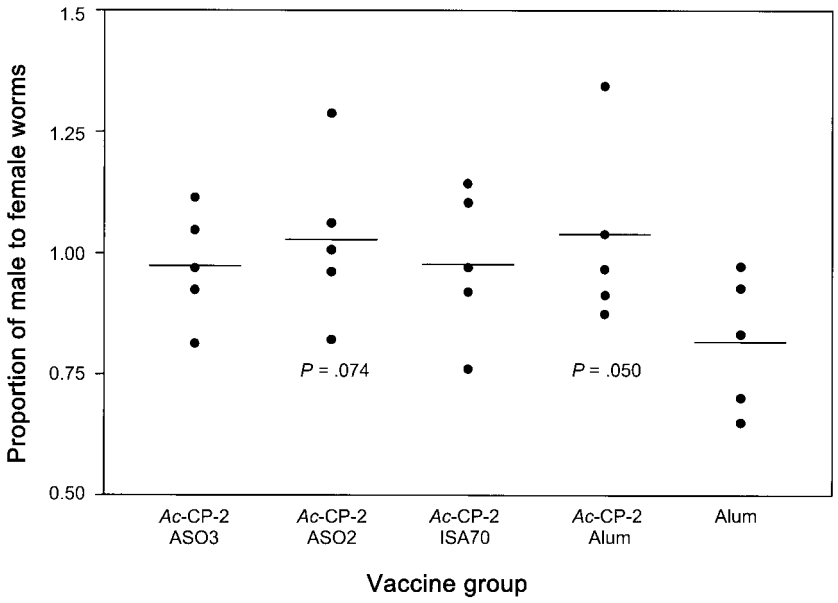

Figure 4. Proportions of male to female worms recovered from dogs vaccinated with *Ac*-CP-2 formulated with different adjuvants or with alum adjuvant alone. Individual proportions are shown for each dog, and the mean value for each group is denoted by a bar. Where the proportions were significantly different ($P < .1$ according to a Wilcoxon signed rank test) between vaccine and control groups, *P* values are denoted beneath the mean.

Table 2. Shorter adult hookworms recovered from dogs that were vaccinated with *Ac*-CP-2 vs. with adjuvant alone.

Vaccine, sex of dogs	Mean ± SD length, cm	*P*
Ac-CP-2/AS03		
Female	0.534 ± 0.22	**.033**
Male	0.384 ± 0.11	**.035**
Ac-CP-2/AS02A		
Female	0.507 ± 0.12	**.003**
Male	0.432 ± 0.12	.844
Ac-CP-2/ISA70		
Female	0.572 ± 0.21	.567
Male	0.465 ± 0.14	.999
Ac-CP-2/Alum		
Female	0.558 ± 0.24	.567
Male	0.471 ± 0.14	1.000
Alum only		
Female	0.612 ± 0.28	…
Male	0.430 ± 0.13	…

NOTE. There were 100 male and female dogs, each, in each vaccine group. *P* values compare the difference between each group that received the vaccine and the adjuvant-alone group, by use of a Dunnett *t* tests in which 1 group was treated as a control and the test groups were compared against it. Statistically significant *P* values are given in bold.

pH range of 5–9 for ES products and somatic extracts of adult *A. caninum* using Z-Phe-Arg-AMC [22].

Hematophagous helminths require blood as a source of nutrients to mature and reproduce. Female schistosomes ingest 13 times as many erythrocytes and ingest them ~9 times faster than male worms [29]. Moreover, mRNAs encoding hemoglobin-degrading proteases of schistosomes are overexpressed in female worms [30]. Although similar studies have yet to be performed for hookworms, female hookworms are bigger than males and lay up to 10,000 eggs/day, which implies that they have a greater metabolism and, therefore, demand for erythrocytes. *Ac*-CP-2 is expressed in the gut, and preliminary data described elsewhere [9] have shown that this enzyme is involved in hemoglobinolysis in the hookworm intestine. It is therefore not surprising that interruption of the function of *Ac*-CP-2 via the action of neutralizing antibodies had a deleterious effect on the growth of female worms and subsequent egg production.

The vaccination of livestock and laboratory animals with cysteine proteases of other nematodes and trematodes has resulted in antifecundity and antiembryonation effects. The vaccination of sheep with the intestinal brush-border complex H-gal-GP confers high levels of protection (both antiparasite and antifecundity) against *H. contortus,* and at least 3 different protease activities, including cathepsin B cysteine proteases, have been detected in this extract. The vaccination of sheep with a cysteine protease–enriched fraction of *H. contortus* membranes resulted in 47% protection against adult worms and a 77% reduction in fecal egg output [18]. To date, the success obtained in vaccinated laboratory animals with cysteine proteases purified from parasite extracts has not been reproduced with the corresponding recombinant proteins expressed in *Escherichia coli,* presumably because the recombinant molecules are incorrectly folded (and catalytically inactive) and thereby fail to induce responses capable of inactivating native proteases [10].

Cysteine proteases are also efficacious as antitrematode vaccines. The vaccination of cattle with cathepsin L cysteine proteases of *F. hepatica* results in decreased embryonation and hatch rates of eggs, in addition to decreased worm burdens [12]. Although these studies were performed with native proteins, trials with yeast-expressed recombinant proteases are in progress [12]. Vaccine trials using a DNA construct for *S. mansoni* Sm32, an asparaginyl endopeptidase that is cysteine protease–like in function but is unrelated in sequence to cathepsins L and B, induced an antifecundity effect in a murine model of schistosomiasis when it was administered as a DNA construct [31].

The data presented in the present article suggest that the adjuvants from GSK Biologicals (AS02A and AS03) perform the best, from a vaccine perspective. The vaccination of dogs with *Ac*-CP-2 formulated with either of these adjuvants resulted in stunted female worms, whereas other adjuvants did not yield significant differences (table 2). CP-2 formulated with all of the adjuvants except for ISA70 resulted in significant reductions in fecal egg counts (figure 3), and alum- and AS02A-formulated vaccines resulted in selective decreases in the numbers of female worms in the intestine (figure 4). AS03 (formerly known as "SBAS3") is an emulsion of oil in water; AS02A (formerly known as "SBAS2") consists of an emulsion of oil in water with the immune stimulants 3D-monophosphoryl lipid A and the saponin derivative QS21 [24]. The immune stimulants in SBAS2 promoted strong Th1 responses in vaccinees vaccinated with the *Plasmodium falciparum* circumsporozoite–derived proteins RTS,S [32]. When RTS,S proteins were formulated with SBAS2, high-titer antibody responses were generated, and protection against malaria was obtained in clinical trials, whereas RTS,S formulated with other adjuvants, including SBAS3 and SBAS4 (alum and monophosphoryl lipid A), did not provide protection [24]. Our data suggest that oil-in-water adjuvants, even in the absence of immune stimulants, induce the highest levels of anti–CP-2 antibodies (figure 2), and these, in turn, are sufficient to drive partially protective immune responses.

Almost all of the pathology and morbidity of human hookworm infection result from intestinal blood loss caused by large numbers of adult hookworms. Depending on host iron and protein stores, a range of hookworm intensities, equivalent to burdens of 40–160 worms, is associated with hemoglobin levels <11

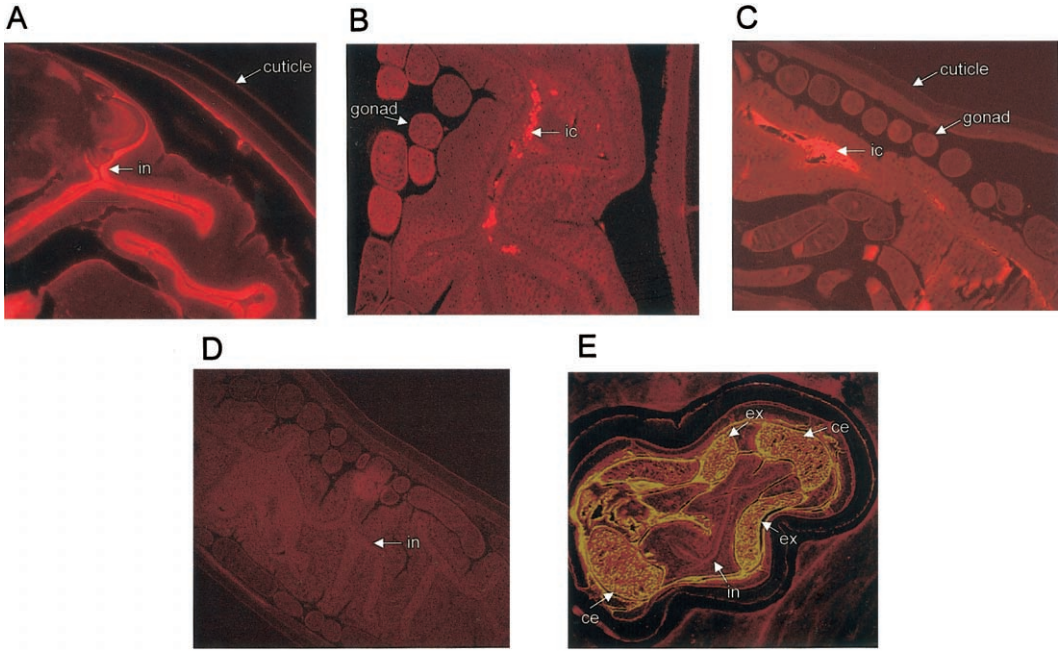

Figure 5. Antibodies binding to the intestinal brush-border membrane (in) and intestinal contents (ic) in vitro and in vivo in dogs vaccinated with *Ac*-CP-2. *A,* Cross-section of an adult *Ancylostoma caninum* probed with dog anti–*Ac*-CP-2 serum followed by Cy-3–conjugated anti–dog IgG. *B-D,* Longitudinal sections of *A. caninum* recovered from a dog that was vaccinated with either *Ac*-CP-2/alum *(B* and *C)* or alum alone *(D)* and proved with Cy3-conjugated anti-dog antibody only (primary antibody was not used). *E,* Recognition of cephalic and excretory glands, but not of intestine, of *Ac*-CP-1 by rabbit anti–*Ac*-CP-1 serum.

g/dL, the World Health Organization threshold for anemia [33]. In Tanzania, Nepal, and Vietnam, where host iron stores are generally depleted, there is a direct correlation between the number of adult hookworms in the intestine and host blood loss [34]. Therefore, the optimal hookworm vaccine will be one that either prevents L3 from developing into adult blood-feeding hookworms or that blocks the establishment, survival, and fecundity of the adult parasites in the intestine [4, 35]. Achieving both goals will likely require a vaccine mixture that is composed of an L3 antigen, such as ASP-2, which is now under clinical development [4], and an adult gut protease, such as CP-2.

An effective hookworm vaccine need not attain 100% efficacy. Unlike many unicellular organisms that reproduce asexually within the host, nematodes need to sexually reproduce. Therefore, smaller numbers of adult worms will generate fewer eggs to contaminate the environment and, subsequently, will reduce transmission. More important, because hookworms are blood feeders, a partial reduction in the adult worm burden equates to a decrease in pathologic symptoms, notably iron-deficiency anemia [34]. The results of mathematical modeling of schistosomiasis in China showed that the elimination of the parasite could be attained using an antifecundity vaccine with 75% efficacy [36], and it is likely that a similar scenario applies to the long-term elimination of other feces-transmitted helminths.

We recently described the partial protection of hamsters against another hookworm, *Ancylostoma ceylanicum,* by vaccination with a larval antigen, *Ay*-ASP-2, as a model of human hookworm disease [6]. The orthologous protein from *A. caninum, Ac*-ASP-2, is expressed by L3 parasites when it is stimulated to feed in vitro [37]. The vaccination of hamsters with *Ay*-ASP-2 resulted in a 32% reduction in the number of worms that reached adulthood [6], and we envisage that a human hookworm vaccine would ultimately consist of multiple antigens targeting both the L3 and the blood-feeding adult stages.

Table 3. Effect of pooled IgGs from vaccinated (*Ac*-CP-2/AS03) and control (adjuvant alone) dogs on the proteolytic activity of recombinant *Ac*-CP-2 against the substrate Z-Phe-Arg-AMC.

Treatment	*Ac*-CP-2 only	*Ac*-CP-2 + αCP-2 IgG	*Ac*-CP-2 + normal IgG	*Ac*-CP-2 + E64
Reduction in proteolytic activity	0 ± 0	73 ± 3	3 ± 2	100 ± 0

NOTE. Values are expressed as the mean percentage reductions ± SD in proteolytic activity from experiments done in triplicate.

The data presented here suggest that cysteine proteases lining the intestinal lumen of hookworms are a valid target in the design of vaccines against hookworm disease. We have identified other proteases of different mechanistic classes that line the intestinal brush border of adult hookworms, where they digest host hemoglobin [9], and some of these molecules might also prove efficacious as recombinant vaccines against hookworm infection.

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